IDENTIFICATION OF MICROTUBULE PROTEINS IN GRISEOFULVIN SENSITIVE AND RESISTANT YEASTS.

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ABSTRACT

The effects of the anti-fungal antibiotic griseofulvin on the growth and cytology of a sensitive yeast Protomyces inundatus were investigated using light, scanning and transmission electron microscopic techniques. This drug was found to inhibit the growth of Protomyces, causing the cells to be arrested in cell division. Electron microscopy revealed that griseofulvin did not cause the disaggregation of nuclear microtubules, however the microtubules appeared to be assembled in a disorganised manner. Differences in sensitivity to griseofulvin between the insensitive Saccharomyces cerevisiae and Protomyces inundatus were found not to be the result of differences in permeability to the drug. Both yeasts appeared to take up the drug very rapidly and to a similar extent. The problems associated with the study of griseofulvin uptake are highlighted and discussed.

Microtubule proteins were investigated in Saccharomyces cerevisiae. Initial attempts to purify microtubules from this yeast by assembly-disassembly were unsuccessful due to the inhibitory activity present in the Saccharomyces extracts. Protease activity, GTP-ase activity and RNA were found to contribute to this inhibitory activity. The addition of glycerol to assembly mixtures also reduced the inhibition. Microtubule proteins were identified from Saccharomyces cerevisiae by co-polymerisation of ^{35}S-labelled cell extract with brain microtubule protein which was either unmodified or depleted in microtubule-associated proteins (MAPs) by assembly in dimethyl sulphoxide (DMSO). Using this technique, a number of radio-active proteins co-polymerising throughout three cycles of assembly-disassembly were detected. These had estimated SDS-molecular weights of 230,000; 200,000; 130,000; 110,000; 73,000; 55,000; 52,000 and 49,000 daltons. The 55,000 and 52,000 dalton proteins eluted in the unbound fraction i.e. behaved as tubulin, during phosphocellulose chromatography of the copolymer. The 55,000 dalton protein co-migrated with brain α-tubulin on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), however no yeast protein co-migrating with brain β-tubulin was observed. Remaining co-polymerised Saccharomyces proteins bound to phosphocellulose and were eluted with 0.25-0.5M salt. Saccharomyces co-polymer preparations assembled with a reduced critical concentration compared with a control, and contained ring-like structures in depolymerised preparations, which were absent in the control suggesting the presence of yeast MAPs. Similar co-polymerisation was performed using Protomyces inundatus. Differences in putative MAPs were observed compared with Saccharomyces cerevisiae.

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ABBREVIATIONS AND CONVENTIONS

AIB	2-amino-iso-butyric acid
CHO	Chinese hamster ovary (cells)
DEAE	Diethyl aminoethyl
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
EAT	Ehrlich ascites tumour (cells)
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis (β-amino ethyl ether) tetra-acetic acid
HMW	High molecular weight
IEF	Isoelectric focusing
IPC	Isopropyl N-phenyl carbamate
MAPs	Microtubule associated proteins
MBC	Methyl benzimidazol-2-yl carbamate
MTOC	Microtubule organising centre
mol. wt.	molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
PIPES	Piperazine-N,N′-bis (2-ethane sulphonic acid)
PEMG(buffer)	0.1M PIPES, 2mM EGTA, 1mM $MgSO_4$, 1mM GTP, pH 6.9
PMSF	Phenyl methyl sulphonyl fluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPB	Spindle pole body
TEMED	N,N,N′,N′tetra methyl ethylene diamine
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone

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INTRODUCTION

Microtubules are found in all eukaryotic cells, in such diverse structures as cilia and flagella , centrioles, spindles and the motile organelles of various protozoa. They are implicated in a variety of functions ranging from cell motility, secretion and intracellular transport, to structural roles in addition to the part - as yet not fully understood - that they play in cell division and chromosome movement.

Microtubules have been extensively investigated with the result that a great deal is known concerning their structure, protein chemistry, biochemistry and physiology both in vivo and in vitro. However this information has been obtained using material from a limited range of sources. There is a variety of reasons for this, the most important being ease of microtubule purification. Thus most of our knowledge of microtubules and their constituent proteins is that of higher eukaryotic cells, mainly mammalian neural tissue or flagellated organisms.

In every location and cell type microtubules appear to be ultrastructurally similar. They are seen as regular tubular structures of approximately 25nm diameter. Their length is indeterminate, usually measured in microns. The major constituent protein is a globular, acidic molecule - tubulin - which, in all sources examined, exists as two species, or protomers, of similar molecular weight and amino acid composition designated α- and β- tubulin (Bryan & Wilson, 1971; Shelanski, Gaskin & Cantor, 1973). The basic building-block of microtubules is a heterodimer of α and β-tubulin protomers, having a sedimentation coefficient of 6S, and a molecular weight of approximately 110,000 daltons. Microtubules may be assembled in vitro under suitable conditions and a variety of agents, including the anti-mitotic drugs (spindle poisons) are known to interfere with this assembly. Inhibition in vitro may be correlated with observed behaviour of microtubules in vivo.

The microtubule consists of 13 protofilaments composed of tubulin subunits arranged in a parallel fashion along the length of the microtubule. The subunits of neighbouring protofilaments are stacked in a helical manner to form a lattice arrangement which has been investigated using techniques such as optical diffraction and image enhancement of negatively stained electron microscope images, (Amos & Klug, 1974; Amos, Linck & Klug, 1976) and X-ray diffraction, (Cohen et. al., 1975). Both of these techniques give similar results and reveal the microtubule to be composed of globular subunits of 4-5nm diameter, arranged to form a helix with an angle of approximately 10^{o}. Assuming the basic building-block to be a dimer of two 4nm subunits, the structure proposed by Amos for flagellar and cytoplasmic microtubules can be described as a left-handed 8-start helical structure composed of 'tilted dumb-bell dimers'. Both the optical and X-ray diffraction patterns detect differences between the two species of tubulin subunits.

The above lattice structure may differ where microtubules are linked together as doublets e.g. in the outer fibres of cilia and flagella - tubulin subunits in the B subfibre (the incomplete microtubule of the doublet) appear to stack in a different manner to that in the A subfibre (the complete tubule of the pair) which resembles cytoplasmic or isolated microtubule lattice structure (Amos & Klug, 1974).

In addition to the arrangement of tubulin molecules in a microtubule, the optical diffraction technique can detect the arrangement of additional components on the surface in the form of a superlattice. Amos (1977) has interpreted this superlattice as the arrangement of high molecular weight microtubule associated proteins (HMW-MAPs) on the surface of the isolated brain microtubules. These are visible in negatively stained preparations

as fibrillar 'arms' or crossbridges extending from the microtubule surface (McIntosh, 1974; Murphy & Borisy, 1975) in a regular fashion.

That tubulin is a versatile molecule capable of association in more than one manner is demonstrated by the variety of polymeric tubulin aggregates observed both *in vitro* and *in vivo*, induced under various conditions. Such polymeric forms include rings, spirals, sheets and paracrystals (Bensch & Malawista, 1969; Kirschner *et al*, 1974; Larsson, Wallin & Edstrom, 1976).

Microtubules are observed as single tubules as in spindles, axons etc, and also in a variety of association patterns and levels of organisation. Immunofluorescence microscopy reveals microtubules ramifying throughout the cytoplasm of mammalian cultured cells (Weber, Pollack & Bibring, 1975; Weber, 1976) in an apparently random manner. Higher levels of microtubule organisation occur in mitotic and meiotic spindles, through to highly ordered structures such as cilia, flagellae and protozoan axostyles in which numerous microtubules are multiply interlinked (McIntosh, Ogata & Landis, 1973).

Tubulin was identified as a soluble protein present in extracts of most eukaryotic cells by its affinity for the anti-mitotic compound colchicine, (Borisy & Taylor, 1967) and also as a major component of cilia and flagella (Shelanski & Taylor, 1967). Colchicine binding protein was correlated with the presence of microtubules and tubulin was assayed, and finally purified by its colchicine-binding activity (Weisenberg, Borisy & Taylor, 1968) using tritiated colchicine. This purification procedure is based on the selection of tubulin by DEAE-Sephadex chromatography in which the acidic tubulin molecule binds to the ion-exchanger and may be eluted using a salt gradient.

Other anti-mitotic drugs have been used to identify and purify tubulin, in particular the plant alkaloid vinblastine. This compound binds to tubulin (Bryan, 1972; Wilson, 1975) forming paracrystals and precipitates (depending on the concentration used) both in vitro and in vivo (Bensch & Malawista, 1968).

Tubulin isolated in the above manner will not form microtubules in vitro, however assembly competent microtubule protein can be prepared by a reversible temperature-dependent assembly-disassembly procedure (Shelanski, Gaskin & Cantor, 1973; Borisy et.al., 1974). This technique has been applied primarily to vertebrate brain tissue which is a rich source of microtubules.

The conditions necessary to obtain microtubule assembly in vitro from brain extract were described initially by Weisenberg (1972) - assembly is favoured in buffers containing millimolar magnesium ions and a calcium chelating agent such as EGTA to ensure a low calcium-ion concentration. Calcium at millimolar concentrations inhibits microtubule assembly. In addition there is a requirement for guanine triphosphate (GTP) at millimolar concentrations. The optimum pH is 6.9. Under these conditions microtubules form from brain extract when warmed to 37^{o}C and may be collected by centrifugation. Protein collected in this way may be purified by further rounds of assembly-disassembly. There is a variety of published procedures for the preparation of microtubule protein from brain by assembly-disassembly. However, the ionic and buffer conditions are similar in all cases, the only differences being the type of buffer used and the presence, (Shelanski et. al., 1973) or absence (Borisy et. al., 1974) of glycerol in the assembly buffer during the preparation.

Brain tubulin isolated in the above manner is an acidic protein with a native molecular weight of approx. 110,000 daltons comprised of two dissimilar peptide subunits designated α- and β-tubulin. These have similar amino acid composition and molecular weight (Luduena & Woodward, 1975) but may be separated, under suitable conditions, by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bryan & Wilson, 1971) or hydroxyapatite chromatography. β-tubulin has the faster mobility on SDS-gels, the separation being on the basis of charge difference.

Cross-linking experiments (Bryan & Wilson, 1971; Luduena, Wilson & Shooter, 1975) have demonstrated the heterodimeric nature of the 6S tubulin molecule. This is also suggested by the fact that microtubules from most sources studied appear to contain α- and β-tubulin in equal quantities.

The sulphydryl content of tubulin is variable depending on the procedure used to isolate the protein. In the presence of glycerol the -SH groups measured by DTNB (5.5′-dithiobis-(2-nitrobenzoic acid)) titration are approx. 7 per tubulin subunit (55,000 daltons), whereas in the absence of glycerol only 4 groups per subunit are titratable, suggesting that glycerol may have some protecting effects in preventing the oxidation of -SH groups (Kuriyama & Sakai, 1974; Mellon & Rebhun, 1976). Tubulin purified in the presence of glycerol is found to have up to 11 molecules of glycerol irreversibly bound (Detrich et. al., 1976). The tubulin molecule possesses binding sites for calcium and magnesium ions and, in addition, binds two molecules of guanine nucleotides per 6S dimer (Weisenberg & Taylor, 1968). These nucleotide binding sites are separate and distinct. One molecule of GTP/GDP is freely exchangeable with the external medium whilst the GTP at the other site is tightly bound and does not exchange, or does so at a rate only slightly greater than the turnover rate of the tubulin molecule itself

(Spiegelman, Penningroth & Kirschner, 1977). These two sites are termed exchangeable and non-exchangeable (E & N) sites respectively.

Assembly-competent tubulin appears to be associated with a certain amount of enzymic activity. GTP-ase activity (hydrolysis of GTP to GDP at the E-site) during assembly is very low and possibly has no relevance to the mechanism of assembly at least in vitro (vide infra). A broad specificity transphosphorylase activity (nucleoside diphosphate kinase) is associated with tubulin, which is capable of transferring the γ-phosphate from ATP or other nucleoside triphosphates to GDP on the E-site of tubulin. It is uncertain whether this transphosphorylase is a separate protein or an activity of tubulin itself. The position regarding a cyclic AMP-activated protein kinase associated with tubulin is not clear, however the activity persists throughout purification cycles of assembly and shows all the characteristics of a true cyclic AMP protein kinase (Soifer et. al. 1975; Lagnado, Tan & Reddington, 1975). The substrate for the activity is also undetermined - it is variously reported to be β- tubulin (Eipper, 1972) or a HMW-MAP, and the implications of this activity for the control of microtubule assembly in vivo are not certain - no correlation between phosphorylation state and assembly competence of microtubule proteins (either tubulin or MAPs) has been made. The method of tubulin purification possibly determines the phosphorylation state of tubulin and whether it will form a substrate for a phosphorylating activity.

The N-terminal of α-tubulin is the substrate for an enzyme known as tyrosine-tubulin ligase which attaches a single tyrosine molecule to the end of the peptide without the use of transfer RNA, in the presence of ATP (Raybin & Flavin, 1977a). This tyrosylation does not appear to have any specific effects upon assembly in vitro, (Raybin & Flavin, 1977b) but may

have some bearing on development in vivo (Rodriguez & Borisy, 1978; Forrest & Klevecz, 1978).

Microtubule protein purified from mammalian brain tissue by assembly-disassembly cycles consists of 80-90% tubulin and 10-20% other proteins which co-purify, and remain in constant stoichiometry with the tubulin throughout numerous rounds of purification. These are designated microtubule associated proteins (MAPs) and in brain they are comprised of two main groups of proteins - the high molecular weight (HMW) MAPs with SDS-molecular weight of approx. 260-280,000 daltons, and a group of inter-related proteins with SDS-mol wt of 58-70,000 daltons (Borisy et. al., 1975; Weingarten et. al., 1975; Penningroth, Cleveland & Kirschner, 1976; Kirschner, 1977) known as tau proteins. Other proteins which have been proposed as candidates for MAPs are a calcium-dependent regulator protein (CDR) (Welsch et. al., 1978) and a heat-stable protein of approx. 68,000 daltons mol wt, distinct from tau and labelled tubulin-assembly protein (TAP) by Lockwood (1978). However CDR does not appear to co-purify in constant stoichiometry with tubulin throughout multiple rounds of assembly, possibly explaining why microtubules appear to be more sensitive to calcium in crude brain extracts than in purified assembly systems. (Nishida & Sakai, 1977). Recently the work of Lockwood concerning TAP may be questioned after the findings of Runge, Detrich & Williams (1979) that the major 68,000 dalton protein in brain microtubule preparations is the major protein of 10nm filaments, which is heat stable and may have a stimulatory effect upon microtubule assembly after heat treatment.

Brain microtubule assembly has been extensively studied in vitro. The kinetics may be monitored in a variety of ways, the most frequently used being viscometry (Borisy et. al., 1975) and turbidimetry (Gaskin, Cantor &

Shelanski, 1974). Sedimentation and electron microscopy (Kirschner, Honig & Williams, 1975) have also been used. Each method has advantages; turbidimetry follows assembly by the increase in turbidity of the protein solution as microtubules form. It is the most convenient method but it cannot distinguish between a situation with many short microtubules and one with few, long microtubules, neither can it discriminate between true microtubule formation and non-specific aggregate formation. Sedimentation monitors the total polymer mass formed, but again this may include non-specific aggregation. Viscosity measurements are more directly related to the quantity of microtubules and their length but it is a less convenient assay in practice. The only unequivocal way to distinguish microtubule formation from non-specific aggregation or precipitation is by electron microscopy - this is however more difficult to quantitate. Bearing this in mind, differences in assay method may be the cause of quantitative differences between results of various groups of workers.

Microtubules will readily assemble in vitro under the ionic and buffer conditions previously described. Assembly can be initiated by warming a solution of microtubule protein from $4^{o}C$ to $37^{o}C$, or by the addition of 1mM GTP to warm microtubule protein. Microtubule formation is dependent on the quantity of protein above a critical concentration (Co) (Olmsted et. al., 1974).

Pure tubulin, separated from MAPs by chromatography on DEAE-sephadex or phosphocellulose, is capable of assembly into microtubules under artificial (non-physiological) conditions such as the presence of high magnesium ion concentrations, between 10-15mM, (Frigon, Valenzuela & Timasheff, 1974) glycerol between 4-8M, (Shelanski et. al., 1973), 10% dimethyl sulphoxide (DMSO) (Himes, Burton & Gaito, 1977) or polycations such as DEAE-dextran

and RNase A (Erickson & Voter, 1976; Erickson, 1976). However, under such conditions aberrant non-microtubule polymers may be formed e.g. double-walled and macro-tubules.

In more physiological conditions MAPs appear to be indispensible for assembly (Murphy & Borisy, 1975), however the role of the MAPs is not clearly established, especially with regard to the particular functions of the two major classes of associated proteins - the HMW and tau MAPs.

The identity of the HMW-MAPs as filamentous material seen to be associated with microtubules in the electron microscope has been verified - tubulin assembled in the absence of MAPs (as above) do not have filamentous side-arms, (Borisy et. al., 1975; Dentler, Grannett & Rosenbaum, 1975; Slobada et. al., 1976) also, when the HMW-MAPs are digested with trypsin the filamentous projections disappear (Vallee & Borisy, 1977). Amos (1977) reports that the projections are regularly spaced on the microtubule lattice, and occasionally are seen to interact with other microtubules. The HMW-MAPs may be responsible for stabilising the tubulin protofilament structure and/or multi-tubule complexes.

MAPs stimulate tubulin assembly into microtubules in vitro. They affect both the rate and extent of microtubule formation and lower the critical concentration for assembly. In whole microtubule protein (i.e. containing MAPs) this value is 0.2mg/ml whereas purified tubulin will not assemble under these conditions at protein concentrations in excess of 5mg/ml (Borisy et. al., 1975). The kinetics of assembly and the role of the MAPs in stimulation have been studied by Murphy, Johnson & Borisy (1977), (also Johnson & Borisy, 1977) who conclude that MAPs are necessary for the initiation but not elongation of microtubules. However, the fact that tubulin will assemble into true microtubules in the absence of MAPs, albeit in artificial conditions, suggests that MAPs may not be essential. It has

been suggested that the MAPs act in a non-specific way as polycations similar to DEAE-dextran etc.

The status of guanine nucleotides during assembly *in vitro* is unclear, especially with regard to whether GTP hydrolysis is essential for assembly and/or disassembly. Contrary to previous reports, (Borisy *et. al.*, 1975) microtubules will form in the presence of non-hydrolysable analogues of GTP (Arai & Kaziro, 1976) suggesting that GTP hydrolysis is not required. ATP and other nucleoside triphosphates will support assembly due to the presence of the nucleoside diphosphate kinase (transphosphorylase). The literature concerning this subject is confusing, however it is thought that the overall function of nucleotides during assembly is allosteric - the binding of GTP or an analogue at the E-site being necessary to stabilise the tubulin molecule in an active, assembly-competent form. GDP inhibits assembly and may stabilise the inactive form of tubulin. GTP at the N-site does not appear to turn over under any conditions, and it is thought that its role is purely structural (Kirschner, 1977).

The requirement for low calcium concentrations to allow assembly *in vitro* was shown by Weisenberg (1972). Micromolar calcium inhibits microtubule assembly and the inhibition is potentiated by the presence of millimolar magnesium ions - however various workers find quantitatively different results, but differences in the preparation procedure or assay method may be the cause of these discrepancies. The concentrations of calcium and magnesium necessary to inhibit assembly *in vitro* in all cases are higher than might be expected to be found *in vivo*, giving rise to doubts as to the physiological significance of the calcium inhibition as a means of intracellular regulation. However, ignorance of the exact intracellular conditions means that the possibility of this form of regulation cannot be precluded.

Solutions of microtubule protein in the cold contain ring-like tubulin oligomers - the exact morphology of the rings depends upon the method of protein preparation, and these oligomers have been variously described as rings, (Olmsted et. al., 1974) double rings (Gaskin et.al., 1974) or spirals (Kirschner et. al., 1974) with sedimentation coefficients of 30-36S. These rings are not seen in tubulin preparations without MAPs, and their number is proportional to the amount of MAP present (Bloodgood & Rosenbaum, 1976). Various schemes for the mechanism of microtubule formation in vitro have been put forward and are clearly reviewed by Kirschner (1977). Most involve the ring-oligomers as an intermediate in the pathway. Kirschner (1975) proposes that the rings are in fact short spirals, and that on warming these unwind and associate laterally to form sheets and finally short stretches of microtubule on to which 6S dimers can assemble. This pathway is substantiated in part by electron microscopic evidence. The presence of a nucleation step, possibly involving rings is suggested by the lag phase which precedes assembly in vitro. No rings are seen after the end of the lag phase. Rings can be removed from protein preparations by high speed centrifugation (Borisy & Olmsted, 1973) or gel filtration and the remaining 6S tubulin and MAPs will not form microtubules - it will however assemble on to pre-existing microtubule seeds such as flagella fragments. Addition of seeds abolishes the lag phase, suggesting that a nucleation step has been bypassed. It is not clear whether rings are an obligate intermediate in assembly - microtubules form in the absence of MAPs and therefore rings. The in vivo significance is similarly obscure.

It is clear that the method of preparation of microtubule proteins, either by assembly or by various affinity or ion-exchange columns, is likely to affect the assembly characteristics and sensitivity to various

treatments. This is illustrated by the difference in protein properties (-SH groups etc) when isolated in the presence and absence of glycerol. Each preparation procedure may also tend to select a particular subclass of tubulin and/or MAPs. It is known that the glycerol assembly method of Shelanski results in reduced quantities of HMW and tau MAPs.

Microtubule assembly in vivo presents a greater problem of defining the control of the system. Microtubule assembly occurs at precise times during the cell cycle, and in specific locations - control of assembly must therefore be both temporal and spatial.

Calcium and guanine nucleotides have been proposed as major controlling factors, however the intracellular status of these agents is not known. If factors such as these do exert control in vivo, there is a need for considerable compartmentation and specific control signals within the cell. Microtubules in one location may be assembling whilst others elsewhere are dissociating.

Control of tubulin availability by controlled synthesis plays only a minor part in the control of assembly during the cell cycle e.g. during cilia regeneration etc. However there is evidence that the tubulin molecule itself may exert a feedback control over its own synthesis, (Ben Ze'ev, Farmer & Penman, 1979; Weeks & Collis, 1976) switching off transcription in cases where assembly is not occurring and a large pool of unpolymerised tubulin is accumulating.

In most cells microtubules appear to be attached to specific organelles or sites from which microtubules originate and grow. These sites have been termed microtubule organising centres (MTOCs), (Pickett-Heaps, 1975) and they include such structures as basal bodies, centrioles and pericentriolar material,

kinetochores of chromosomes, and spindle pole bodies. Often the MTOC appears to be nothing more than electron-dense areas of cytoplasm such as the pericentriolar material (Gould & Borisy, 1977). Evidence for the MTOC activity of such sites has been obtained from *in vitro* evidence; brain tubulin has been demonstrated to assembly on to kinetochores of isolated chromosomes, (Telzer, Moses & Rosenbaum, 1975; Gould & Borisy, 1978) isolated centrioles and pericentriolar material (Gould & Borisy, 1977) and yeast spindle pole bodies (Hyams & Borisy, 1977). The isolated pericentriolar material of HeLa cells seems to have retained some vestiges of *in vivo* control - it will only initiate the assembly of brain microtubules *in vitro* if isolated from pro- or metaphase cells (Telzer & Rosenbaum, 1979) indicating that there is cell cycle dependent control of microtubule organisation inherent in such MTOCs.

A number of drugs and chemicals which display anti-mitotic activity have been shown to bind specifically to tubulin and/or interfere with microtubule assembly both *in vivo* and *in vitro*. These drugs have played an important role in both the isolation and identification of tubulin and the elucidation of microtubule function.

Colchicine

The best known and most extensively studied of this class of drugs - known as the microtubule poisons - is the plant alkaloid colchicine (Fig. 1). This drug has been shown to inhibit spindle formation and a variety of microtubule-mediated cellular functions such as maintainance of cell shape, motility and secretion. However some microtubule structures within cells appear to be insensitive to colchicine e.g. cilia, basal bodies and centrioles.

In addition to its effects on microtubules, colchicine may affect cell functions apparently unrelated to microtubules and/or mitosis e.g. purine and pyrimidine metabolism, effects on blood clotting and glycolysis in muscle.

Tubulin was isolated and characterised (Borisy & Taylor, 1967) as the macromolecule binding tritiated colchicine in the soluble fraction of a variety of cells, and correlated with the presence of microtubules in these cells.

The binding of the drug to tubulin is stoichiometric, there being one specific binding site per 110,000 daltons i.e. one site per $\alpha\beta$ dimer (Sherline, Leuing & Kipnis, 1975). Binding studies using bromocolchicine - a photoaffinity label and competitive inhibitor of colchicine binding - indicate that the binding site is on the α-subunit of the dimer. (Schmitt & Atlas, 1976). Estimation of the affinity of tubulin for colchicine is rendered difficult by the fact that tubulin is unstable in vitro (Garland & Teller, 1975) especially at elevated temperatures. As colchicine binding is slow and only takes place at temperatures greater than 15-20^{o}C, correction must be made for tubulin denaturation during incubations with radio-labelled drug. Rate constants for association were found to be $k = 0.37 \times 10^{6}$/M/h, and k-1 = 0.009/h; Kd = 5×10^{-7}M. Binding is therefore tight with a half-life of approx. 36 hours (Sherline, Leung & Kipnis, 1975).

Colchicine disrupts spindle microtubules in vivo at concentrations of approx. 1μM or less, and it can also be shown to inhibit microtubule assembly in vitro (Borisy, Olmsted & Klugman, 1972) at 10-100μM. A sub-stoichiometric poisoning mechanism was proposed by Olmsted & Borisy (1973) on observation that the concentration of colchicine needed to half-saturate the available binding-sites was greater than that required to inhibit assembly. Intact microtubules do not bind the drug, (Wilson et. al.,

1974) however assembled microtubules *in vivo* are observed to disappear when treated with colchicine. This may be explained by considering microtubules as a dynamic equilibrium system (Inoué & Sato, 1967) where assembled microtubules are continually being broken down and reformed. Blocking of assembly would bring about eventual disassembly of the structure. However, whilst this may explain the sensitivity of cytoplasmic and spindle microtubules it is more difficult to understand the sensitivity of microtubules in platelets and axopodia. In resistant structures such as cilia, basal bodies etc. where microtubules are highly interlinked, it is possible that no such dynamic equilibrium exists. This is borne out by the fact that such structures are only sensitive to colchicine during their regeneration. Wilson (1975) proposes a mechanism for colchicine action in which assembly is blocked by the addition of a colchicine-tubulin complex on to the growing end of a microtubule thereby preventing further subunit addition. This model assumes a number of things about the mechanism of assembly i.e. that microtubule growth is polar, and that growth occurs by the addition of tubulin molecules singly in a sequential manner. Both of these assumptions have been substantiated experimentally (Margolis & Wilson, 1977, 1978). Moreover, assembly and disassembly have been shown to take place at opposite ends of a microtubule *in vitro*.

Olmsted *et. al.*, (1974) state that colchicine destroys the 30S ring oligomers, however Kirschner (1975) reports that 1mM colchicine does not affect the formation of the 36S ring component of microtubule protein and that the protein in the ring-containing peak from a bio-gel column has a lower affinity for colchicine than 6S protein.

It has recently been reported that MAPs - both HMW and tau - competetively inhibit colchicine - binding to tubulin and may in some way alter the

affinity of the protein for the alkaloid (Nunez et. al., 1979). As rings are thought to sequester MAPs, this may be the cause of lowered colchicine binding to this fraction. Alernatively the association of tubulin in an oligomeric structure such as a ring or microtubule may result in the colchicine site becoming inaccessible, thus reducing its affinity for the drug.

Colchicine binding has been used as a measure of native tubulin in various tissues (Frigon & Lee, 1972; Shelanski et. al., 1973), however there is evidence that the colchicine binding activity of tubulin and the ability to assemble in vitro from extracts are two different properties of the molecule. Barton (1978) finds that on storage of microtubule protein in sucrose, polymerising ability is lost in an exponential fashion whereas the colchicine-binding activity remains at 100% over the same period. A similar situation is found in C_6 glial cell microtubule protein (Wiche, Honig & Cole, 1977). The half-life for polymerising ability and colchicine binding ability were found to be 25 min and 230 min respectively. Their data suggest that the decay of polymerising ability is a reflection of the loss of integrity of an endogenous factor other than tubulin, possibly a MAP.

Sherline, Leung & Kipnis (1975) in their studies of colchicine-binding detected differences in binding between crude brain extracts and purified tubulin preparations. They suggest that this is an indication of the presence of factors in tissues such as brain which may influence the binding of colchicine either by altering the conformation of tubulin, or perhaps by competing with the drug for its binding site on tubulin. This prediction seems to be borne out by the investigations of Lockwood (1979), and independently by Sherline, Schiavone & Brocato (1979) in which endogenous

inhibitors of tubulin-colchicine binding have been isolated from mammalian brain. In both cases the active moiety is a protein or peptide. Such endogenous factors may have some relevance to the control of the polymerisable pool of tubulin *in vivo*.

Podophyllotoxin

Podophyllotoxin is also a complex plant alkaloid (Fig 2) which demonstrates similar effects on mitosis and microtubule assembly *in vitro* to those of colchicine. Podophyllotoxin competes with colchicine in binding to tubulin (Pfeffer, Asnes & Wilson, 1976) suggesting that these two drugs share the same binding-site on the tubulin molecule. However the mechanism of drug binding is different from that of colchicine - podophyllotoxin binds rapidly in a non-temperature-dependent manner (Wilson *et. al.*, 1975) with an association rate constant (k) ten times greater than that of colchicine. One mole of podophyllotoxin is bound per 110,000 daltons (Cortese, Bhattacharyya & Wolff, 1977). Effects of this drug *in vivo* are not restricted to microtubule poisoning effects (c.f. colchicine) e.g. nucleoside uptake has been shown to be affected in HeLa cells (Loike & Horowitz, 1976).

Catharanthus (Vinca) alkaloids

This group of compounds, also derived from extracts of plants, exhibit anti-mitotic activity *in vivo* similar to that shown by colchicine but generally at much lower concentrations and the effects are more persistant. These properties have made them useful drugs in the treatment of cancer. The two most important molecules in this group are vinblastine (Fig 3) and vincristine, both of which are used clinically.

Brain microtubule assembly *in vitro* is prevented by vinblastine concentrations of approx. 0.4μM, a concentration which correlates well with the effective concentration *in vivo* (Owellen *et. al.*, 1976). The kinetics of assembly inhibition *in vitro* are similar to those exhibited during colchicine inhibition.

At higher vinblastine concentrations i.e. 1-10μM, birefringent crystalline structures are formed in some cells e.g. sea urchin eggs (Bensch & Malawista, 1969). These crystals are composed of α- and β- tubulin subunits in equal quantities (Marantz, Ventilla & Shelanski, 1969) and this phenomenon has formed the basis of a purification method for tubulin. The protein in vinblastine paracrystals appears to be exceptionally stable (Bryan, 1971).

At even higher concentrations vinblastine will precipitate acidic proteins, including tubulin, from solution. However Wilson (1970) has attributed this to non-specific cation-like precipitation as similar patterns of protein may be precipitated by calcium ions.

Vinblastine and vincristine are reported to affect pre-formed microtubules, forming aberrant macro-tubule and spiral structures when treated with drug at 1-10μM. MAPs have been implicated in this activity (Donoso, Haskins & Himes, 1979).

Vinblastine binds specifically to tubulin at a site distinct from the colchicine site, (Bryan, 1972) one molecule of drug being bound per tubulin dimer. Vinblastine-induced tubulin crystals bind colchicine as efficiently (or more so) than intact, free tubulin, possibly due to the stabilizing effect of vinblastine on the tubulin molecule. Detailed experiments on the binding of the vinca alkaloids to tubulin have revealed two binding sites for these drugs on brain tubulin - a high affinity site corresponding to the concentration of drug necessary to prevent assembly; and a low

affinity site corresponding to that which is needed for non-microtubule aggregation including paracrystal formation (Bhattacharyya & Wolff, 1976).

At low concentrations, where the effects of vinblastine are solely on microtubule assembly, it is thought that the mechanism of inhibition by this drug is similar to that of colchicine i.e. a sub-stoichiometric poisoning of microtubule growth by capping of the growing end by a drug-tubulin complex (Wilson et. al., 1975).

Benzimidazole carbamates

The benzimidazole carbamate compounds are a commercially important group of microtubule poisons. Different derivatives have characteristic spectra of target organisms e.g. the group includes fungicides (Benomyl, Methyl benzimidazol-2-yl carbamate (MBC) (Fig 4) and Thiabendazole (Fig 5)) also anthelmintics (Mebendazole, Parbendazole etc).

MBC inhibition of fungal mitosis appears to be of the colchicine type i.e. pro-metaphase arrest of the cell-cycle, however it is approx. one thousand times less effective than colchicine on brain microtubule assembly in vitro. Other derivatives such as parbendazole and oncodazole (Fig 6) are strong inhibitors of brain microtubule assembly in vitro.

Mebendazole and oncodazole competetively inhibit colchicine binding to brain tubulin (Ireland et. al., 1979; Hoebeke, van Nijen & De Brabander, 1976; Friedman & Platzer, 1979) therefore benzimidazole carbamate compounds exert their various effects by interaction with tubulin in a similar way to colchicine. However there appears to be no strict correlation between anthelmintic activity and inhibition of brain microtubule assembly in vitro.

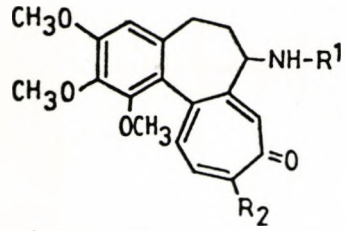
Griseofulvin

All of the previously mentioned anti-mitotic drugs exert their effects by interaction with tubulin. There is evidence that griseofulvin does not act in this way and may be a novel type of microtubule inhibitor.

Griseofulvin is an antifungal antibiotic which is widely used in medicine as a treatment of dermatophytic fungal infections. It has been shown to disrupt cellular functions involving microtubules including mitosis in fungi (Gull & Trinci, 1973; 1974), also cilia regeneration during differentiation in the protozoan Stentor coeruleus (Margulis, 1975). Mitosis in many tissues is sensitive to griseofulvin including fibroblasts (Adair, 1974), fungi (Gull & Trinci, 1973) and slime moulds (Gull & Trinci, 1974). In all cases mitosis is reversibly inhibited. Griseofulvin appears to be less effective than colchicine where both drugs have effects on mitosis e.g. in mouse 3T3 cells (Weber, Wehland & Herzog. 1976).

Griseofulvin has been shown to inhibit brain microtubule assembly in vitro (Roobol, Gull & Pogson, 1976) contrary to previous reports (Wilson & Bryan, 1975). Concentrations of 10-100μM inhibit both rate and extent of assembly and in addition substantially lengthen the lag phase preceding assembly. This result suggests that this drug may interact with an initiating factor i.e. MAPs. Specific binding to MAPs has been reported (Roobol, Gull & Pogson, 1977a, Grisham, 1976; Nardi, 1976). On addition of griseofulvin to microtubule protein in the cold a non-microtubule aggregate is formed which consists of approx. 90% of the MAPs originally present in the microtubule protein (Roobol, Gull & Pogson, 1977b).

Griseofulvin is therefore a novel microtubule inhibitor, being the only substance so far described which has as its target the MAPs rather than the tubulin.



$R^1 = CO\text{-}CH_3$ $R_2 = O\text{-}CH_3$

Fig. 1 Colchicine

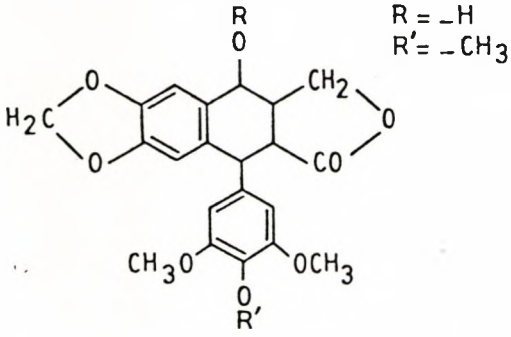


Fig. 2 Podophyllotoxin

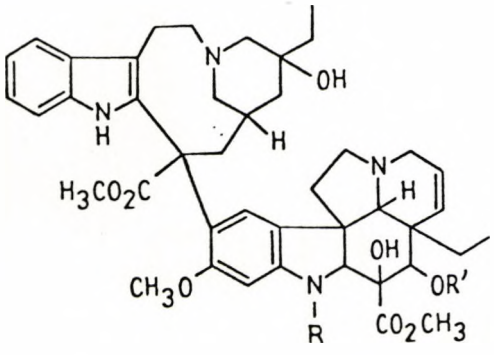


Fig. 3 Vinblastine

VBL: $R = -CH_3$ $R' = -CO-CH_3$
VCR: $R = -COH$ $R' = -CO-CH_3$

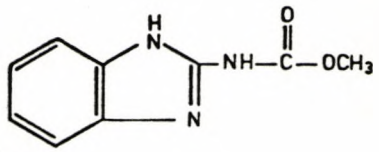


Fig. 4 Methyl benzimidazol-2-yl carbamate (MBC)

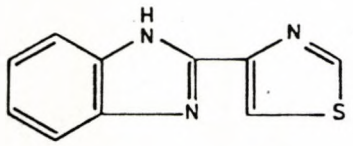


Fig. 5 Thiabendazole

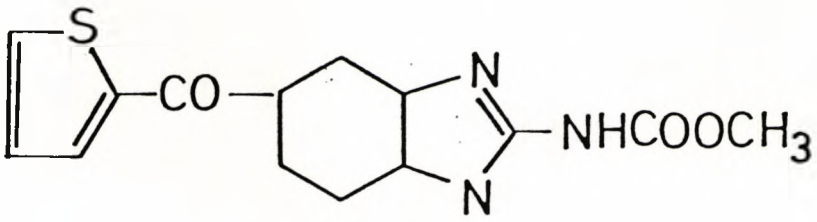


Fig. 6 Oncodazole

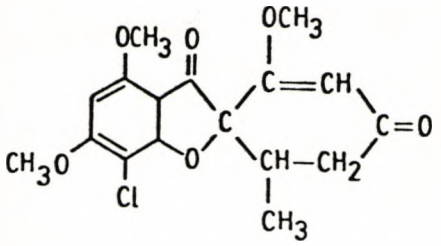


Fig. 7 Griseofulvin

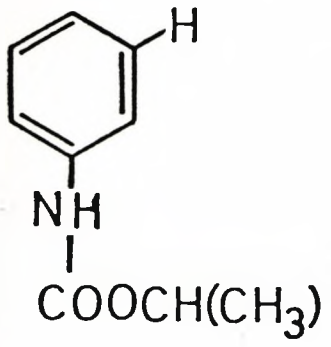


Fig. 8 Isopropyl N-phenyl carbamate

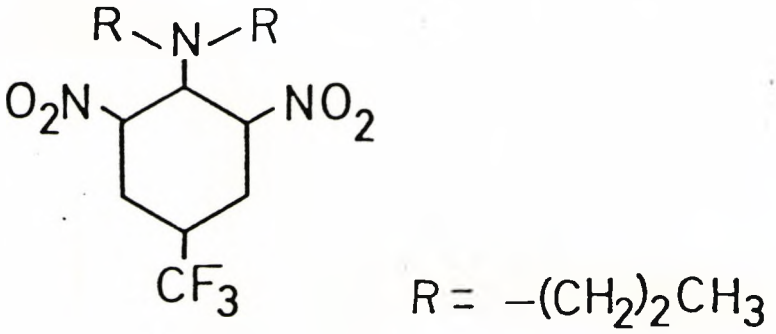


Fig. 9 Trifluralin

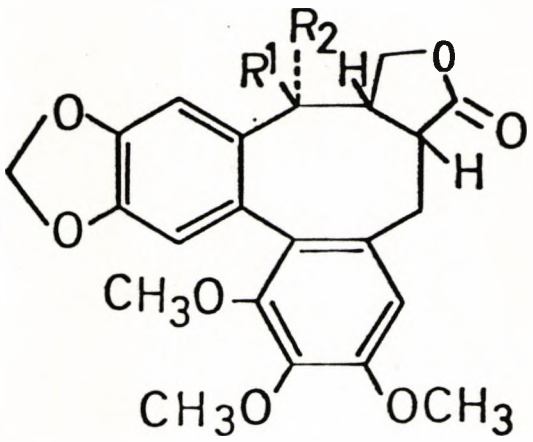


Fig. 10 Steganacin

$R^1 = OCOCH_3$

$R_2 = H$

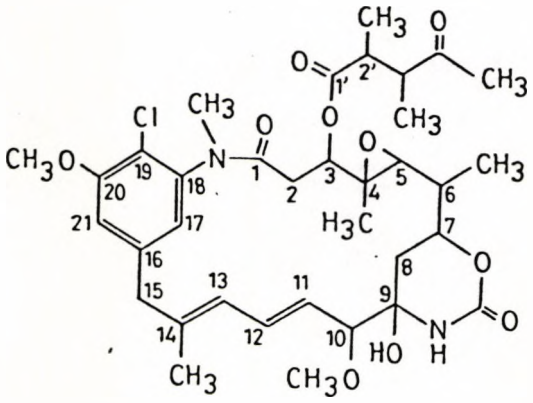


Fig. 11 Maytansine

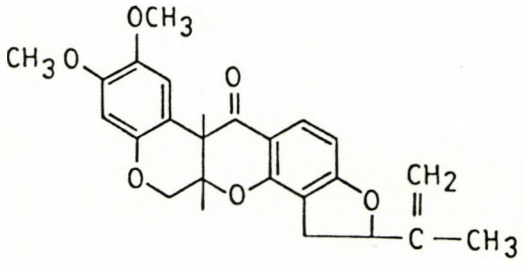


Fig. 12 Rotenone

A variety of other chemicals and antibiotics are known to have anti-mitotic and anti-microtubule effects *in vivo* but their effects *in vitro* and tubulin binding characteristics are not so well characterised as those of colchicine and vinblastine. Such drugs include compounds such as the herbicides Trifluralin (α, α, α-trifluoro-2, 6-dinitro-N, N-dipropyl-p-toluidine) (Fig 9) and IPC (Isopropyl-N-phenyl carbamate) (Fig 8). Neither of these compounds affects brain microtubule assembly *in vitro* or binds to brain tubulin (Bartels & Hilton, 1973). They do, however, exhibit anti-mitotic activity in lower eukaryotic organisms and plants (Banerjee & Margulis, 1969; Banerjee, Kelleher & Margulis, 1975; Margulis, Banerjee & Kelleher, 1976), and bind specifically to tubulin from lower eukaryotes e.g. *Chlamydomonas* (Hess & Bayer, 1977). Such differences in species specificity may reflect differences in tubulins from different sources.

Maytansine is an ansa macrolide similar in structure to the antibiotic rifamycin (Fig 11). It is a very powerful anti-microtubule agent *in vivo*, inhibiting mitosis in sea urchin eggs at 0.06μM (Remillard *et. al.*, 1975). It is slightly less active than colchicine *in vitro*.

Other molecules affecting microtubules either *in vivo* or *in vitro* are substances such as the respiratory inhibitor rotenone (Fig 12) (Barham & Brinkley, 1976) and a lactone steganacin, (Fig 10), another colchicine-like inhibitor which is reported to bind to the same site on tubulin (Wang, Rebhun & Kupchan, 1977).

The major evidence for tubulins being identical in all organisms comes from data on partial sequencing, immunology and co-polymerisation. Partial sequence analysis has been performed on tubulins from chick brain and sea urchin sperm flagella and shows a close correlation in the first

25 N-terminal amino acids, but by far the largest body of evidence in favour of tubulin being a highly conserved protein comes from antibody studies. Dales (1972) finds that antibodies to tubulin from vinblastine paracrystals will cross-react with tubulins from a wide range of organisms, from human tissue-culture cells to turtles, snakes and diptera - as tested by immunofluorescence staining. Antibodies to sea urchin sperm will react with mammalian cytoplasmic and spindle tubulin and vinblastine paracrystals (Weber, 1975). However, the fact that one can prepare antibodies to tubulins from different organisms means that there must be differences in antigenic determinants between species.

More evidence is appearing suggesting that tubulins and microtubule systems may not be universally identical, especially with respect to lower organisms. Most of this evidence is indirect i.e. from drug binding and sensitivity data and immunology, however the development of more sensitive protein analysis techniques e.g. two-dimensional gel-electrophoresis and peptide mapping means that small differences in proteins may be detected.

The knowledge gained by the study of brain microtubules has been applied to non-neural tissues, and microtubule protein has been isolated in an assembly-competent form i.e. by cycles of assembly-disassembly *in vitro* from other higher eukaryote tissues, namely platelets (Castle & Crawford, 1975), and a variety of mammalian cell lines including HeLa cells (Bulinsky & Borisy, 1979; Weatherbee, Luftig & Weihing, 1978), Ehrlich ascites tumour (EAT) cells (Doenges, *et. al.*, 1977), mouse 3T3 fibroblasts (Weber *et. al.*, 1977; Wiche, Lundblad & Cole, 1977), C_6 glial cells (Wiche & Cole, 1976; Wiche, Honig & Cole, 1979), neuroblastoma and Chinese hamster ovary (CHO) cells (Nagle, Doenges & Bryan, 1977).

In most cases the tubulin isolated by assembly appears to be identical to brain tubulin when analysed by the usual criteria including one-dimensional PAGE, however recently Doenges et. al. (1979) have obtained evidence for an additional band on SDS-gels between the α- and β-tubulin bands from EAT cells. They find a similar band, with a slightly different mobility, in their brain tubulin preparations. This same group also finds that assembly of EAT microtubule preparations in vitro is insensitive to calcium ions - the presence of EGTA in preparation buffers being unnecessary (Doenges, 1978). Assembled microtubules are not depolymerised by calcium.

Where such cell-line microtubule preparations differ from brain preparations is in the MAP content. Only in C_6 glial cell preparations are HMW proteins found that may correspond to the HMW brain MAPs on SDS-gels (Wiche & Cole, 1976). Nagle et. al. (1977) find the major MAP in their material from neuroblastoma, C_6 and CHO cells to be a protein of about 49,000 daltons (SDS mol wt). This can also be seen in EAT preparations, along with other proteins co-purifying through two cycles of assembly-disassembly. Such proteins have SDS-molecular weights of about 90,000, 70,000, 45,000 and 30,000 daltons (Doenges et. al., 1977).

In HeLa cells the species of MAPs obtained appears to depend upon whether glycerol is present in the assembly buffers. Weatherbee et. al. (1978) find the major associated protein in their preparations obtained in the absence of glycerol to be a protein migrating with a mol. wt. of approx. 68,000 daltons. However, in the presence of glycerol this protein is very much reduced. In similar work using HeLa cells Bulinski & Borisy (1978) find associated proteins of 210,000 and 120,000 daltons remaining with the tubulin throughout four cycles of assembly-disassembly in the absence of glycerol. In this system the 68,000 dalton protein fails to

co-purify. Recently, evidence has been obtained that a 10nm filament protein, which has a mol. wt. of approx. 68,000, may associate with microtubules during their purification (Runge, Detrich & Williams, 1979) under certain conditions, and that this protein may stimulate assembly of microtubules, thus mimicing the activity of a true MAP. Bulinsky & Borisy (1978) discuss the criteria for the identification of a true MAP.

Microtubule preparations from cell lines have been reported to contain few (Wiche et. al., 1979), or no rings (Doenges et. al., 1979) in cold depolymerised protein. This may be a reflection of quantitative or qualitative differences in MAP content, or a characteristic of the tubulin. In any case it is by no means certain that rings are an obligate intermediate in assembly.

Polymerising ability of cell-line microtubule protein appears to decay more rapidly than brain preparations even when care is taken to abolish any proteolytic activity (Wiche et. al., 1977). This may be a feature of tubulin or of the MAPs.

In all of the cell-lines and non-neural tissues studied, tubulin has been estimated to comprise 2-5% of the soluble proteins of the cell, and this is the major factor which has enabled the assembly-disassembly purification protocol to be applied to these cells. This is not the case with the lower eukaryotes where tubulin seems to be a much smaller proportion of cellular proteins. However, the flagellated and ciliated organisms have received some attention as cilia and flagella are relatively easy to obtain. In addition flagellar synthesis is frequently inducible at specific points of the cell cycle, enabling tubulin synthesis from messenger RNA, and its control, to be studied using in vitro translation systems.

Olmsted et. al. (1971) have compared porcine brain and Chlamydomonas flagellar tubulin on SDS-urea-PAGE and show that only one tubulin is common

to both sources - the β-tubulin. The α-tubulins from brain and Chlamydomonas flagella differ in their electrophoretic mobility under these conditions. Further analysis by iso-electric focusing (IEF) on urea-acrylamide gels resolved the Chlamydomonas outer fibre tubulin into at least 5 bands (Witman, Carlson & Rosenbaum, 1972). The dissimilarity between Chlamydomonas and brain α-tubulins is borne out by work of Piperno & Luck (1977) who combined SDS-PAGE with 'rocket' immunoelectrophoresis in the second dimension. Results show that antiserum to Chlamydomonas β-tubulin cross-reacts with brain protein, but brain α-tubulin antiserum will not react with Chlamydomonas tubulin thus confirming a difference in the α-tubulins from these two sources. It is interesting to note that on the SDS-PAGE system used in this work there appears to be no migrational difference between the α-tubulins of brain and Chlamydomonas as is seen on the SDS-urea gels of Olmsted et. al. (1971). Piperno & Luck report the separation of the α-tubulin subunit of Chlamydomonas flagella into 'at least' 5 components by IEF-PAGE, of which the major and two minor components were phosphorylated. In contrast to the above results, Weeks, Collis & Gealt (1977) have studied the products of in vitro protein synthesis after experimental deflagellation of Chlamydomonas. They found that proteins were induced which both co-polymerised and co-migrated with chick brain tubulin in their SDS-PAGE system.

The flagella of sea urchin sperm (Strongylocentrotus purpuratus) have been extensively investigated. Stephens (1978) has detected primary structural differences between tubulins from central pair, A and B subfibres of sperm flagella . These components also differ in their solubility properties, however this may be a result of differences in inter-tubule bonding etc. Stephens' results indicate that multiple types of α- and

β- subunits may exist in flagellar outer fibres, and in addition that the tubulins of flagella , cilia and vinblastine-induced crystals of cytoplasmic tubulin of this organism differ in their peptide maps. Kobayashi & Mohri (1977) resolve the α- and β-tubulins from starfish Asterias amurensis flagellae into at least 4 bands each on IEF gels c.f. the work of Weeks & Collis with Chlamydomonas.

Bibring et. al. (1976) also find that tubulins from flagellae, cilia and cytoplasm (in this case the mitotic apparatus) in sea urchins differ, specifically in their α-subunits. They have also shown that the resolution of multiple tubulins on PAGE depends, to a large extent, upon the system employed i.e. they find that a urea-SDS polyacrylamide system resolved the tubulin into multiple bands better than SDS or urea-acrylamide alone. This emphasises that PAGE evidence (especially in only one dimension) should be interpreted with caution, and that it is not possible to compare results analysed on different gel systems.

From this work, it appears that tubulin or tubulin subunits may be specific for a particular organelle. Further evidence for this hypothesis comes from the antibody work of Kowit & Fulton (1974) on the slime mould Naegleria gruberi. This organism undergoes a transformation from an amoeboid to a flagellated form in poor nutrient conditions. Despite the presence of a large tubulin pool in the amoebae it was found that flagellar tubulin was synthesised de novo during differentiation. In addition, antibodies to the flagellar protein synthesised did not cross-react with cytoplasmic tubulin from the same organism or with any other species tested including Tetrahymena cilia. Fulton & Simpson (1976) evaluate the literature and propose a 'multi-tubulin hypothesis' which suggests that in an organism, or even within a single cell, there exist different tubulins, produced by

different genes and different in primary structure, used to assemble specific organelles and microtubular structures. This implies a multiplicity of tubulin pools within a cell with equilibrium within each one but not between different pools. Their hypothesis also predicts that the lower eukaryotes should show this phenomenon more clearly but that throughout evolution tubulin has become more conserved, thus less differences would be expected between the tubulins of higher eukaryotes.

Cytoplasmic tubulin from lower eukaryotes has proved more difficult to isolate, and in some cases even to identify. Various purification methods have been successful with certain ciliated organisms e.g. Tetrahymena and eggs or embryonic stages of starfish and sea urchins where large tubulin pools mean that tubulin comprises a reasonably large proportion of soluble cell proteins.

In Tetrahymena pyriformis (Maekawa & Sakai, 1978) tubulin is estimated to be approx. 2% of a soluble protein extract and could be purified by DEAE-Sephadex chromatography and ammonium sulphate precipitation. However the tubulin obtained in this way was unable to assemble into microtubules in vitro unless ciliary outer fibre doublets were added as seeds. When examined by SDS-PAGE, no difference was obvious between cytoplasmic and ciliary tubulin from the same organism, however on reduction and carboxy-methylation treatment of samples brain and cytoplasmic Tetrahymena tubulin were observed to behave differently on SDS-gels - the β-tubulins of brain and Tetrahymena co-migrated, but Tetrahymena α-tubulin had a faster mobility than that of brain. Tetrahymena tubulin could be co-polymerised with brain microtubules, however in the heterologous assembly system the DEAE tubulin fraction of Tetrahymena appeared to inhibit the rate but not the extent of brain microtubule assembly as measured by viscometry.

Tubulin has been isolated from sea urchin and starfish eggs (Kuriyama, 1977) by DEAE - Sephadex chromatography followed by cycles of temperature dependent assembly-disassembly. The protein obtained was observed to co-migrate on SDS-PAGE with porcine brain and sea urchin flagellar tubulin, and behave in a similar fashion to brain microtubules during assembly in vitro.

The anti-microtubule drugs, in particular colchicine, have been widely used as a tool to identify and purify tubulin from mammalian and other higher eukaryote tissues. However there is evidence that many lower eukaryotes, especially micro-organisms, are considerably less sensitive to colchicine. The concentraion of colchicine necessary to inhibit cell division in various tissues and organisms is shown in table 1. These differences in sensitivity appear in most cases to be due to the insensitivity of the microtubule systems i.e. uptake phenomena have been discounted.

Colchicine-stable microtubules are a feature of many cells e.g. cilia and flagella of mammalian cells are not disassembled by the colchicine concentration which inhibits mitosis. In this case the increased stability is probably caused by inter-tubule cross-bridging resulting in a lack of turnover of tubulin in these organelles. Cilia and flagella are sensitive to anti-microtubule drugs during their regeneration after experimental deflagellation or during differentiation.

Cytoplasmic tubulin of Tetrahymena has been shown to have a very low affinity for colchicine (Maekawa, 1978) - the dissociation constant (Kd) for the colchicine-tubulin complex being 2.7×10^{-3}M compared with 2.0×10^{-7}M for porcine brain tubulin. This explains the high concentrations of the drug necessary to block both cell division and cilia regeneration in Tetrahymena.

Table 1

Tissue/organism	Colchicine conc. to inhibit mitosis	Reference
Mammalian cells	0.1μM	Taylor 1965
Pea roots	0.4mM	
Chlamydomonas reinhardii	5 mM	Adams & Warr 1972
Tetrahymena pyriformis	12.5mM	Rosenbaum & Carlson 1969 Wunderlich & Speth 1970
Schizosaccharomyces pombe	100 mM	Lederberg & Stetton 1970
Saccharomyces cerevisiae	50 mM	Haber *et. al.* 1973

Binding of [^{3}H]-colchicine in cell extracts has frequently been used as a diagnosis of the presence and quantity of tubulin in cells. Burns (1973) found no colchicine binding in post-ribosomal supernatants from a variety of organisms and tissues including Schizosaccharomyces pombe, Chlamydomonas reinhardii and Tetrahymena pyriformis. Flanagan & Warr (1977) however, report low levels of colchicine binding in extracts of Chlamydomonas which shows some of the characteristics of binding to tubulin i.e. non-competition by lumicolchicine or vinblastine, competition by admittedly very high concentrations of podophyllotoxin. The discrepancy between this work and the data of Burns regarding Chlamydomonas may be the result of differences in binding assay used; Burns used DEAE-cellulose columns, eluted with an NaCl gradient, whereas Flanagan & Warr used a more rapid filter-disc method using triethylaminoethyl-cellulose (TEAE-cellulose).

It is interesting to note that despite the earlier finding, in a later publication Farrel & Burns (1975) isolated a tubulin-like protein from Chlamydomonas using a colchicine affinity column after DEAE-cellulose chromatography of extract. However this protein was unable to assemble into microtubules in vitro, even in the presence of seeds, and was apparently unable to co-polymerise with gerbil-brain microtubules in vitro. (N.B. no precautions were taken to eliminate any possible proteolytic or other inhibitory activity from the preparation.)

Further evidence of the difference between Chlamydomonas tubulin and that of mammalian brain comes from data concerning the anti-microtubule herbicide trifluralin. This compound does not bind to brain tubulin or inhibit brain microtubule assembly in vitro (Bartels & Hilton, 1973), but it is, however, very active in inhibition of mitosis and flagellar regeneration in Chlamydomonas (Hess, 1979). Trifluralin inhibits flagellar regeneration at concentrations of 3.75 - 5μM, whereas colchicine concentrations

of 2.5 - 3.75mM are necessary to achieve similar effects, also trifluralin has been shown to bind specifically to Chlamydomonas flagellar central pair tubulin (Hess & Bayer, 1977). Trifluralin has been shown to be ineffective against animal cell mitoses, but active in blocking mitosis in plant tissues e.g. 0.3μM is sufficient to disrupt cell division in Haemanthus (blood lily) endosperm (Jackson & Stetler, 1973), whereas colchicine at 0.1mM is necessary to achieve a similar effect (Heppler & Jackson, 1969). This situation is reversed in the case of animal cell mitosis, colchicine being effective against HeLa cells at 0.1μM (Taylor, 1975) whereas trifluralin has no effect at concentrations greater than 10μM which is the limit of solubility (Hess & Bayer, 1975).

Trifluralin has been claimed to be an anti-microtubule drug specific for plant microtubules, however, the drug has effects which suggest that other lower eukaryotes may be included within its spectrum of activity e.g. it is by far the most effective drug of all the anti-microtubule agents tested in delaying cilia regeneration during oral differentiation in the ciliate protozoan Stentor coeruleus (Banerjee, Kelleher & Margulis, 1975).

A similar situation exists in the case of IPC (Isopropyl-N-phenyl carbamate), also a herbicide with complex actions on dividing plant cells. It has been shown to disrupt mitosis in Haemanthus katherinae (Heppler & Jackson, 1969), and decay cilia regeneration in Stentor (Banerjee & Margulis, 1969) whilst not effecting brain microtubule assembly in vitro, nor binding to brain tubulin (Bartels & Hilton, 1973).

The effect of various drugs on Chlamydomonas flagellar regeneration illustrates clearly the sensitivity of lower eukaryotic systems to a different spectrum of anti-microtubule agents; colchicine inhibited regeneration at 4mM, colcemid at 0.7mM, vinblastine at 0.1mM and IPC at 0.3mM.

Tubulin has been identified from the slime mould Dictyostelium discoideum (Cappuccinelli, Martinotti & Hames, 1978) by co-polymerisation with porcine brain, and appears to be very similar in SDS-PAGE behaviour, however there is evidence from their immunological data that there may not be complete homology - small spurs are detectable on the immunoprecipitin lines on immunodiffusion plates. The growth and development of Dictyostelium is sensitive to colchicine at all stages of its life-cycle, in contrast to a similar organism Physarum polycephalum. This slime mould appears to be resistant to the anti-mitotic effects of colchicine, podophyllotoxin and maytansine in both the plasmodial and amoeboid stages of the life-cycle, however oncodazole, MBC and griseofulvin are all effective, oncodazole being the most potent, inhibiting the growth of amoebae at 10uM. MBC and griseofulvin are effective at 50 and 100uM respectively (Mir & Wright, 1978). It has been reported that colchicine does not bind to extracts of Physarum (Jokush, Brown & Rusch, 1971). Recently microtubule protein has been isolated and assembled in vitro from myxamoebae of Physarum (A. Roobol, this laboratory, unpublished data), confirming that assembly is not inhibited by colchicine at 100uM, nor does colchicine bind to amoebal tubulin.

In contrast to the flagellated lower eukaryotes e.g. Tetrahymena, sea urchins, Chlamydomonas etc, the non-flagellates, in particular the fungi, have received very little attention with respect to their microtubule proteins. There is evidence, mostly from work involving anti-microtubule drugs which suggests that the micrtotubules of such organisms may differ substantially from those of higher eukaryotes.

The antibiotic griseofulvin has been recognised as an antifungal agent for many years (Brian, Curtis & Hemming, 1949), however its site of action remained undetermined. Recently it has been shown that griseofulvin

inhibits mitosis in fungi (Gull & Trinci, 1973) and also that it is an inhibitor of brain microtubule assembly *in vitro* (Roobol, Gull & Pogson, 1976; 1977a) of a novel nature, binding specifically to brain MAPs rather than to tubulin. The target molecule(s) of this drug in fungi has not been determined, however it has been reported that the greater sensitivity of dermatophyte fungi compared to yeasts is due to differential uptake into dermatophytes (El-Nakeeb & Lampen, 1965a). These authors also report that griseofulvin is selectively bound to nucleic acids (El-Nakeeb & Lampen, 1965b), a report contradicted by Huber & Gottlieb (1968). Griseofulvin literature has been extensively reviewed up to 1967 (Bent & Moore, 1966; Huber, 1967).

Cell division in fungi and yeasts does not appear to be sensitive to colchicine, and no colchicine-binding activity has been detected in soluble protein extracts (Burns, 1973; Haber *et. al.*, 1972; Heath, 1975). Yeast are insensitive to colchicine but sensitive to a colchicine analogue colcemid (desacetyl-N-methyl-colchicine) (Haber *et. al.*, 1972; Lederberg & Stetton, 1970), however relatively high concentrations i.e. 2-10mM are required to inhibit yeast growth, compared with the colchicine concentration which blocks mammalian cell mitosis (approx. 0.1μM). Uptake of both colchicine and colcemid is equivalent in *Saccharomyces cervisiae*. Affinity constants for yeast extract protein were determined for colchicine at 4×10^2 1/mol, and colcemid at 5×10^3 1/mol (Haber *et. al.*, 1972). A colcemid-binding activity could be identified by DEAE-sephadex fractionation, eluting at 0.5M KCl - conditions diagnostic for brain tubulin in this system. Tubulin content of *Saccharomyces cerevisiae* was estimated, in this paper, at 0.2 - 0.4% of the soluble proteins.

Of the mycelial fungi *Aspergillus nidulans* has been most thoroughly studied. The growth of this fungus is sensitive to the fungistatic

benzimidazole compounds Benomyl and its hydrolysis product MBC (Davidse, 1973), also to Thiabendazole (Davidse & Flach, 1978). The intracellular binding of MBC in sensitive and resistant mutant strains of the organism has been studied by Davidse (1975; 1977). The MBC-binding protein was identified as a protein similar to brain tubulin as determined by electrophoretic analysis. Binding constants for the MBC-tubulin complex was estimated to be 4.5×10^5 1/mol in a wild-type strain, and much lower i.e. 3.7×10^4 1/mol in an MBC-resistant mutant. Super-sensitive strains gave a value of 1.6×10^6 1/mol. MBC binding was competitively inhibited by oncodazole.

MBC-binding activity was partially purified by DEAE-sephadex chromatography and analysed by PAGE under a variety of conditions. Two proteins with similar mobilities to brain tubulin on SDS-gels were identified from wild-type and MBC super-sensitive strains, whereas these proteins were obtained in markedly reduced quantities, using the same procedure, from resistant strains.

Further evidence that the MBC-binding protein is in fact a microtubule component protein, was obtained by studying growth of diploids containing wild-type and resistant characteristics. Colonies of such diploids showed a high level of sectioning on solid media - such behaviour is diagnostic of chromosome non-disjunction during cell division due to impaired spindle function/assembly. MBC-binding proteins were capable of co-polymerisation with brain microtubules, despite the differences in drug sensitivity.

Aspergillus has been studied in greater detail by Morris's group. Proteins co-polymerising with chick-brain microtubules were identified by Sheir Neiss et. al. (1976). A protein co-migrating with α-tubulin was

identified, but no protein corresponding to brain β-tubulin was present; however a protein in the co-polymer with a greater mobility than brain β-tubulin was identified from Aspergillus. Isoelectric focusing with double-labelled material gave a similar result in that one Aspergillus protein co-electrophoresed with brain tubulin, but other proteins from brain and Aspergillus had dissimilar isoelectric points.

Sheir-Neiss, Lai & Morris (1978) have used Benomyl-resistant strains of Aspergillus (similar to those used by Davidse) and IEF/SDS two-dimensional PAGE to identify a gene for β-tubulin in this fungus. In the two-dimensional system four α- and four β-tubulin spots are identified, all of which co-polymerise with brain microtubules. In benomyl-resistant strains, two of the four β-tubulin spots are altered in mobility in the IEF dimension, indicating a difference of one charge unit. The α-tubulins were normal. Post-translational modification of these β-tubulins was ruled out by analysis of diploids of wild-type and resistant genes, which possessed both normal and altered β-tubulins.

The gene for α-tubulin in Aspergillus has been identified in a similar manner (Morris, Lai & Oakley, 1979) using supressor mutations of benomyl resistance in which the major α-tubulin was altered, as determined by two-dimensional PAGE. Thus a mutation in the α-subunit compensates for a change in the β-tubulin during supression of benomyl resistance in Aspergillus. These findings, in addition to suggesting that fungal tubulin may be different from mammalian brain protein, provoke interest in the association of α- and β-tubulin subunits in both native microtubules and co-polymers in vitro - this will be discussed later.

This work on Aspergillus indicates that this fungus possesses multiple forms of tubulin. There is also evidence that multiple forms of α- and β-tubulins exist in brain and mammalian neural tissue. Lu & Elzinga

(1977) report the separation of DEAE-purified tubulin into three species, α_1, α_2 and β-tubulin, on one dimensional SDS or Urea-acrylamide gels, α_1 and α_2 being indistinguishable by amino acid analysis.

Marotta, Harris & Gilbert (1978) using IEF/SDS two-dimensional PAGE resolve brain α_1 and α_2 into a further three components each, representing acidic and basic forms, and in addition they detect a second minor β-subunit. In a subsequent publication (Marotta, Strocci & Gilbert, 1979) they provide evidence that the pattern of the subunit forms varies depending on the source of material. An additional α-subunit, α_3, is detected in synaptosomal tubulin and identified as a tubulin by radio-iodinated tryptic peptide mapping.

It would seem that Fulton's predictions concerning multiplicity of tubulins in lower but not in higher eukaryote systems are not fulfilled, however the question arises as to whether multiple forms of tubulin subunits separated by two dimensional gel analysis are, in fact, separate gene products. Considering the work with Aspergillus where alteration in only two β-tubulins out of four results in benomyl-resistance, if each tubulin were a separate, functional gene-product an explanation of drug inhibition of assembly would be very complex. The alternatives are (i) that the multiple forms of α- and β-tubulins are post-translational modifications i.e. that a pro-tubulin may exist in Aspergillus and only the 'functional' tubulins are detected as altered due to mutation, (ii) that the multiple tubulins are in fact separate gene products but are normally sequestered as separate, non-interacting pools e.g. only the tubulins involved in spindle formation show the mutation for benomyl resistance, or (iii) that the proteins detected in this particular area of the two dimensional gel map, and designated tubulins, are not tubulins at all - other proteins are known

to be associated with tubulin and to migrate in a similar area of the gel e.g. tau proteins. However, the work on multiple tubulins from brain was performed using DEAE-purified tubulin i.e. supposedly free from MAPs.

There are several advantages to the possession of multiple tubulins within a cell or organism, separate genes would enable independent control of synthesis of a particular organelle, especially in an organism with a complex life-cycle. This situation appears to exist in Naegleria. Organelle-specific microtubules with different optimum conditions for assembly and/or disassembly may afford a fine control over microtubule organisation within the cell, and this may involve the different tubulins possessing specific binding sites for different species of MAPs.

Yeast have been less extensively studied than Aspergillus despite the well-defined differences in drug sensitivities. Proteins from Saccharomyces cerevisiae have been identified as tubulin almost exclusively by their ability to co-polymerise in vitro with brain tubulin from various sources. Water & Kleinsmith (1976) describe proteins which co-migrate with rat brain tubulin on SDS-PAGE gradient gels after two cycles of co-polymerisation in the presence of glycerol. In contrast, Shriver & Byers (1977) find that the major co-polymerising proteins have estimated molecular weights quite different from brain tubulin i.e. 50 and 48,000 daltons, and that these proteins show little homology with brain tubulin on fingerprint analysis. In addition they find their predominant yeast MAP with a mol. wt. of approx. 130,000 daltons. In a study of temperature sensitive cell division cycle mutants they find differences in the MAP content depending on the position of the block in the cell cycle. The yeast proteins described as tubulins by Baum, Thormer & Honig (1978) also have lower estimated molecular weight than brain tubulins on SDS-gels. Additional evidence from immuno-precipitation experiments show a cross-reactivity with antibody to brain

tubulin and Chlamydomonas tubulin, with a greater efficiency of precipitation with the Chlamydomonas antibody.

The identification of microtubule proteins in the yeast appears to be tentative and controversial, especially as only one means of identification has been employed - i.e. that of co-polymerisation with brain microtubules.

Evidence for tubulins being identical in all organisms comes mainly from data on partial sequence analysis, immunological cross-reactivity and co-polymerisation data. Partial sequencing however is limited to only the first 25 N-terminal amino acids from brain tubulin and sea urchin sperm. Full sequencing of protein from a range of organisms is required before claims to total homology can be verified. Antibody cross-reactivity indicates that tubulins from different species have similar antigenic determinants, but there is immunological data indicating that lower eukaryote tubulins of flagella are not the same as brain, or even lower eukaryote, cytoplasmic tubulin. Immunofluorescent staining has been performed using antibodies to brain tubulin to stain microtubules in tissue-culture cells, and there is a single instance of immunofluorescence in plant cells - carrot cell cultures (Lloyd et.al., 1979) - using brain tubulin antibody. No lower eukaryote material has been immunofluorescently stained using higher eukaryote antibody.

The fact that tubulins from a wide range of organisms co-polymerise in vitro with brain microtubules indicates that microtubule proteins in general require similar optimum conditions for assembly, or that one is selecting only those molecules which will co-polymerise in vitro, i.e. those similar to brain proteins. It has been shown that lower organisms, and indeed brain tissue, may contain multiple forms of tubulin.

The main body of evidence that microtubule proteins in lower eukaryotes may not be identical to higher eukaryote proteins comes from anti-microtubule sensitivity and binding data. A synopsis of such data from a range of organisms and tissues can be found in table 2. In general the lower eukaryotes appear to be insensitive or less sensitive to colchicine than higher eukaryote tissues. This insensitivity seems to be a result of a reduced affinity of the tubulin for this drug, however the lower organisms are sensitive to a different range of anti-mitotic substances, many of which have no effect on mitosis or assembly in vitro of microtubule proteins from mammaliam cells.

There is direct evidence from gel-electrophoretic analysis of tubulins from different species, that proteins from various sources differ in either their α- or β-tubulin subunit proteins, rarely both. However, data from different research groups is often contradictory and this may be a consequence of the multitude of different electrophoretic conditions and gel systems used throughout this field of research.

The development of two-dimensional PAGE techniques has increased the sensitivity of this method of protein analysis, and revealed the fact that tissues, or even single cells, may contain multiple forms of tubulin molecules. The precise nature and function of these multiple tubulins (if they are tubulins), especially with respect to in vivo control of microtubule assembly and function, remains to be determined.

The aim at the outset of this project was to identify, purify and polymerise in vitro microtubule proteins from the yeast Saccharomyces cerevisiae and to determine their sensitivity to microtubule poisons in vitro. In the process of this investigation some of the difficulties

involved in looking for such proteins in yeast, and other lower eukaryotes, are described and, to some extent overcome.

Recently attention has been turned to the investigation of microtubule systems in tissues other than mammalian brain, and the problems involved in working with this material have been recognised. Microtubule protein competent to assemble in vitro has been isolated from mammalian tissue culture cells but as yet no such system has been developed for non-flagellar proteins from lower organisms.

The development of more sensitive PAGE techniques, in particular two-dimensional PAGE, has enabled the detection of small quantities of protein and also small differences in migrational characteristics between tubulins from different sources. Such techniques have been applied to the filamentous fungus Aspergillus nidulans with the result that multiple tubulins have been described in this organism. The implications of these findings have already been discussed.

Saccharomyces cerevisiae has a number of advantages over other lower eukaryotes. Microtubule functions in this organism appear to be limited to their role in cell division i.e. spindle formation. The presence of a cell wall means that no cytoskeleton is necessary for maintainance of cell shape c.f. animal cells, in addition yeast do no possess flagella or cilia at any stage in their life cycle. All of these facts reduce the possibility that this organism synthesises different pools of tubulin which would complicate any efforts to identify microtubule proteins, however it may mean that the tubulin content of the cell is likely to be very low.

The work presented in this thesis attempts to clarify the identification of microtubule proteins in Saccharomyces cerevisiae, and to attempt to use the same criteria with a different species of yeast, Protomyces inundatus

which is sensitive to the anti-mitotic drug griseofulvin. Effects of this drug in this sensitive yeast are described using light and electron microscopy. Drug uptake is also examined in an attempt to identify the basis of differences in sensitivity between Saccharomyces and Protomyces.

Griseofulvin was chosen for this study as it appeared to be the most effective antimicrotubule drug against fungi, and in addition it is a novel type of antimicrotubule drug. Fungi and yeast are generally resistant to colchicine, therefore this drug cannot be used as a tool for investigating tubulin in these organisms. It has, however, become apparent from work published during the course of the present study, that MBC is also an effective antimitotic drug, showing colchicine-like effects in fungi. This drug may be used as a colchicine substitute in future work on microtubules in yeast. Such differences in drug sensitivity in themselves suggest that microtubules in fungi may be different from those of higher eukaryotes.

MATERIALS AND METHODS

Micro-organisms

Saccharomyces cerevisiae 20B-12, a mutant deficient in proteases A, B and C (Jones, 1977)

Saccharomyces cerevisiae cdc 13 (Hartwell *et. al.*, 1973)

Protomyces inundatus strain 115295 (-) obtained from the Commonwealth Mycological Institute

Cultures of these organisms were maintained on solid media, growing at 25°C with regular subculturing, or stored on slopes of complex medium at 4°C.

Media

Yeast cultures were maintained on solid complex medium, either MYPG or YM-1 (Hartwell, 1967), and transferred to liquid medium as required.

MYGP medium

	final concentration g/litre
Malt extract	3
Yeast extract	3
Glucose	10
Mycological peptone	5

YM-1 medium

	final concentration g/litre
Succinic acid	10
Sodium hydroxide	6
Yeast extract	5
Mycological peptone	10
Yeast nitrogen base	6.7

Defined media were prepared as follows:

Defined medium for Protomyces inundatus (Trinci, 1971)

Solution A

	final concentration g/litre
KH_2PO_4	3.4
$Na_2HPO_4.12H_2O$	8.9
NH_4NO_3	6.0

pH 6.8, made up in 918 mls to give 1 litre of medium when all the components are added

Solution B: Trace elements

	final concentration g/litre		final concentration g/litre
EDTA	0.6	$CuSO_4.5H_2O$	0.005
$MgSO_4.7H_2O$	0.25	$FeSO_4.7H_2O$	0.1
$CaCl_2$	0.05	Na_2SO_4	0.5
$ZnSO_4.7H_2O$	0.2	$NaMoO_4.2H_2O$	0.005
$MnSO_4.4H_2O$	0.02		

The trace element solution was made up and stored as a stock solution at 20 x the final concentration, and diluted accordingly, as required.

Solution C: Vitamin solution

	final concentration mg/litre
m. inositol	1.25
Thiamine HCl	0.25
Riboflavin	0.25
Calcium pantotheinate	0.25
p.amino benzoic acid	0.125
Pyridoxin	0.25
D-biotin	0.0125

The vitamin solution was made up and stored at 80 x the final concentration and diluted accordingly, as required

To make the complete medium - the above components were mixed in the following amounts:

- 918 mls Solution A
- 50 mls Solution B
- 12.5 mls Solution C
- 20 mls 50% (w/v) glucose solution

Defined medium for Saccharomyces cerevisiae 20B-12

Solution A

	g/litre
KH_2PO_4	4.65
Na_2HPO_4	5.75
$(NH_4)_2SO_4$	6.0

pH 6.8, made up in 910 mls to give 1 litre of medium when all components added.

To make the complete medium, components were mixed in the following amounts:

Solutions B and C were as for Protomyces inundatus

910 mls Solution A
50 mls Solution B
12.5 mls Solution C
20 mls 50% (w/v) glucose solution
10 mls 0.4% (w/v) tryptophan

All solutions were sterilised by autoclaving at 115°C for 20 min, except for vitamin Solution C which was sterilised by filtration through a 0.45µm Millipore membrane.

Measurement of growth

For experiments involving the effect of various drugs or chemicals on yeast growth, cultures were incubated in 250 ml conical flasks equipped with test-tube side-arms to fit an EEL colorimeter. Absorbance of the culture was read using an appropriate filter with sterile medium as a blank.

For estimations of dry weight, replicate samples of culture were vacuum-filtered onto dry, pre-weighed Whatman GF/A glass fibre filters and washed with 20 mls of sterile distilled water. The filter was then dried for 48 hours in an oven at 120°C, and re-weighed immediately after removal. This method gave a reproducible estimate of dry weight.

Cell number was estimated using a haemocytometer.

Drugs

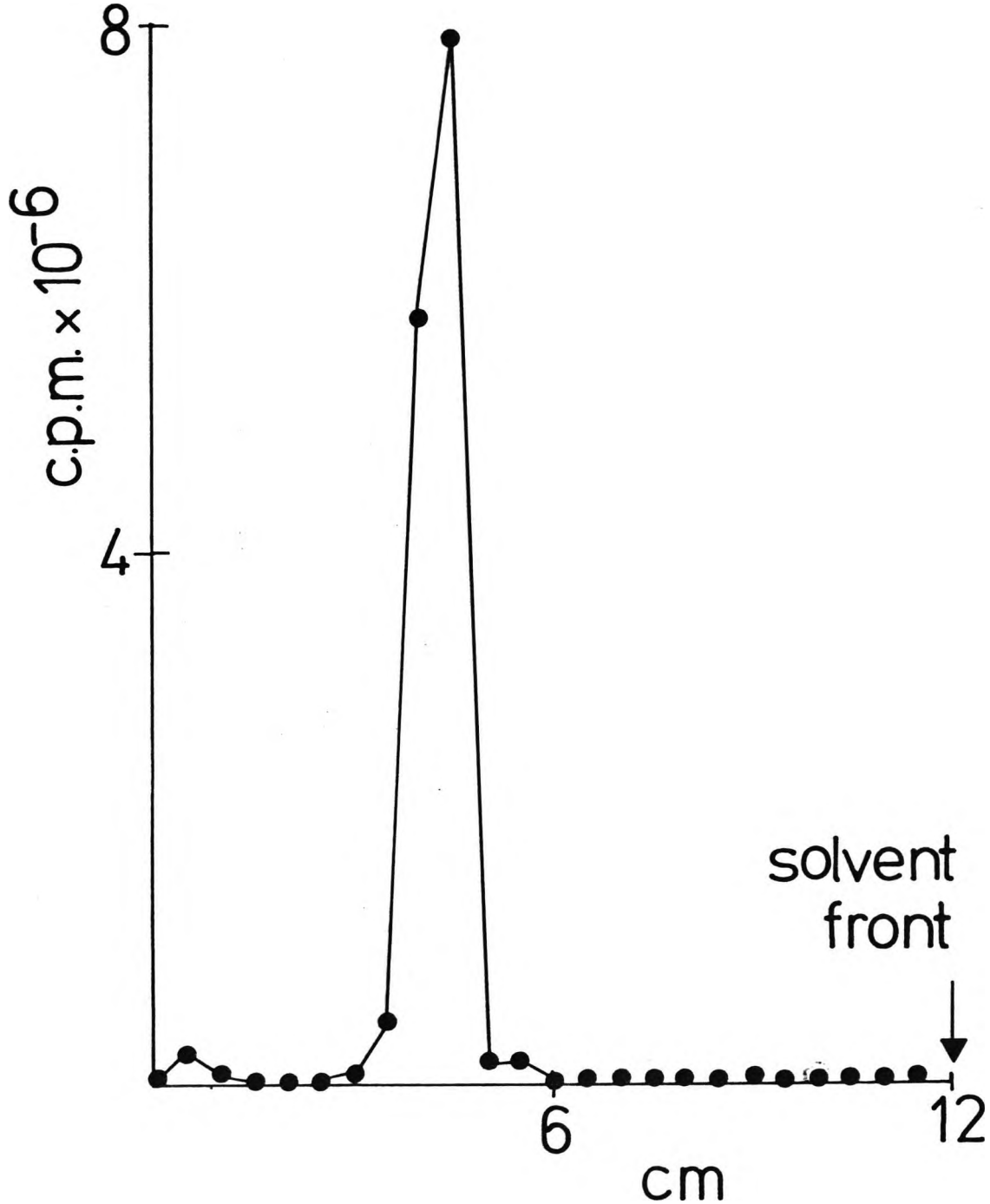
Griseofulvin (7-chloro-2′,4,6-trimethoxy-6′-methyl spiro {benzfuran 2(3H), 1′-[2]cyclohexene}-3,4′-dione) was obtained from Sigma (London) Chemical Co, Poole, Dorset, England.

Randomly tritiated griseofulvin was prepared by the Radiochemicals Centre, Amersham, England and purified by thin layer chromatography by Dr. C.I. Pogson according to the method of Cole, Kirksay & Holaday (1970). It was eluted and stored in dimethyl formamide (DMF) (Fisons SLR grade) as a 4.4 mM solution (225 Ci/mole) at -30°C. Dilutions of this stock were made into unlabelled griseofulvin in DMF as required. The purity of the radioactive griseofulvin stock was checked by thin-layer chromatography according to Cole et. al. (1970) as above. Radioactive griseofulvin and freshly prepared unlabelled drug solutions were applied to Polygram Sil G/U.V. 254 nm thin-layer plates (Camlab Ltd, Cambridge, England) using a microcap pipette, with air drying between applications. Chromatograms were developed using 7% (v/v) acetone in chloroform in a sealed tank (approx. 30 min), air dried and viewed under long-wave ultraviolet light. Griseofulvin could be identified as a bright fluorescent spot. 0.5 cm portions of the chromatogram were scraped off into PCS scintillation cocktail (Hopkin and Williams Ltd, Chadwell Heath, Essex, England) and radioactivity determined in a Packard 3375 scintillation counter (Fig. 13).

Griseofulvin is soluble in water to a limit of approx. 35μM. To obtain concentrations greater than this an organic carrier solution was necessary; consequently stock solutions of the drug were made up in 100% DMF and diluted into aqueous media to give the required drug concentration in either 2% or 0.5% (v/v) DMF.

Fig. 13: Thin layer ascending chromatography of [^{3}H] griseofulvin.

Radioactivity in 0.5cm sections of the developed chromatogram


8
4
c.p.m. x 10⁻⁶
solvent
front
6
12
cm

Ring C-methoxy-[^{3}H] colchicine, 2-amino [1-[^{14}C]] iso butyric acid and N-acetyl-D-[1-[^{3}H]] glucosamine were all obtained from the Radiochemical Centre, Amersham. Unlabelled colchicine was obtained from Sigma.

Drug uptake experiments

Cells were incubated with radioactive drug in an appropriate volume in 50ml conical flasks treated with Repelcote (Hopkin and Williams). Flasks were shaken at 25°C between sampling.

Cells used for uptake experiments were grown in batch culture at 25°C, and used during the logarithmic or, in certain experiments the stationary phase of growth.

Drugs were added either directly to aliquots of the culture, or the cells were harvested by centrifugation at 1000 rpm on a bench centrifuge (5 min at 25°C), washed twice in warm buffer and resuspended in defined medium Solution A. This suspension was then used for experiments.

In some experiments cells were separated from medium at the end of the incubation with radioactive drug by centrifugation through a layer of silicone fluid in a centrifuge tube. At timed intervals, aliquots of yeast-drug suspension, usually 1 ml, were removed and layered over 0.25mls of silicone fluid in a 1.5ml Eppendorf centrifuge tube which was then spun immediately at 12,000 x g for 30 seconds in an Eppendorf microfuge. The remaining medium and silicone were aspirated away from the cells which were dissolved in Toluene butyl-PBD scintillation fluid for determination of radioactivity.

The silicone fluid in the tubes was a mixture of Silicon Öl AR 200 (Serva): Silicon fluid DC 200/CS (Hopkin and Williams) in the ratio 3:1, a composition which allowed the passage of yeast cells but not medium during the centrifugation.

An alternative method was used in which 1 ml aliquots of incubation mixture were taken at timed intervals, vacuum filtered onto Whatman GF/A glass-fibre filters and washed immediately with 15ml of ice-cold distilled water (Grenson et. al., 1966). Filters were placed in plastic scintillation vial inserts and dried for 1-2 hours at 40°C, after which 3 ml of scintillation fluid (Toluene butyl-PBD, or 'Cocktail T' (Hopkin and Williams)) were added.

Determination of radioactivity

Scintillation fluids used were:

PCS and Cocktail T, both from Hopkin and Williams; Toluene butyl PBD scintillant, a 'cocktail' consisting of:

6 gm Butyl-PBD(2-(4′-6-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole)

80 gm Naphthalene

600 ml Toluene

400 ml 2-methoxy ethanol

Corrections for quenching were by an internal standardisation method.

Electron microscopy

Negative staining

Samples were absorbed onto carbon-coated, formvar coated grids. Excess was removed by suction and grids were washed with aqueous saturated uranyl acetate (Polaron).

Fixing and embedding

Yeast cells were fixed in 2.5% (v/v) glutaraldehyde in the growth medium for 15 min, then in 2.5% glutaraldehyde in cacodylate buffer pH 7.2 for 2½ hours. Cells were post-fixed in 1% (w/v) osmium tetroxide in

veronal/acetate buffer for 90 min, dehydrated through a graded ethanol series and embedded in Spurr's resin (Spurr, 1969).

Sections were cut using a diamond knife on a Reichert OMU3 ultramicrotome, stained with 5% (w/v) uranyl acetate in 1% (v/v) acetic acid followed by lead citrate (Reynolds, 1963), and examined using an AEl 801A electron microscope at an accelerating voltage of 60kV.

Scanning electron microscopy

Cells were fixed overnight in 2.5 (w/v) glutaraldehyde in cacodylate buffer, pH 7.4, and dehydrated through aqueous acetone; 25, 50, 75 and 100% (v/v). Samples were critical-point dried (Polaron model E3000) with liquid carbon dioxide, sputter coated with gold (Polaron model E5000) and examined in a Cambridge S600 scanning electron microscope at either 15 or 25 kV.

Preparation of microtubule protein

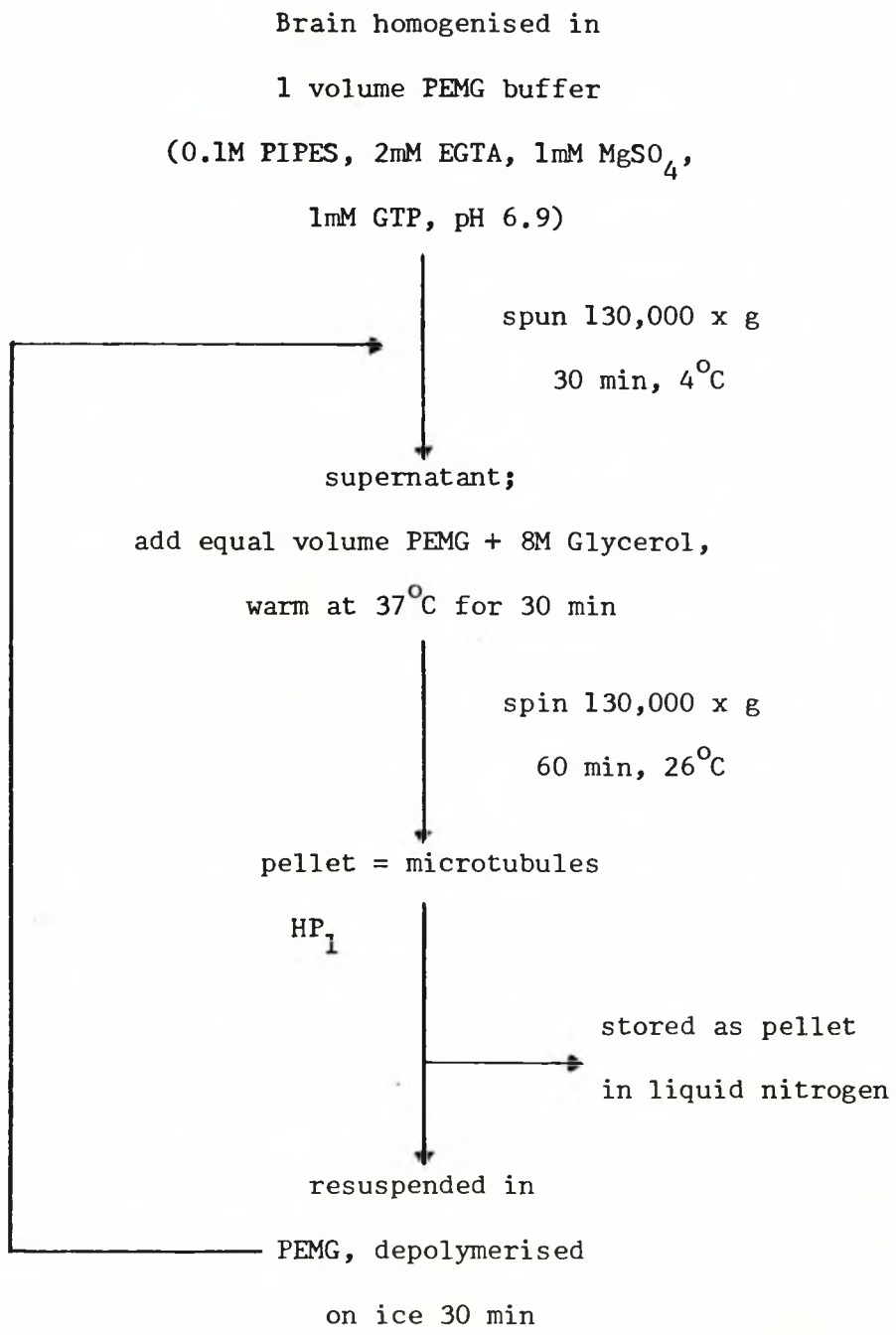
Microtubule protein was prepared from sheep brains by a modification of Dentler et. al. (1975) as described in Roobol, Gull & Pogson (1976). The procedure is outlined in Fig. 14.

In some cases 0.1mM GTP + 1mM ATP was substituted for 1mM GTP in the homogenising buffer and steps up to the production of the HP_1, with no apparent loss of yield.

HP_2 pellets were stored in liquid nitrogen for periods up to six weeks during which time no loss of assembly activity was detected.

Immediately before use the HP_2 pellets were resuspended in cold PEMG buffer (0.1M PIPES, 2mM EGTA, 1mM $MgSO_4$, 1mM GTP pH 6.9), depolymerised on ice for 30 min and then centrifuged at 130,000 x g at $4^{o}C$ to remove

Fig. 14: Purification of microtubules from sheep brain by cycles of temperature-dependent polymerisation and depolymerisation



non-cold-labile aggregates. The supernatant was subsequently used for experiments. Polymerising ability was retained, without decay, for at least 6 hours after resuspension.

The products of the above purification procedure analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Fig. 15, along with MAP-depleted microtubule protein prepared by assembly in Dimethyl sulphoxide (DMSO) and tubulin prepared by phosphocellulose chromatography.

MAP-depleted microtubule protein was prepared using a modification of the method of Himes (1977) by assembly of microtubules in DMSO.

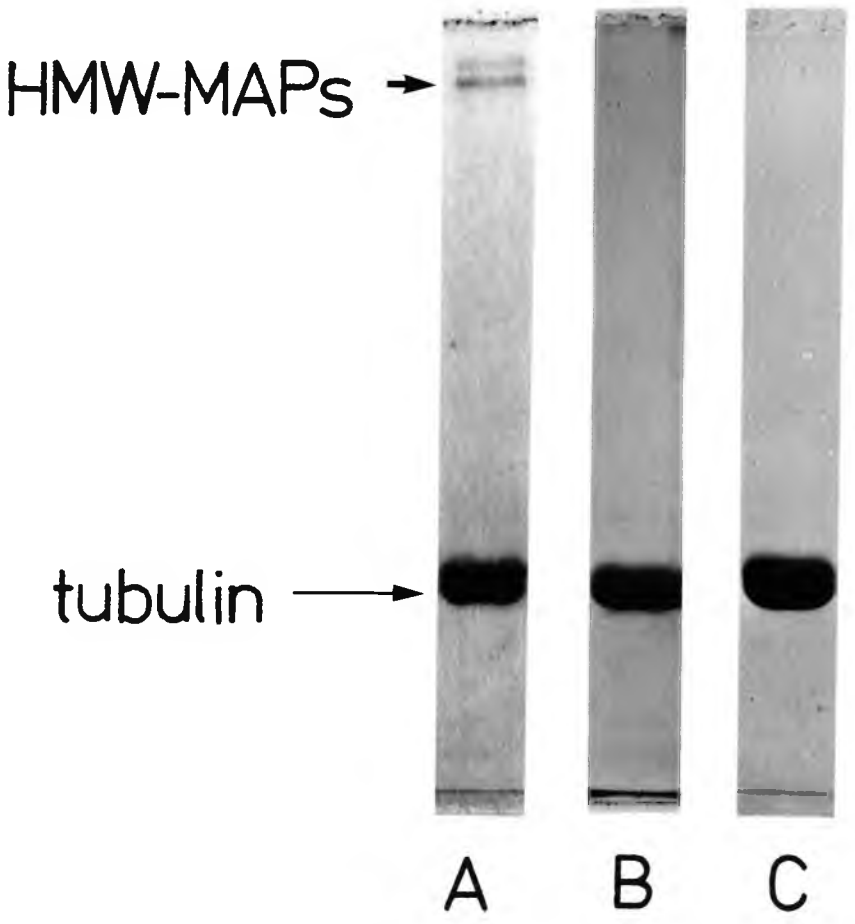
A HP_2 microtubule pellet was resuspended, depolymerised and spun in 0.1M PIPES, 1mM EGTA, 0.5mM $MgSO_4$, 0.5 mM GTP pH 6.9 at $4^{o}C$, and the resulting supernatant diluted with $\frac{1}{3}$ volume of this buffer containing 4M glycerol, followed by an equal volume of 0.8M PIPES, 20% (v/v) DMSO pH 6.9. This mixture was incubated at $37^{o}C$ for 20 min and the assembled protein collected by centrifugation at 130,000 x g for 20 min. Most of the MAPs remained in the supernatant and were discarded. The tubulin-enriched pellet was resuspended in PEMG buffer, depolymerised and centrifuged as for whole microtubule protein, and the supernatant used for experiments.

Preparation of phosphocellulose

Phosphocellulose powder (Whatman P11) was stirred with distilled water (200ml per gram of powder) until all particles were wet, then allowed to settle for 30 min after which time the fine particles were removed. This de-fining procedure was repeated once more.

The ion-exchanger was pre-cycled by resuspending the de-fined phosphocellulose in 0.5M NaOH (150 mls per 10g) and allowing it to stand for 1 hour. It was then filtered onto Whatman No. 541 filter paper over a

Fig. 15: A, microtubule protein prepared by the assembly-disassembly procedure;
B, microtubule protein depleted in HMW-MAPs by assembly in DMSO;
C, tubulin prepared by phosphocellulose chromatography.


HMW-MAPs
tubulin
A
B
C

vacuum and washed to neutrality. This washed material was then resuspended in 0.5M HCl and allowed to stand for 1 hour before being filtered and washed as above. The pre-cycled phosphocellulose was resuspended in 0.1M PIPES buffer re-adjusted to pH 6.9 with sodium hydroxide, and stored in this buffer at 4°C.

Tubulin preparation by phosphocellulose chromatography

Microtubule protein pellets were resuspended, depolymerised and cleared by centrifugation as described. The supernatant was loaded onto a phosphocellulose column of dimensions 1.5 x 11.5cm (bed volume 20ml) equilibrated in the appropriate buffer. The separation was carried out at 4°C at a flow rate of 40-50ml per hour. The tubulin emerging from the column was detected at 280nm using an MSE Spectroplus recording spectrophotometer equipped with a flow-through cuvette. Fractions of tubulin were collected in tubes cooled by liquid nitrogen and either thawed and used immediately, or stored in liquid nitrogen for later use.

Assays of microtubule assembly in vitro

Microtubule assembly was assayed by turbidmetry (Gaskin, Cantor & Shelanski, 1974) at 400nm using a Gilford 240 or 250 recording spectrophotometer equipped with a temperature-controlled automatic cuvette changer. Assays were performed at 37°C.

Preparation of yeast extracts

Where large volumes of cultures were used, cells were harvested by centrifugation at 10,000 x g at 4°C for 5 min, including one wash with cold, distilled water. The yeast pellet was resuspended in an equal volume of PEM buffer containing 0.1mM GTP (or buffer appropriate to the experiment),

and cells were broken by a single passage through a French Pressure Cell (Aminco) at 16,000 p.s.i. Cell breakage was monitored by light microscopy and was usually 80-90%. The broken cell suspension was centrifuged at 130,000 x g for 30 min at 4°C to produce an extract.

Where small quantities of cells were used, or for radioactive cultures, cells were broken by vortex mixing with an equal volume of 0.45mm diameter glass beads for 5 min with intervals of cooling on ice. The broken cell suspension was pipetted away from the beads and centrifuged as above.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel-electrophoresis was perfomed essentially according to the method of Laemmli (1970) in 1mm thick slabs of 6% or 7.5% acrylamide with a 3% acrylamide stacking-gel.

Stock solutions were as follows:

Solution A: 3M Tris, 0.48N HCl

Solution C_1: 24% (w/v) acrylamide, 0.6% (w/v) bis-acrylamide

Solution C: 28% (w/v) acrylamide, 0.735% (w/v) bis-acrylamide

Solution D: 1M Tris, 0.8% (w/v) SDS pH 7.0

Ammonium persulphate 0.28% (w/v) in distilled water - freshly prepared

6% gel solutions were prepared by mixing:

4.95 ml Solution C_1

2.5 ml Solution A

5.0 ml ammonium persulphate

0.1 ml 20% (w/v) SDS

7.2 ml distilled water

This solution was degassed for 1-2 min on a vacuum pump after which 11.5µl of N,N,N′,N″-tetramethyl-ethylenediamine (TEMED) were added, and the solution allowed to polymerise to form the running gel of composition; 6% acrylamide, 0.15% bis-acrylamide, 0.1% SDS, 0.38M Tris, 0.07% persulphate. Bis-acrylamide : acrylamide ratio was 1:40. The gel solution was overlayed with distilled water during the polymerisation to ensure a flat surface.

A 3% acrylamide stacking-gel solution was prepared by mixing;

3.0 ml Solution D

2.55 ml Solution C

6.45 ml distilled water

12.0 ml ammonium persulphate

After degassing as above, 12.5µl TEMED were added and stacking-gel polymerised onto the running gel. The stacking-gel had the composition; 2.9% acrylamide, 0.08% bis-acrylamide, 0.1% SDS, 0.125M Tris, 0.14% persulphate Electrode buffer was that of Laemmli i.e. 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.4. Where buffer of pH 9.2 was used it was 192mM glycine, 0.1% SDS, adjusted to the correct pH with NaOH.

Apparatus was constructed in the department to our own specifications based on Matsudaira et. al. (1978).

Sample preparation

Protein samples were diluted into Laemmli sample buffer (62.5mM Tris HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue, pH 6.8) to give protein concentrations of 0.5-1mg/ml. Maximum dilution of sample buffer used was 1:2, protein solution : buffer. The diluted samples were heated at 100°C for 90 sec, cooled and loaded onto gels. Acetone precipitates of protein were heated in Laemmli buffer in the same way.

Samples were loaded onto gels using a Hamilton micro-syringe. Volumes used were usually 10-20µl, giving a protein loading of 5-10µg. For a sample containing a large number of different proteins a loading of 10-20µg was used.

6 cm or 20 cm gels were routinely used. The longer gels gave a better resolution of bands in the 50-60,000 molecular weight region.

Samples were subjected to electrophoresis at a constant current of 12mA. Under these conditions 6 cm gels were complete in 2 hours. 20 cm gels were run for varying lengths of time.

Gels were stained with 0.1% (w/v) Coomassie Brilliant blue R250 (Gurr) in glacial acetic acid : isopropanol : water (10:25:65) for 1-2 hours, destained for 1 hour in the above solvent mixture without Coomassie blue, and finally stored in 7% (v/v) glacial acetic acid.

Gels were dried using a Bio-rad gel-drier. Dried radioactive gels were exposed to x-ray film (Kodirex or Kodak X-Omat H1) between heavy glass plates in a light-tight box for the required length of time. Films were developed using Kodak developer DX-80 and Fixer FX-40 according to the instructions.

Molecular weight determination using SDS-PAGE

Marker proteins of known molecular weight were prepared for gel-electrophoresis as described. 2µg of each protein were loaded to ensure a sharp band on stained gels.

Proteins used as markers were: Bovine serum albumin (BSA), 68,000 mol wt; β-galactosidase, 130,000 mol wt; Glutamate dehydrogenase, 53,000 mol wt and the β, β′ and α subunits of RNA-polymerase, 165,000, 155,000 and 39,000 mol wt respectively.

Materials and sources

Acrylamide and N,N′-methylene-bis-acrylamide were especially purified for electrophoresis, obtained from BDH Chemicals Ltd, Poole, England. SDS was SLR grade from Fisons.

All other chemicals including molecular weight marker proteins were obtained from Sigma.

Assay of Protease activity

Protease activity in yeast extracts was assayed by testing the effect of extracts on brain microtubule proteins using SDS-PAGE. Extract (enzyme) and microtubule protein (substrate) were incubated in various ratios at 37°C for 30 min i.e. microtubule polymerising conditions. Where protease inhibitors were used they were pre-incubated with extract on ice for 10 min before addition of the substrate. Substrate concentration was 2 mg/ml and extract concentrations were in the range 1-5 mg/ml giving substrate : extract ratios of 0.5-2 in the assay. Total assay volume was 250µl in PEMG buffer.

Assay mix: 100µl substrate (5mg/ml stock)

10-50µl extract (1-5mg/ml final concentration)

140-110µl PEMG buffer to make 250µl final volume

The incubation was terminated by the addition of 750µl of Laemmli sample buffer with or without 1mM PMSF, and the samples were then boiled for 90 sec and subjected to electrophoresis as previously described.

Protease inhibitors used were:

TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone HCl)

PMSF (Phenyl methyl sulphonyl fluoride)

both obtained from Sigma

Leupeptin, Chymostatin and Pepstatin were a gift from Professor H. Umezawa

Measurement of GTP-ase activity

The GTP-hydrolytic activity in yeast extract was measured using a timed incubation of yeast extract with 1mM GTP. Various concentrations of yeast extract protein were used, the incubation was started by the addition of GTP and warming to 37°C. Incubations were stopped at timed intervals up to 90 min by the addition of ice-cold perchloric acid (PCA) to 2% (w/v) final concentration, and the tubes were then stored on ice until the end of the experiment.

The PCA precipitate was removed by centrifugation at full speed on a bench centrifuge at 4°C. 1ml of the supernatant was removed and neutralised, to stabilise the GTP, by addition of a pre-determined volume of 0.5M Tri-ethanolamine pH 7.4, 2M KOH, whilst rotamixing. Samples were stored at -30°C at this stage, before assay of nucleotides.

Assay of GTP and GDP

Both nucleotides were assayed by the oxidation of NADH in a coupled enzyme assay.

GDP

Stock buffer of 100mM Tris, 50mM KCl, 10mM Mg acetate, pH 7.4, containing 200μM phosphoenol pyruvate (PEP) and 150μM NADH.

Assay mix: 930μl buffer + PEP + NADH
50μl sample
10μl of 1mg/ml lactate dehydrogenase solution (E.C. 1.1.1.27)

Initial OD 340nm was recorded.

10ul of 1mg/ml pyruvate kinase solution was added and the final OD 340nm was determined after 20 min.

GTP

Stock buffer was as for the GDP assay containing 150μM NADH and 10μl β-mercaptoethanol.

Assay mix: 850μl buffer + NADH + mercaptoethanol
50μl sample
100μl 50mM phosphoglyceric acid

Initial OD 340nm was recorded and the reaction started by addition of 10μl of enzyme cocktail consisting of:

20μl 3-phosphoglycerate kinase (E.C. 2.7.2.3)
80μl Glycerinaldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12)
100μl water

The final OD 340nm was read after 20 min. The ΔOD_{340} gives a measure of the nucleotide present in the sample. (1mM GTP gives a ΔOD_{340} of 6.22)

Co-polymerisation of Saccharomyces cerevisiae and brain microtubules

Saccharomyces cerevisiae 20B-12 was grown in defined medium as described, shaking at 25°C. Cultures were harvested by filtration onto a sterile 1.2µ Millipore, washed with warm sulphate-free medium (a medium in which all the sulphates were replaced by chlorides) and resuspended in 50mls of sulphate-free medium containing 1mCi (1.88mgs) of $Na_2{}^{35}SO_4$ (Radiochemicals Centre, Amersham, England).

Cells were incubated in this medium for 4 hours at 30°C in a reciprocal shaking waterbath. Labelled cells were collected by filtration, washed in sulphate-free medium and finally suspended in twice the pellet volume of PEMG buffer containing 1mM PMSF in 1% (v/v) DMF; 200µM TLCK; 50µg/ml leupeptin. The cells were broken by vortex mixing with an equal volume of cold, 0.45mm diameter glass beads as described previously. The broken cell suspension was pipetted from the beads and centrifuged as described.

MAP-depleted microtubule protein was prepared as previously described and mixed with the ^{35}S-labelled yeast extract. Alternatively whole microtubule protein was used in some experiments.

RNase A (E.C. 3.1.4.22) at 2µg/ml and DNase 1 (E.C. 3.1.4.5) at 1µg/ml were added, protease inhibitors maintained at the above concentrations and the mixture adjusted to 4M glycerol.

Co-polymerised microtubules were formed by warming the mixture at 37°C for 30 min, and collected by centrifugation at 130,000 x g at 26°C for 60 min. This first co-polymer pellet (HP_1) was resuspended in cold PEMG buffer, depolymerised on ice for 30 min and centrifuged at 130,000 x g at 4°C for 30 min. The supernatant was diluted with an equal volume of PEMG 8M glycerol buffer, warmed at 37°C for 30 min and microtubules collected by centrifugation as before to give a second co-polymer pellet (HP_2). This procedure was repeated to give a HP_3 co-polymer pellet. Protease

inhibitors were maintained throughout at the above concentrations. Samples for SDS-PAGE were taken at each stage.

Co-polymerisation experiments using Protomyces inundatus were essentially similar except that cells were incubated in ^{35}S-labelling medium for approx. 16 hours at 25°C.

Phosphocellulose chromatography of the co-polymer

A HP_3 co-polymer pellet was resuspended in cold 0.025M PIPES, 0.5mM EGTA, 0.25mM $MgSO_4$, 0.1mM GTP, depolymerised on ice and applied to a 3 x 1cm phosphocellulose column equilibrated in this resuspension buffer and run at 4°C. The column was washed with 5 column volumes of buffer to remove all unbound material. Protein bound to the column was eluted with a stepwise gradient of KCl in column/resuspension buffer using 2 column volumes of each batch of eluant. Fractions were collected using an LKB 7000 fraction collector. Aliquots of each fraction were mixed with PCS scintillation cocktail and radioactivity determined. Protein was precipitated from the fractions for SDS-PAGE by addition of an equal volume of ice-cold acetone, and the precipitate allowed to form overnight at -20°C. The resulting precipitate was collected by centrifugation at full speed in a bench centrifuge, 50ul of Laemmli sample buffer was added and samples prepared as previously described. Electrophoresis was performed also as described, in 6% acrylamide gels.

Chromatography of Saccharomyces cerevisiae extract on phosphocellulose

Unlabelled extract of Saccharomyces cerevisiae 20B-12 were prepared and treated with protease inhibitors, RNase and DNase as described, except that cells were resuspended and broken in 0.025M PIPES, 0.5mM EGTA, 0.25mM $MgSO_4$, 0.1mM GTP. This ensured that the ionic strength of the

extract was low enough to allow the MAP-like proteins to bind to the ion-exchanger. 300mg of extract protein in 10mls were loaded onto a 2 x 6.6cm (20ml bed volume) column, equilibrated with the above resuspension buffer at 4°C. The column was washed with 5 column volumes of buffer to remove unbound proteins. Protein peaks emerging from the column were monitored at 280nm using an MSE Spectroplus with a flow-through cuvette.

Bound proteins were eluted using 50ml each of 0.1M, 0.25M, 0.5M and 1M KCl in column buffer. 10ml fractions were collected and stored in liquid nitrogen.

Before use, each fraction was dialysed against 2 changes of 20 volumes of column buffer at 4°C, to remove the salt. These fractions, and brain tubulin previously prepared by phosphocellulose chromatography, were dialysed separately against two volumes of PEMG, 8M glycerol buffer for 90 min, on ice. This procedure served to concentrate the protein in the fractions and to adjust them to approx. 4M glycerol. The dialysed, concentrated fractions were then mixed with the tubulin, 3mg tubulin : 1mg fraction protein, warmed at 37°C for 30 min and the microtubules collected by centrifugation as usual. Pellets were stored in liquid nitrogen.

Estimation of protein concentration

Protein was determined by the method of Lowry et. al. (1951) using bovine serum albumin as a standard.

RESULTS

The effect of the anti-mitotic antibiotic griseofulvin on the growth of various species of yeast was tested. Firstly, preparatory investigations were necessary to determine the optimum conditions for the study.

Solubility of griseofulvin

Griseofulvin is a very hydrophobic molecule and is relatively insoluble in aqueous solvents, the maximum concentration obtainable in water being approx. 35μM. If higher concentrations are required, the presence of an organic solvent is necessary to maintain the griseofulvin in solution. The solvent routinely used was dimethyl formamide (DMF) in which the drug was dissolved to give a stock solution which was then diluted into aqueous buffer to give the required concentration in the lowest possible organic solvent concentration. The maximum DMF concentration used was 2% (v/v). This was the highest concentration which had no effect upon growth of Saccharomyces cerevisiae but which maintained griseofulvin in solution at a high concentration.

The absorption spectrum of griseofulvin in DMF was determined (Fig. 16). The major absorption peak occurred at 296nm, and the spectrum remained the same when diluted into aqueous buffers. The solubility of griseofulvin in aqueous media in the presence of 0.5% and 2.0% (v/v) DMF was determined by plotting the absorbance at 296nm for increasing concentrations of the drug. Fig. 17 shows that the relationship between absorbance and drug concentration is linear between 10-100μM griseofulvin with 0.5% and 2% DMF.

Griseofulvin binding to growth medium

Griseofulvin binding to yeast growth medium was tested to determine if the drug bound to any component in the medium thus lowering the free

Fig. 16: Absorption spectrum of griseofulvin in 100% DMF, showing a major peak at 296nm

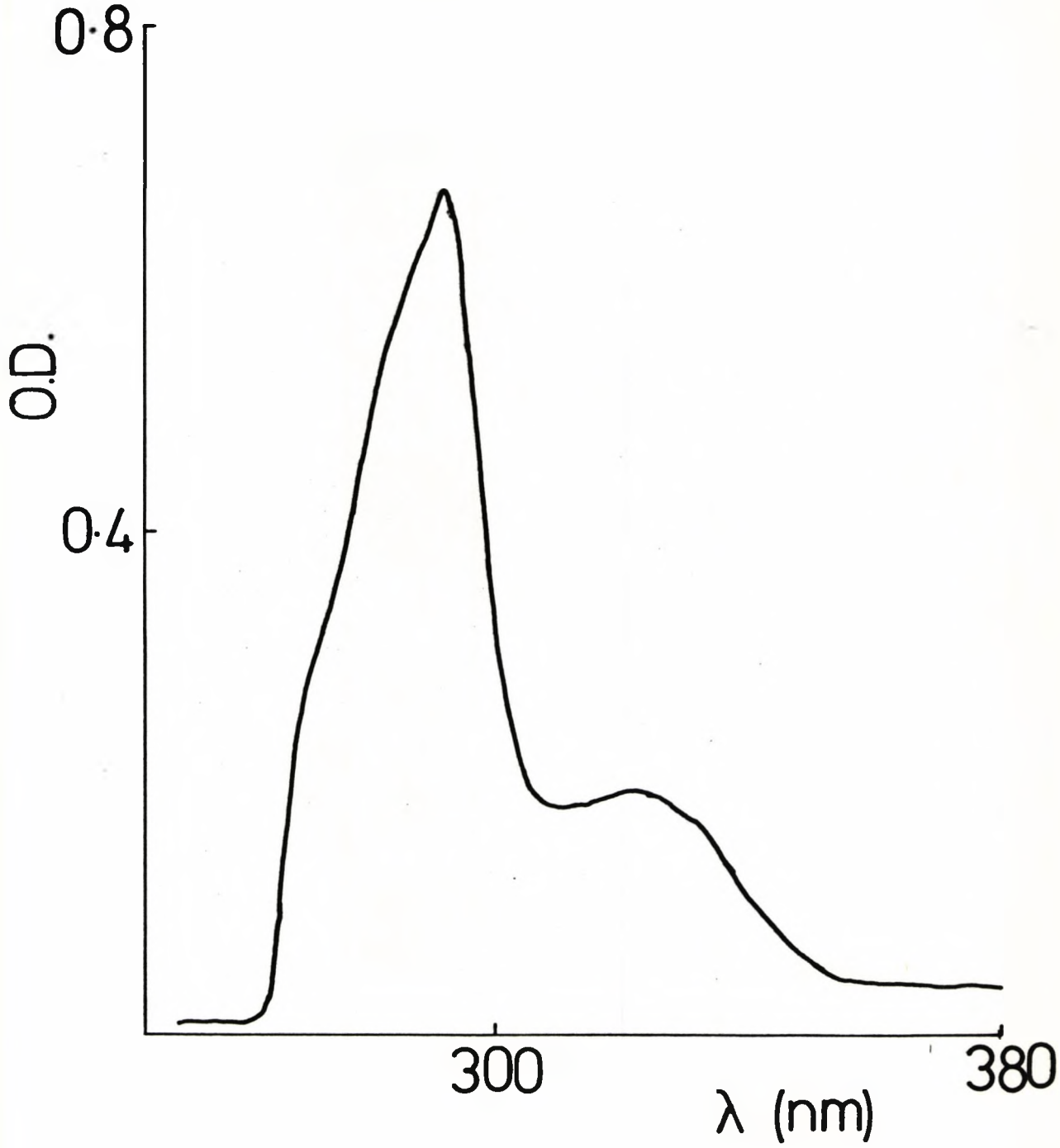
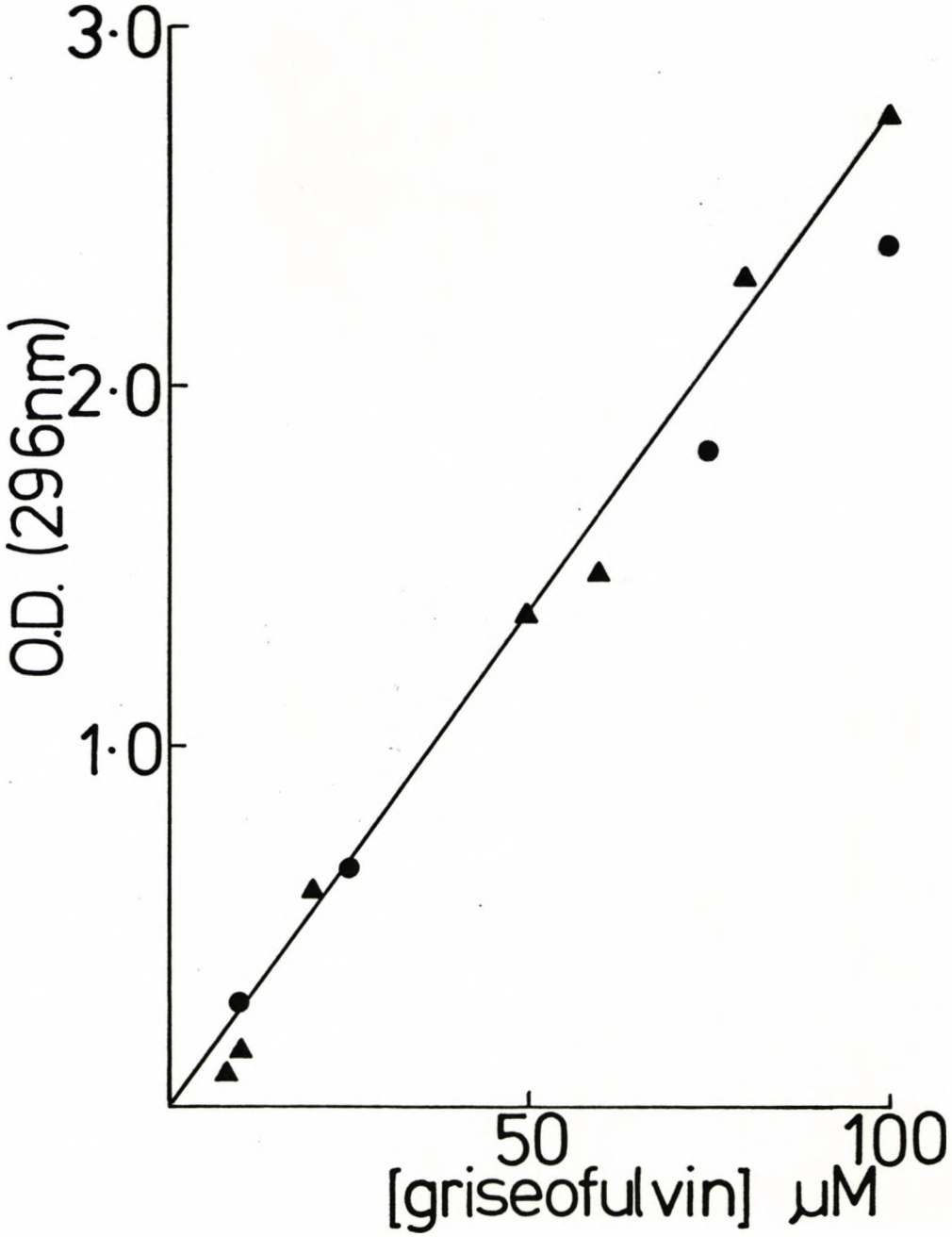

0·8
0·4
O.D.
300
380
λ (nm)

Fig. 17: Solubility of griseofulvin in aqueous buffer (defined medium solution A) measured by comparing O.D.(296nm) with drug concentration in the range 10-100μM, in the presence of; (▲), 0.5% (v/v) DMF; (●) 2.0% (v/v) DMF.


3·0
2·0
1·0
O.D. (296nm)
50
100
[griseofulvin] µM

drug concentration in solution. Binding was measured using an equilibrium dialysis method against ^{3}H-labelled griseofulvin. The study was restricted to concentrations of drug below saturated aqueous concentrations as, in excess of this concentration griseofulvin in 0.5% and 2% DMF precipitated in the presence of dialysis membrane. 1ml aliquots of MYGP medium and a 0.1M citrate buffer pH 5.5 (the pH of the MYGP medium) were dialysed for 16 hours at room temperature against 20 volumes of buffer containing ^{3}H-labelled griseofulvin in either 0.5% or 2% DMF, at concentrations between 5-30μM.

Table 6 shows the results of this binding study. Radioactivity in the external dialysis medium and the control was equivalent, demonstrating that the drug had equilibrated across the dialysis membrane. There was no concentration of radioactive drug in the MYGP sample over and above the control, indicating that griseofulvin does not bind to any component in the medium at the concentrations used.

Effects of griseofulvin on growth of Saccharomyces cerevisiae and Protomyces inundatus

The effects of griseofulvin on the growth of Saccharomyces cerevisiae and Protomyces inundatus were examined. Cultures in the logarithmic phase of growth were inoculated into fresh medium containing griseofulvin, and growth was monitored by increase in culture turbidity or dry weight.

Figs. 18 & 19 show growth of Saccharomyces cerevisiae in the presence of griseofulvin. In both minimal and complex media this drug does not appear to inhibit the growth of this yeast as measured by culture turbidity. The DMF concentration in these experiments was 2% (v/v), a concentration which had no effect upon the growth of this yeast.

Table 6 Measurement of [^{3}H] griseofulvin binding to yeast growth medium MYGP by equilibrium dialysis. The control was 0.1M citrate buffer pH 5.5.

Table 6

	[Griseofulvin] µM	Radioactivity (cpm per 1.0ml) Dialysis medium	Control	MYGP medium
0.5% DMF	24.5	27395	27300	27390
	10	13290	13206	13640
	5	6991	6573	6995
2.0% DMF	29.7	72133	66830	70573
	9.9	24128	24072	22478
	4.9	13120	12956	12320

Fig. 18: Effect of griseofulvin on the growth of Saccharomyces cerevisiae in defined medium, measured by culture turbidity

(●), control containing 2% (v/v) DMF

(○), 100μM griseofulvin)
) in 2% (v/v) DMF
(▲), 50μM griseofulvin)

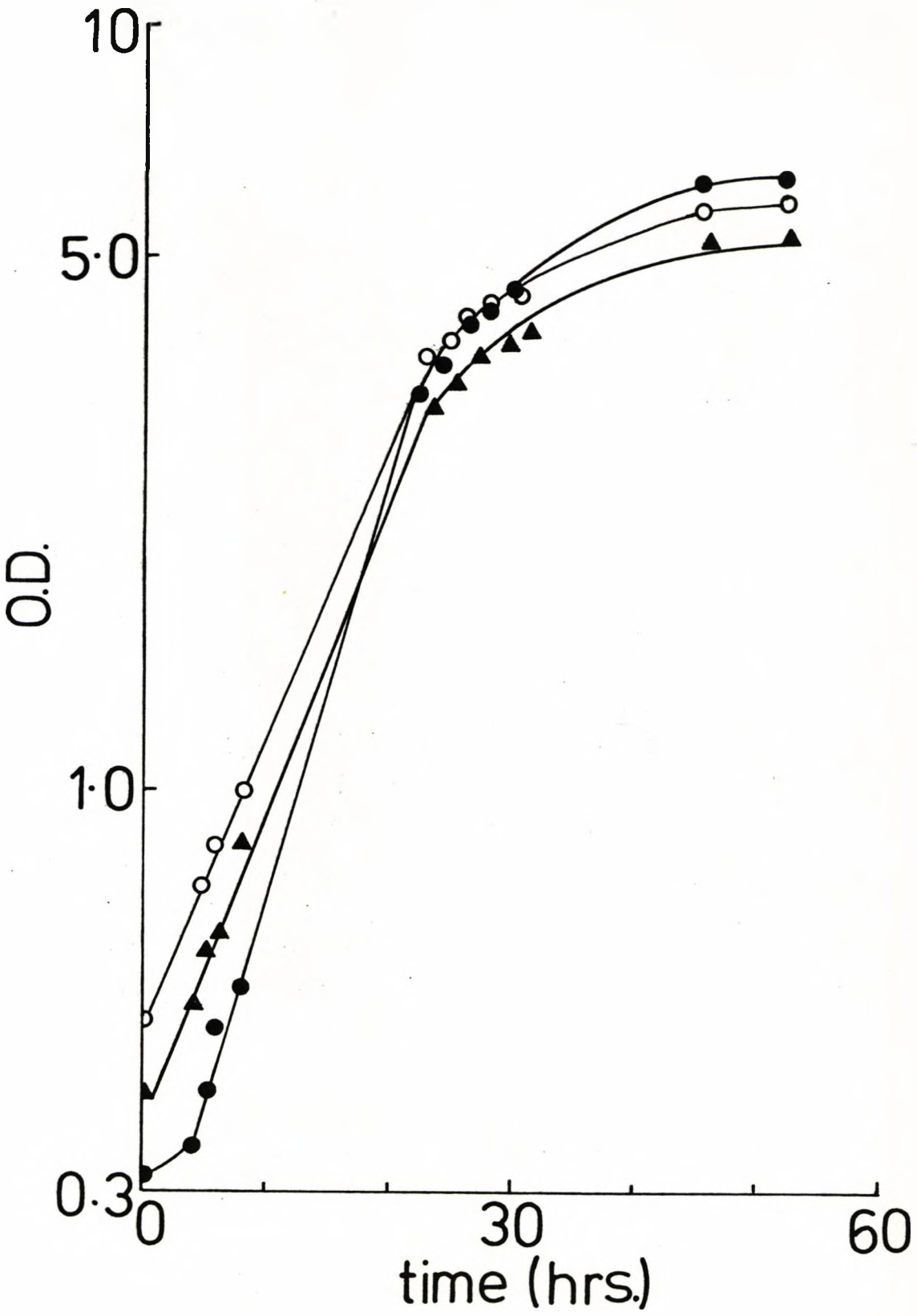
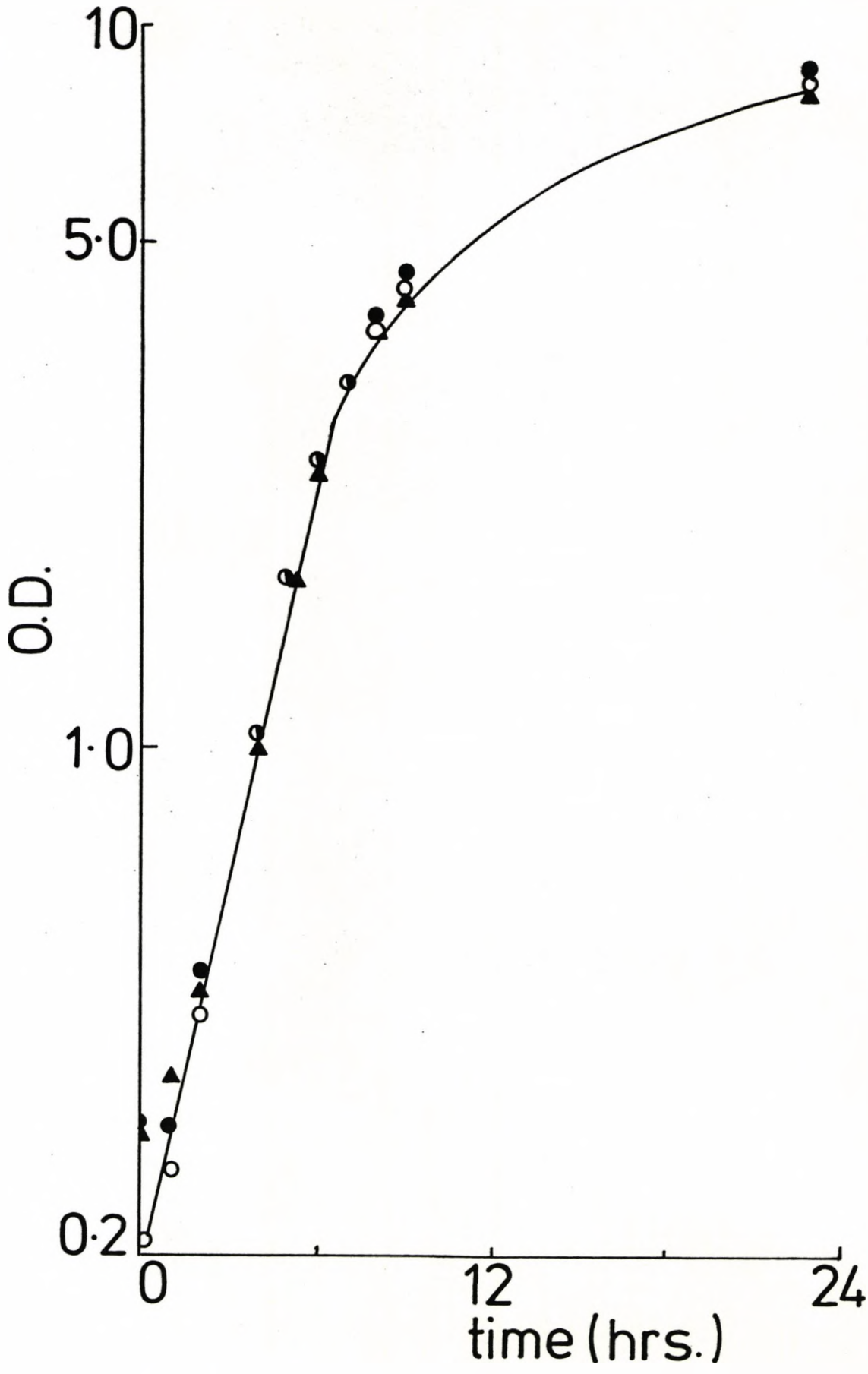

10
5·0
1·0
0·3
O.D.
0
30
60
time (hrs.)

Fig. 19: Effect of griseofulvin on the growth of Saccharomyces cerevisiae in MYGP medium, measured by culture turbidity

(●), control

(○), control + 2% (v/v) DMF

(▲), 100μM griseofulvin in 2% DMF


10
5·0
1·0
0·2
O.D.
0
12
24
time (hrs.)

Figs. 20 & 21 on the other hand show that Protomyces inundatus is sensitive to griseofulvin in minimal and, to a lesser extent, in complex medium. The concentration of DMF in these experiments is 0.5% (v/v), as the growth of Protomyces is somewhat inhibited by 2% DMF. The results from culture turbidity and dry weight measurements are similar (Figs. 22 & 23). Griseofulvin at 50μM strongly inhibits growth, allowing approx. one doubling of culture density/mass before growth slows and is eventually arrested. The characteristics of inhibition in minimal medium are independent of inoculum density.

Samples were taken during growth of Protomyces inundatus in the presence of griseofulvin for light microscopy, also for scanning and transmission electron microscopy.

Light microscopy

Protomyces inundatus grows as an elongated, apically budding yeast when grown aerated at 25°C. Usually only one bud is attached to each parent cell (Figs. 24, 25, 30 & 31). In defined medium, griseofulvin at concentrations as low as 10μM appear to have little effect upon cell morphology, (Fig. 26 & 27), however in 50μM griseofulvin, after 10-20 hours cells appear to be budding in an abnormal fashion. Daughter cells do not detach themselves from the parent cell, giving rise to either chains of unseparated cells or Y-shaped configurations. Also, cells appear to be larger in drug-treated cultures (Figs. 28 & 29). These abnormal features can also be seen clearly in cells grown in YM-1 medium containing 100μM griseofulvin (Figs. 32-35). These cells eventually die on prolonged incubations with the drug, e.g. approx. 40-60 hours, as evidenced by the loss of phase-brightness and apparent degeneration of cell contents seen in the light microscope.

Fig. 20: Effect of griseofulvin on the growth of Protomyces inundatus in defined medium, measured by culture turbidity

(●), control

(○), control + 0.5% (v/v) DMF

(△), 10μM griseofulvin)
) in 0.5% DMF
(▲), 50μM griseofulvin)

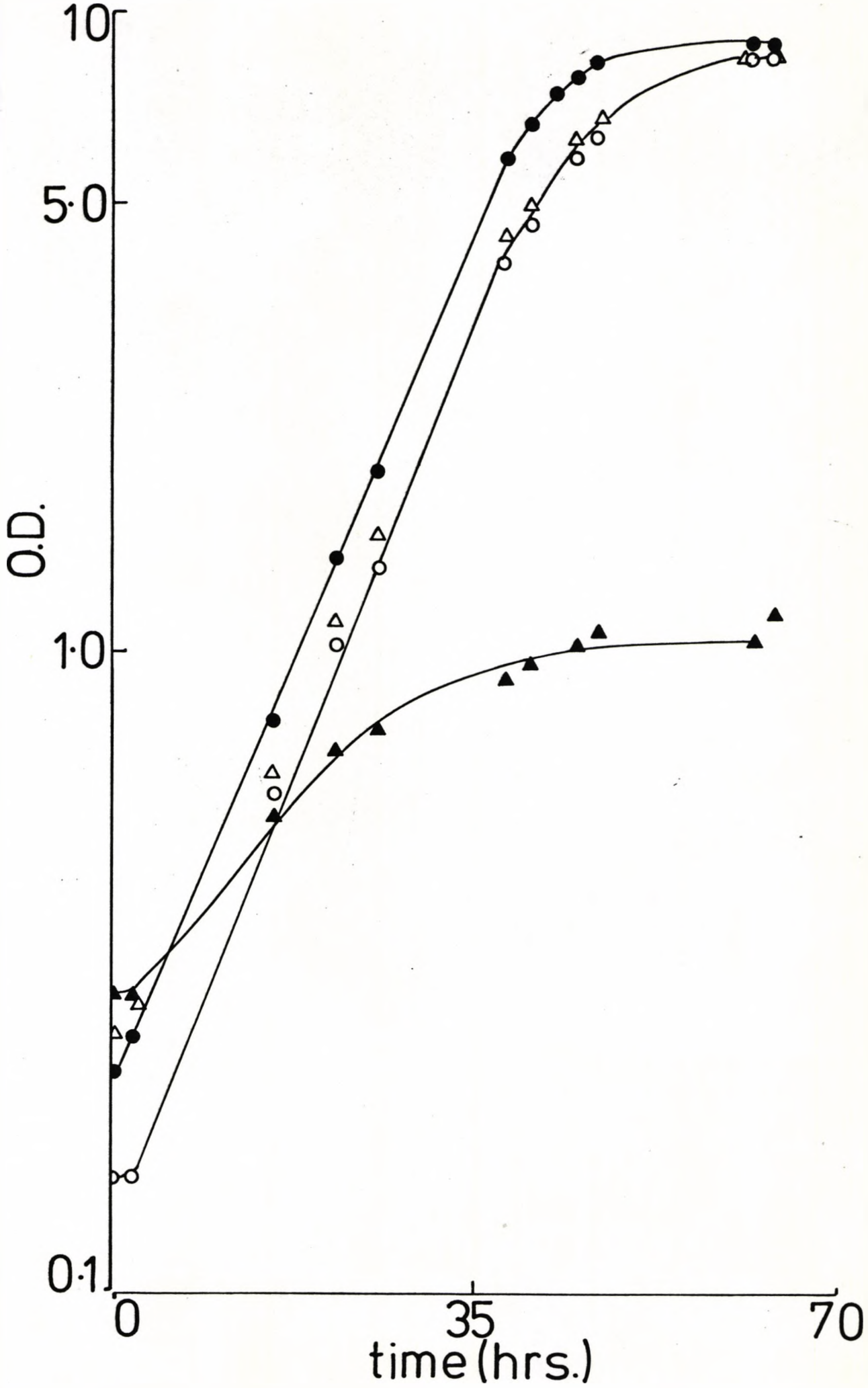

10
5·0
1·0
0·1
O.D.
0
35
70
time (hrs.)

Fig. 21: Effect of griseofulvin on the growth of Protomyces inundatus in YM-1 medium, measured by culture turbidity

(●), control

(○), control + 2% (v/v) DMF

(▲), 10μM griseofulvin

(△), 50μM griseofulvin

(▼), 100μM griseofulvin

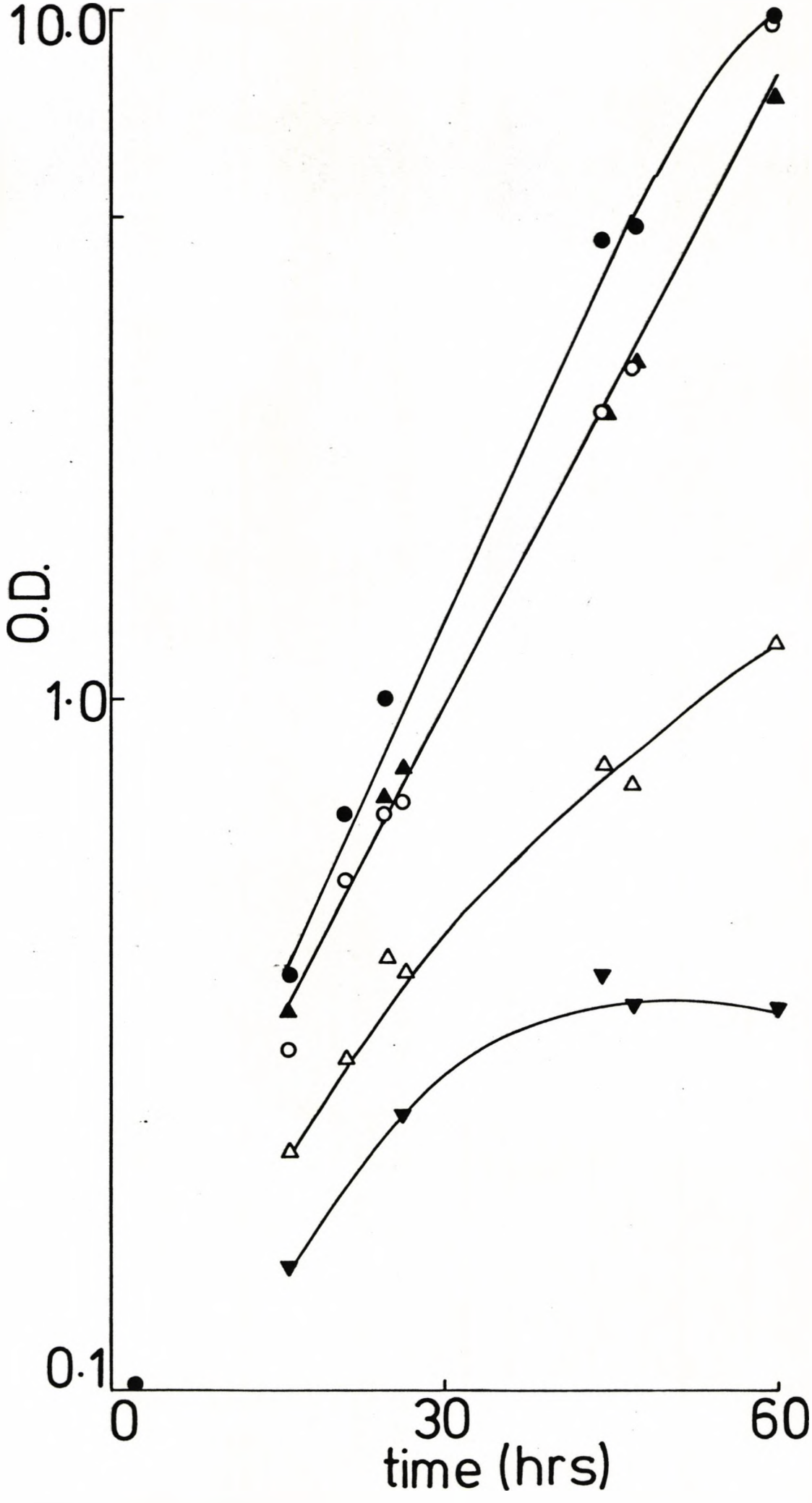

10·0
O.D.
1·0
0·1
0
30
60
time (hrs)

Fig. 22: Effect of griseofulvin on the growth of Protomyces inundatus in defined medium, measured by culture dry weight.

(●), control

(○), 50μM griseofulvin

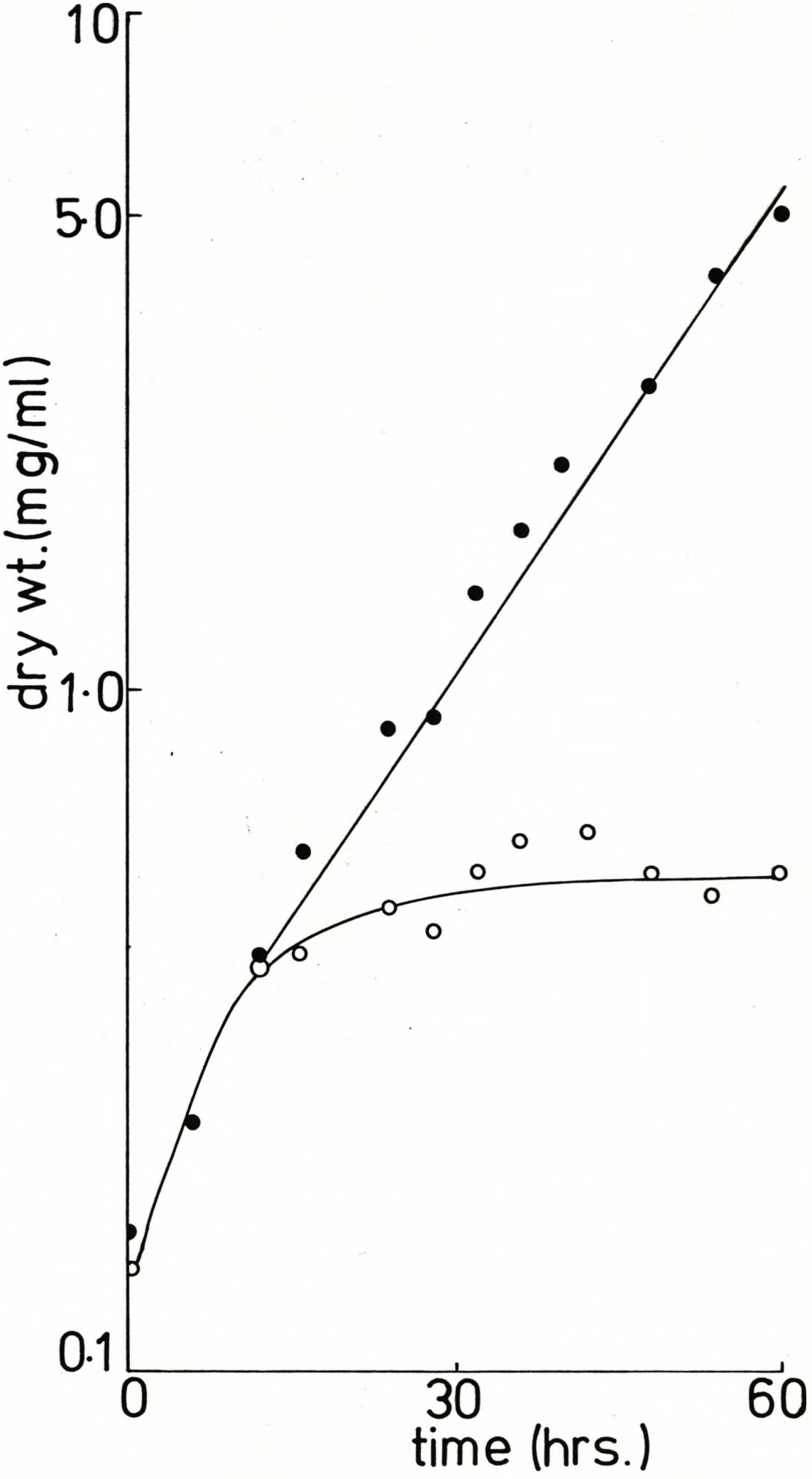
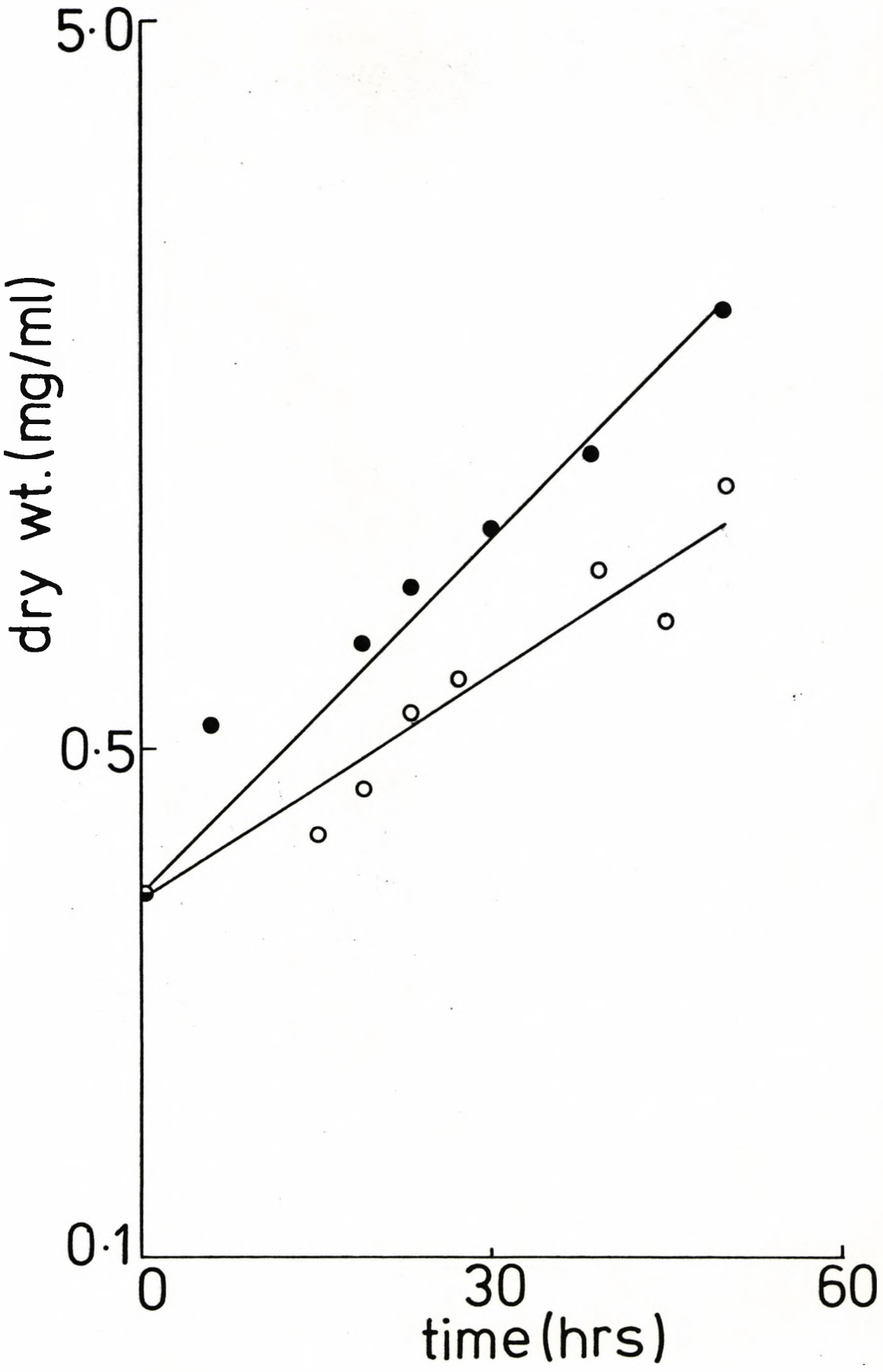

10
5·0
1·0
0·1
dry wt.(mg/ml)
0
30
60
time (hrs.)

Fig. 23: Effect of griseofulvin on the growth of Protomyces inundatus in MYGP, measured by culture dry weight.

(●), control

(○), 50μM griseofulvin

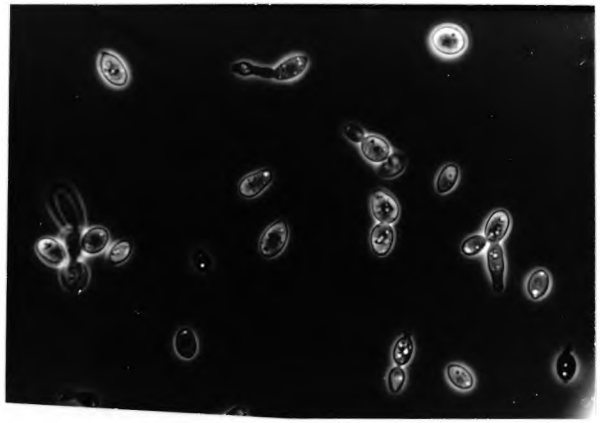
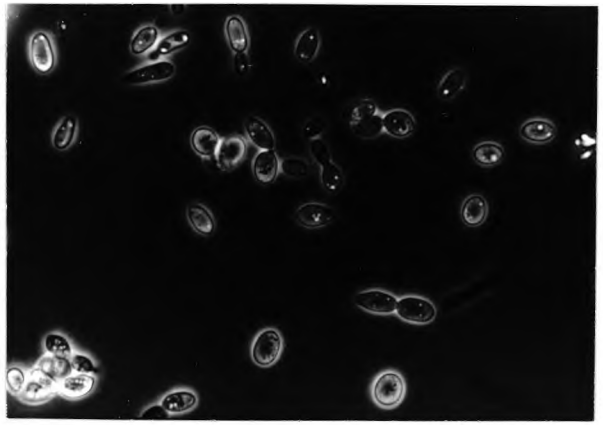
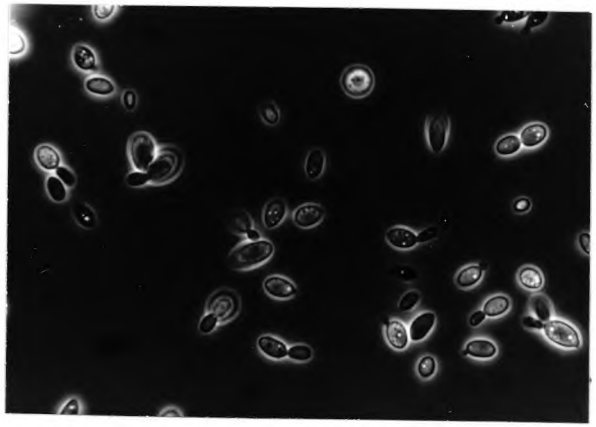
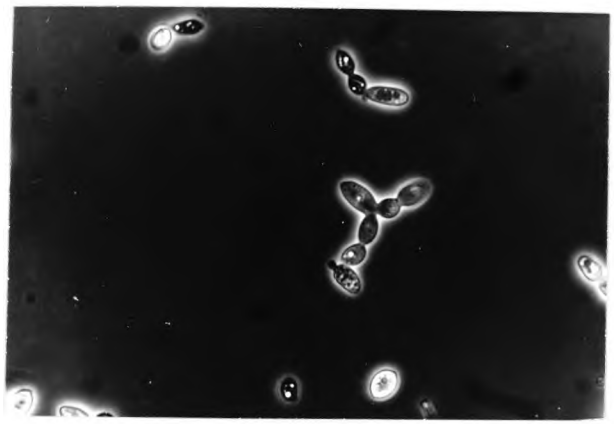
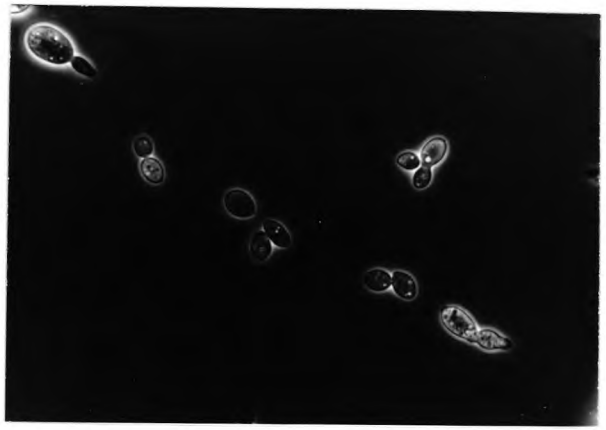
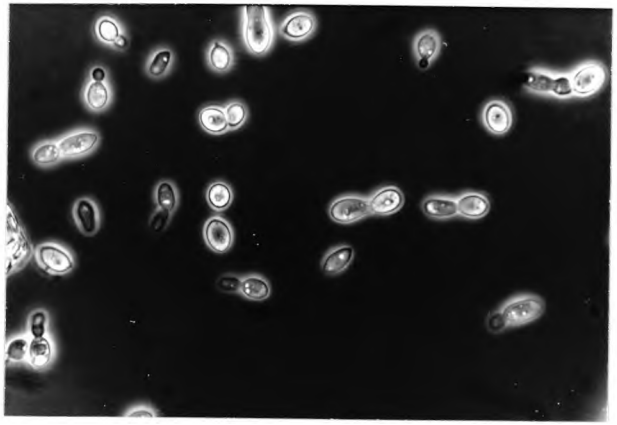

5·0
0·5
0·1
dry wt.(mg/ml)
0
30
60
time(hrs)

Light micrographs of Protomyces inundatus grown in defined medium for 24 hours (x 490)

Figs. 24 & 25: Control cells

Figs. 26 & 27: Cells grown in the presence of 10μM griseofulvin

Figs. 28 & 29: Cells grown in the presence of 50μM griseofulvin

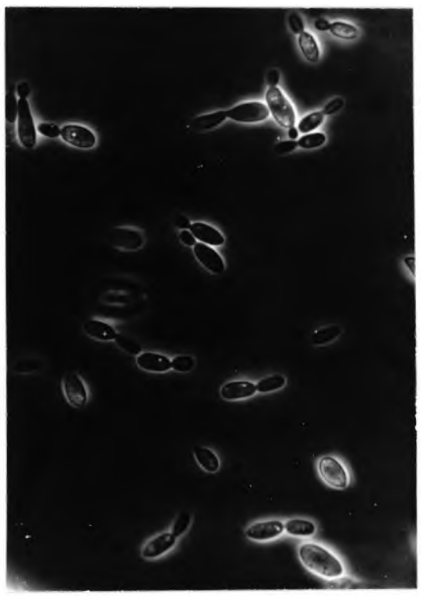







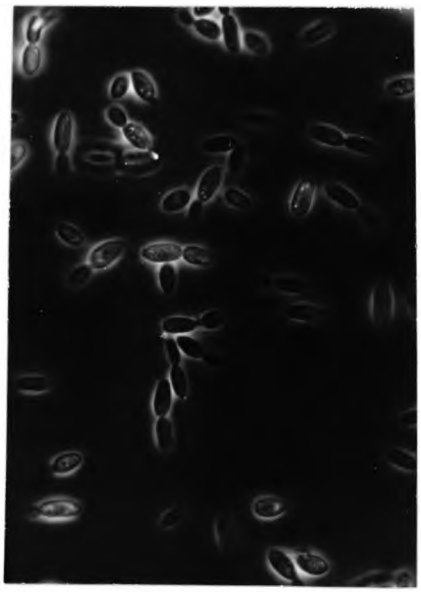
Light micrographs of Protomyces inundatus grown in YM-1 complex medium for 48 hours

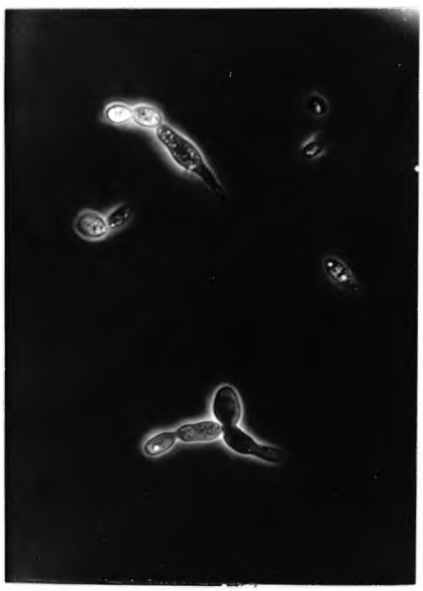
Figs. 30 & 31: Control (x 490)

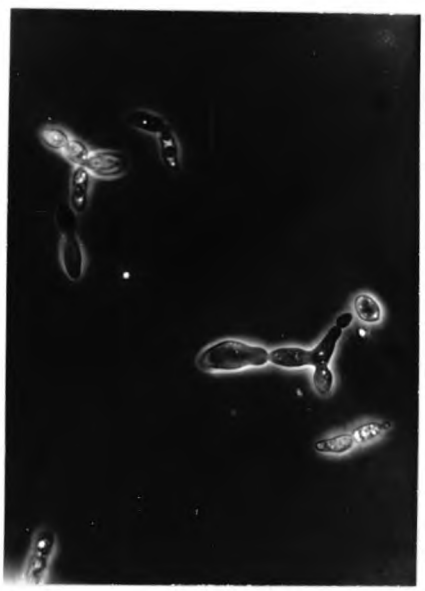
Figs. 32, 33 & 34: Cells grown in the presence of 100µM griseofulvin (x 490)

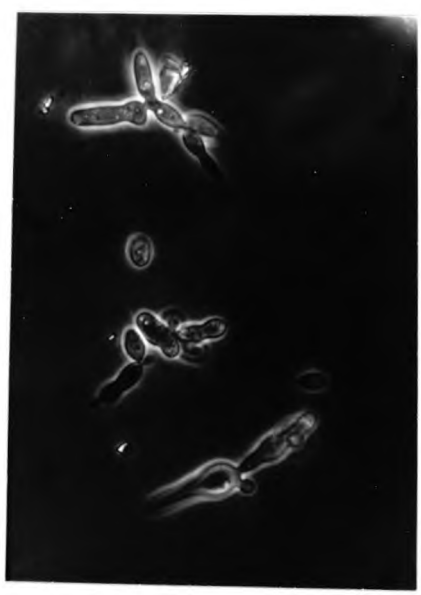
Fig. 35: Cells grown in the presence of 100µM griseofulvin (x 620)

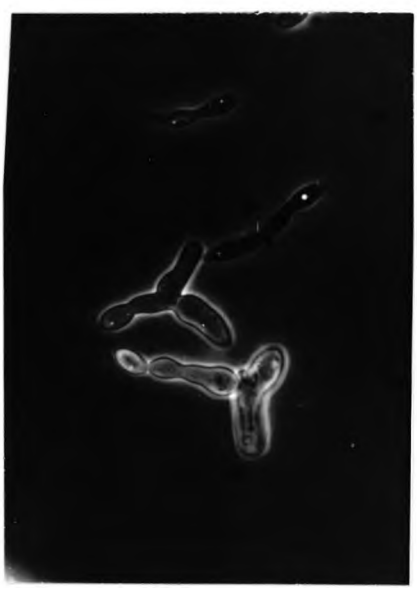












Scanning electron microscopy

Scanning electron microscopy shows these characteristics more clearly. Control cells are oval, 'bottle-shaped' cells, budding at the apices with the daughter cells detaching after division is complete (Figs. 36-38). Cells treated with griseofulvin for 22 hours begin to show abnormal characteristics e.g. elongation of cells and retention of buds attached to the parent cell.

Transmission electron microscopy

Normal and griseofulvin treated cells of Protomyces inundatus were prepared for electron microscopy after approx. 48 hours of growth, and the cells, in particular the nuclei, were examined for abnormalities due to drug treatment.

Examples of control nuclei are shown in Figs. 42-45. In section they appear as normal, rounded nuclei with a single prominent nucleolus. Spindle pole bodies were also occasionally seen. Griseofulvin treated cells demonstrated the abnormal morphology previously seen using light and scanning electron microscopy. Again, drug-treated Protomyces cells have the appearance of groups of cells. Low magnification micrographs (Figs. 46 & 47) show that no septa have formed to divide the buds from the parent. Only one enlarged nucleus is apparent per group of cells, suggesting that bud emergence and duplication of cytoplasmic components has occurred, but that nuclear division has not taken place.

When examined in greater detail, the nuclei of griseofulvin treated cells appear to be irregular and lobed in shape (Figs. 49, 50, 56 & 57). Spindle pole bodies are prominent and frequently encountered (Figs. 48-52), suggesting that these cells are arrested at some stage of nuclear division. Microtubules are visible associated with, and radiating from, spindle pole

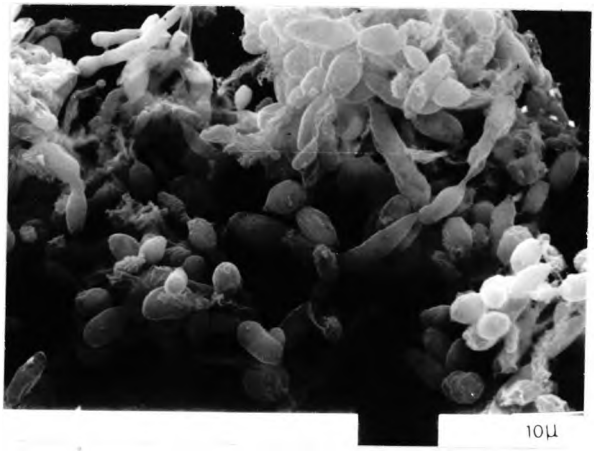
Figs. 36, 37 & 38: Scanning electron micrographs of Protomyces inundatus cells grown for 22 hours in defined medium

4μ

2μ

4μ

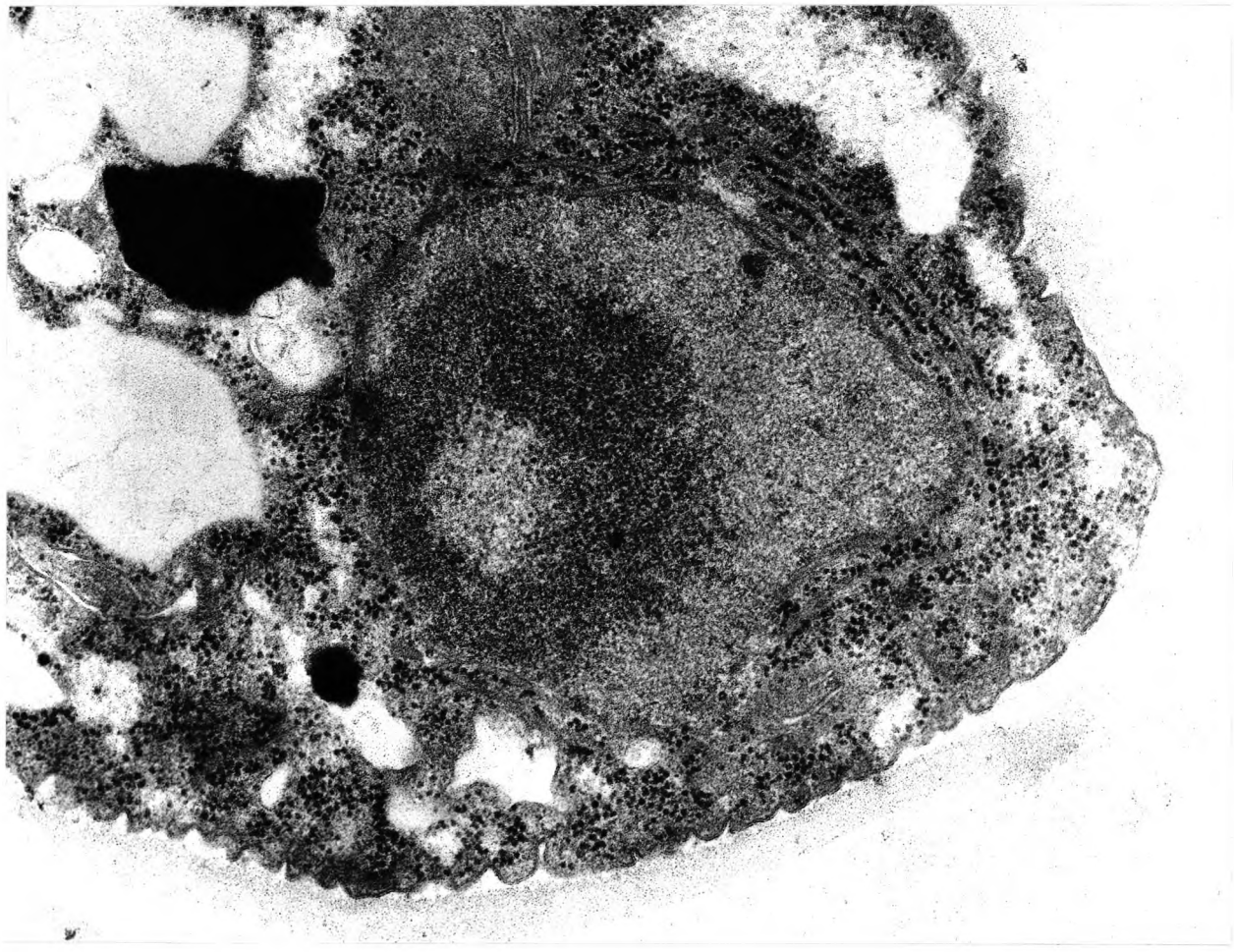
Figs. 39, 40 & 41: Scanning electron micrographs of Protomyces inundatus cells grown in defined medium for 48 hours in the presence of 50μM griseofulvin


10μ


4μ


4μ

Figs. 42 & 43: Nuclei of control cells of Protomyces inundatus (x 55,000)



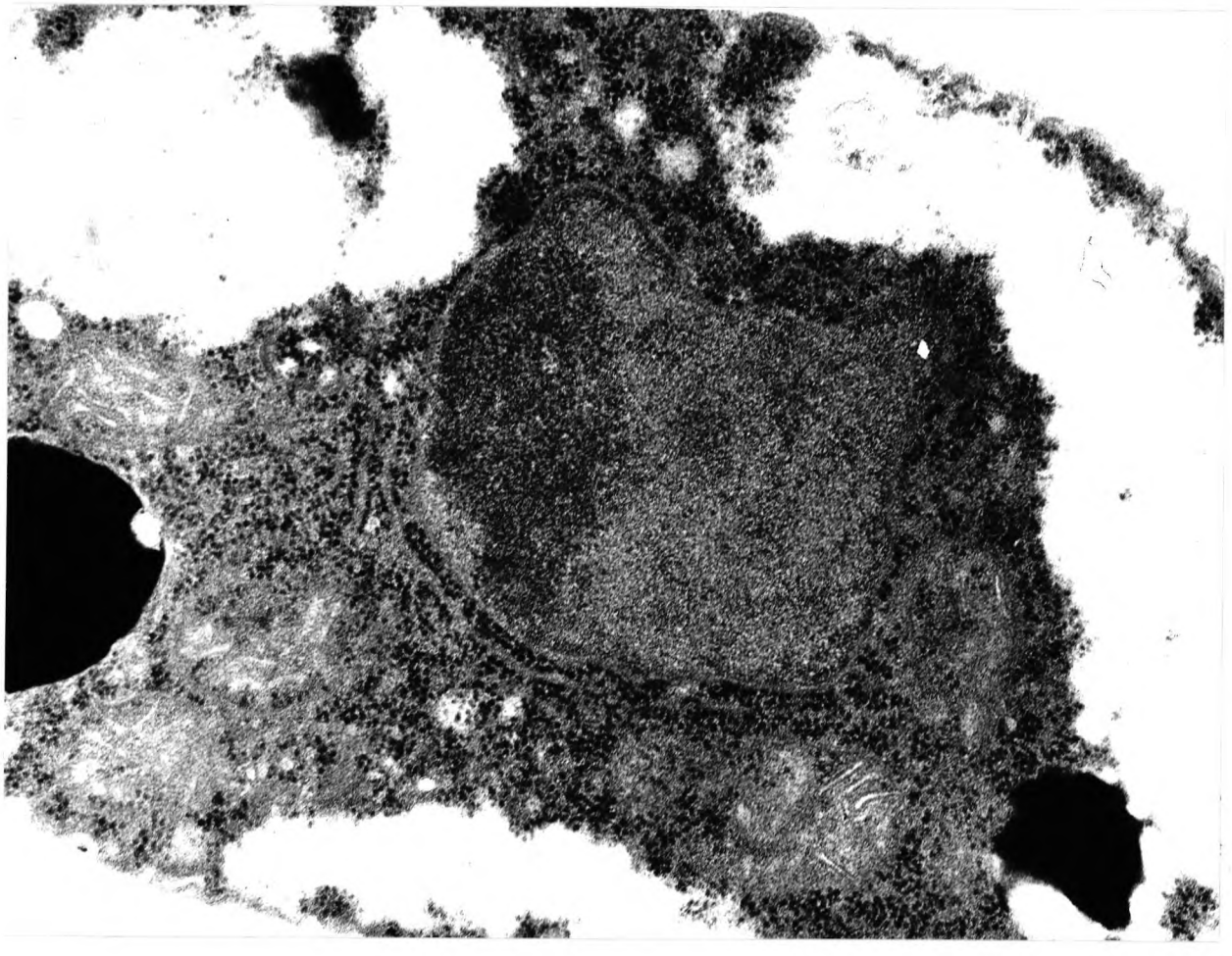
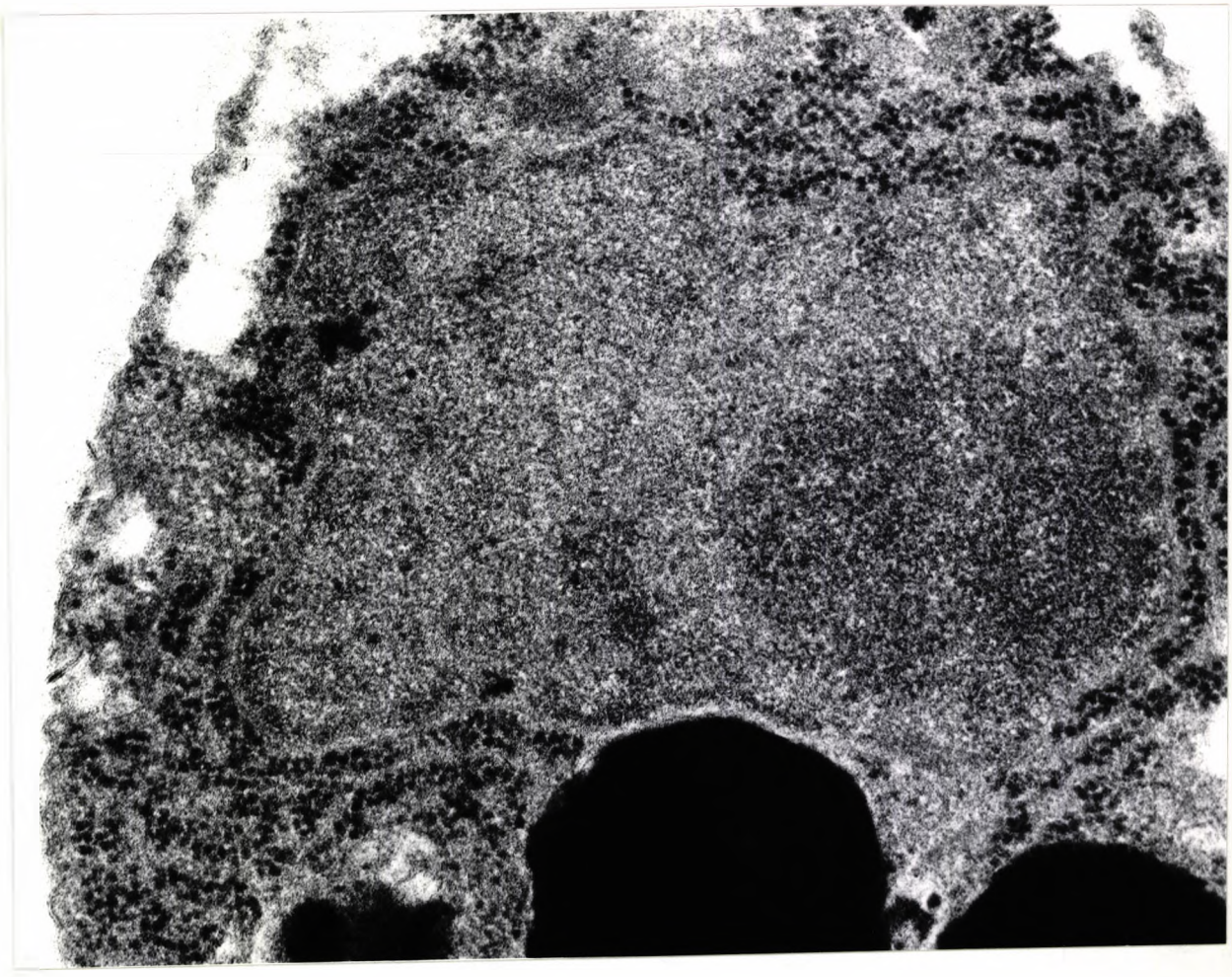
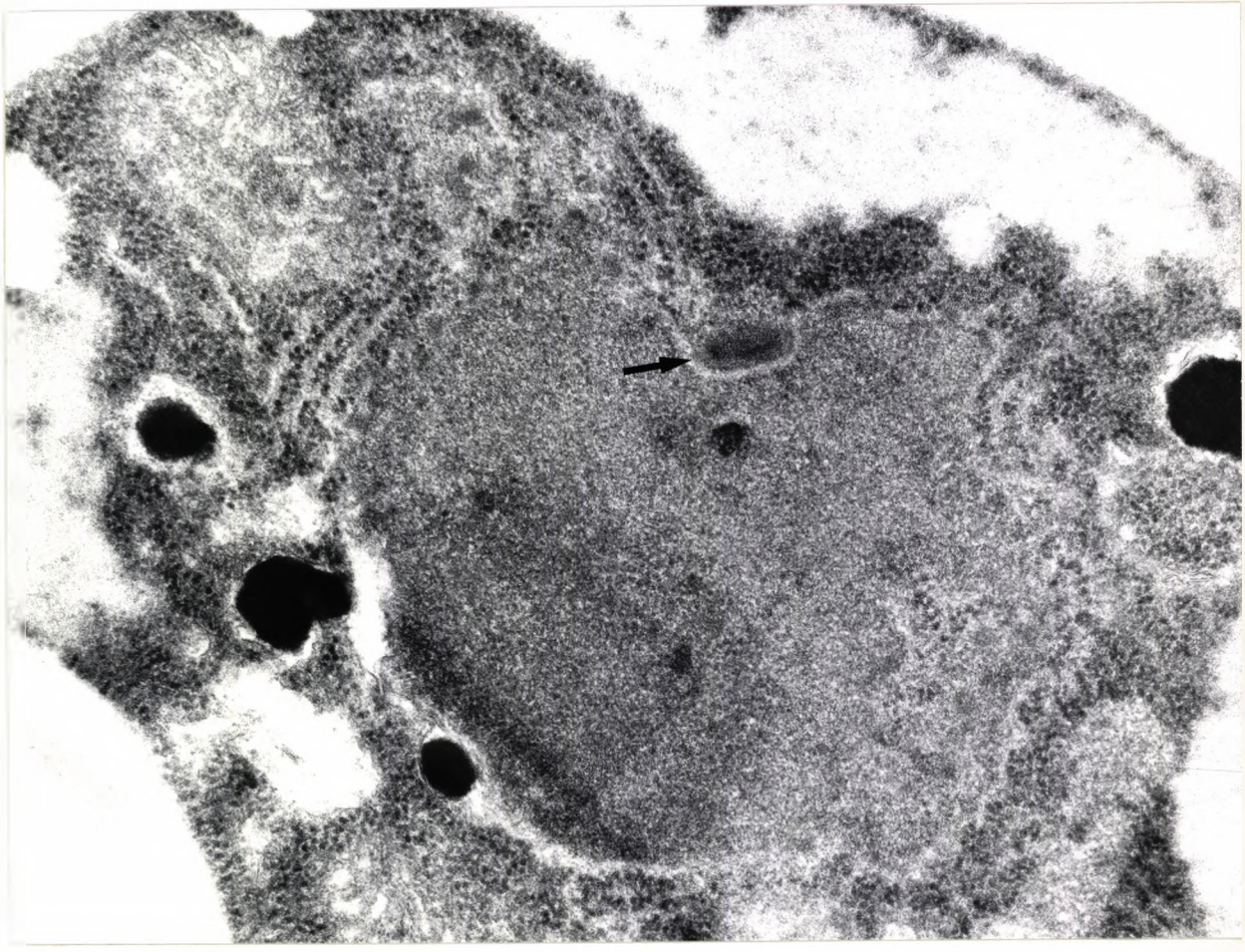


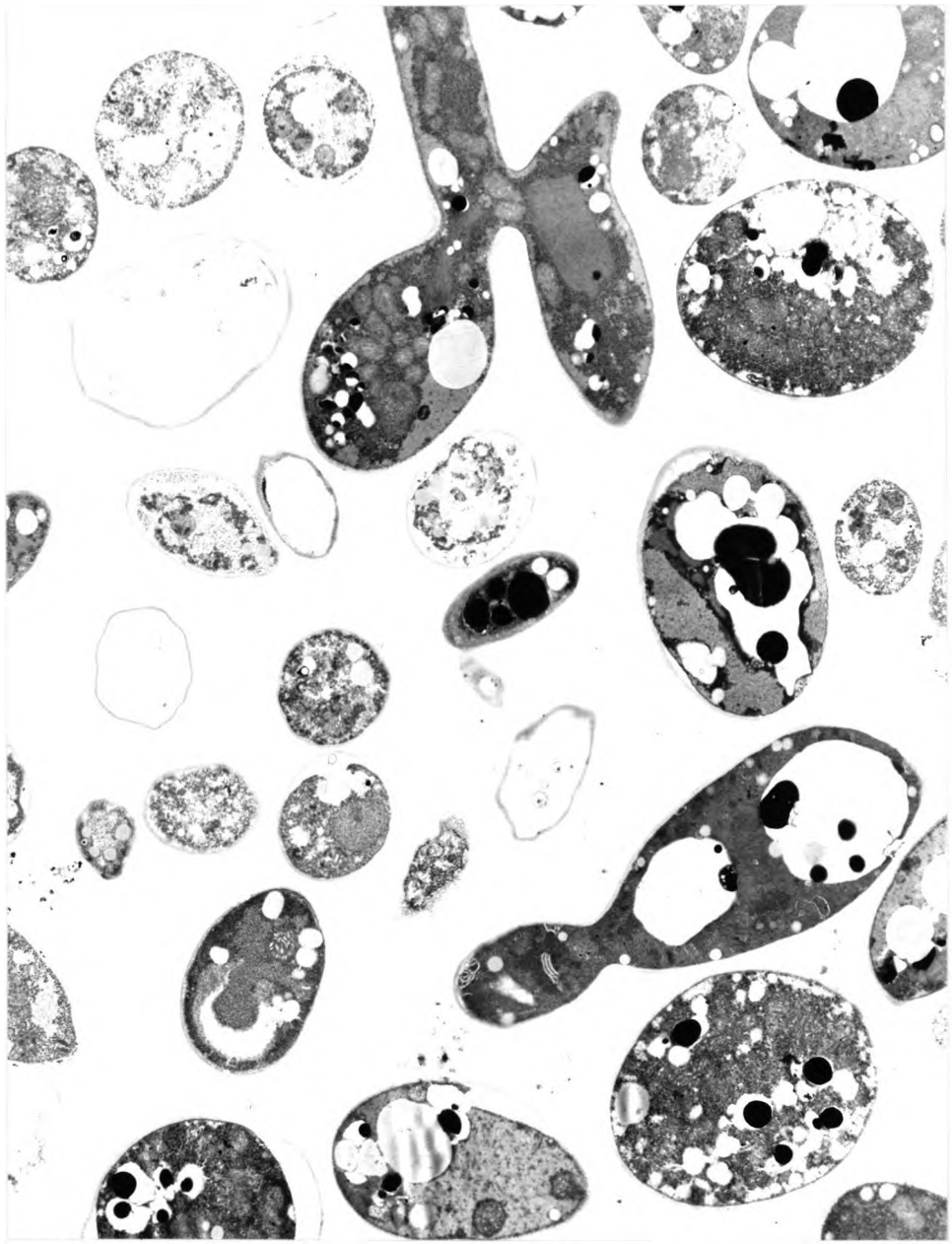
Fig. 44: Control nucleus (x 87,000)

Fig. 45: Control nucleus, showing spindle pole body (arrowed) (x 56,000)





Figs. 46 & 47: Griseofulvin-treated cells of Protomyces inundatus, showing abnormal morphology (x 5,500)



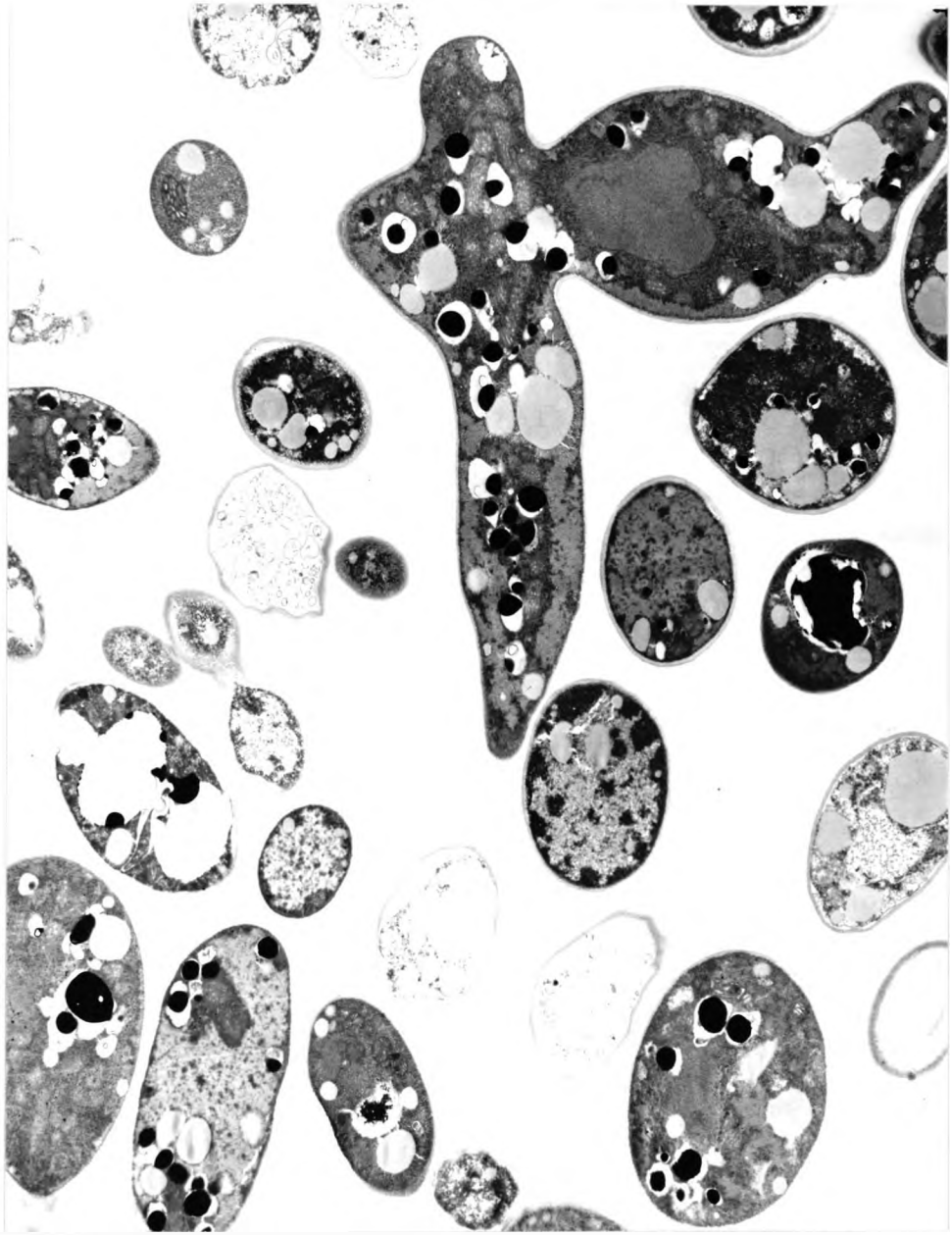
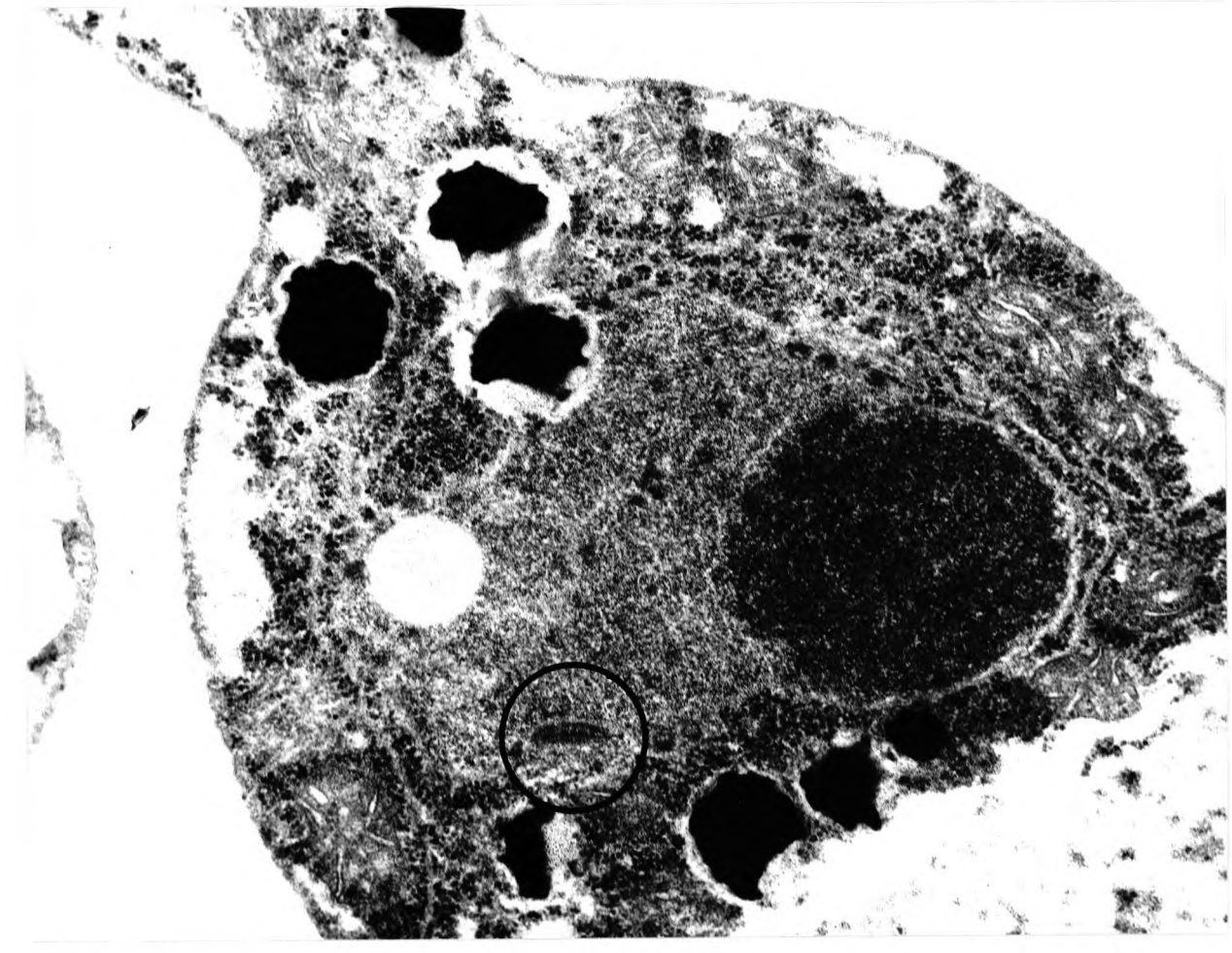
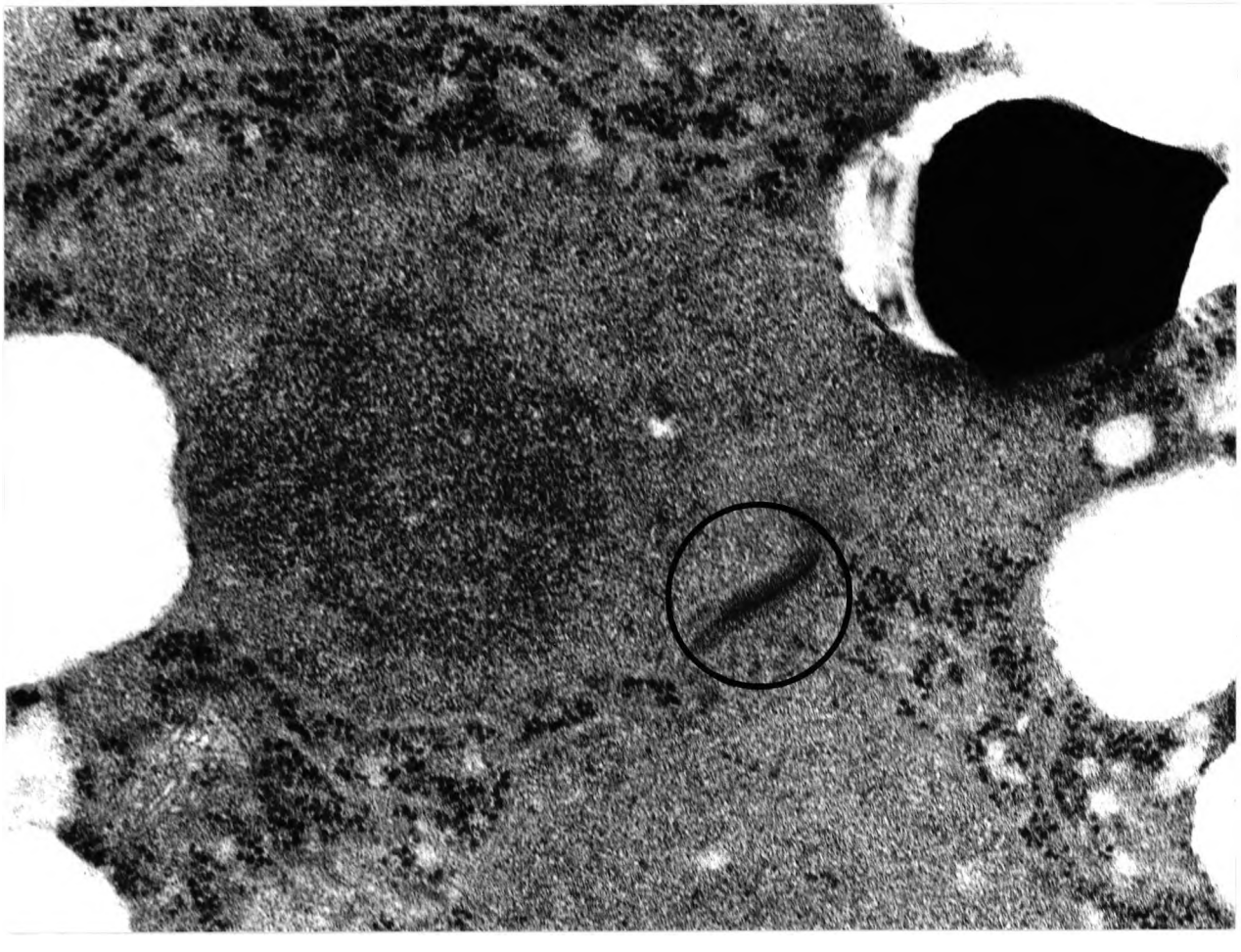


Fig. 48: Nucleus of a griseofulvin-treated cell, showing spindle pole body (x 42,000)

Fig. 49: As above (x 68,000)





Figs. 50 & 51: Non-consecutive serial sections of a griseofulvin-treated nucleus, showing three aspects of the spindle pole body (circled) (x 67,000)

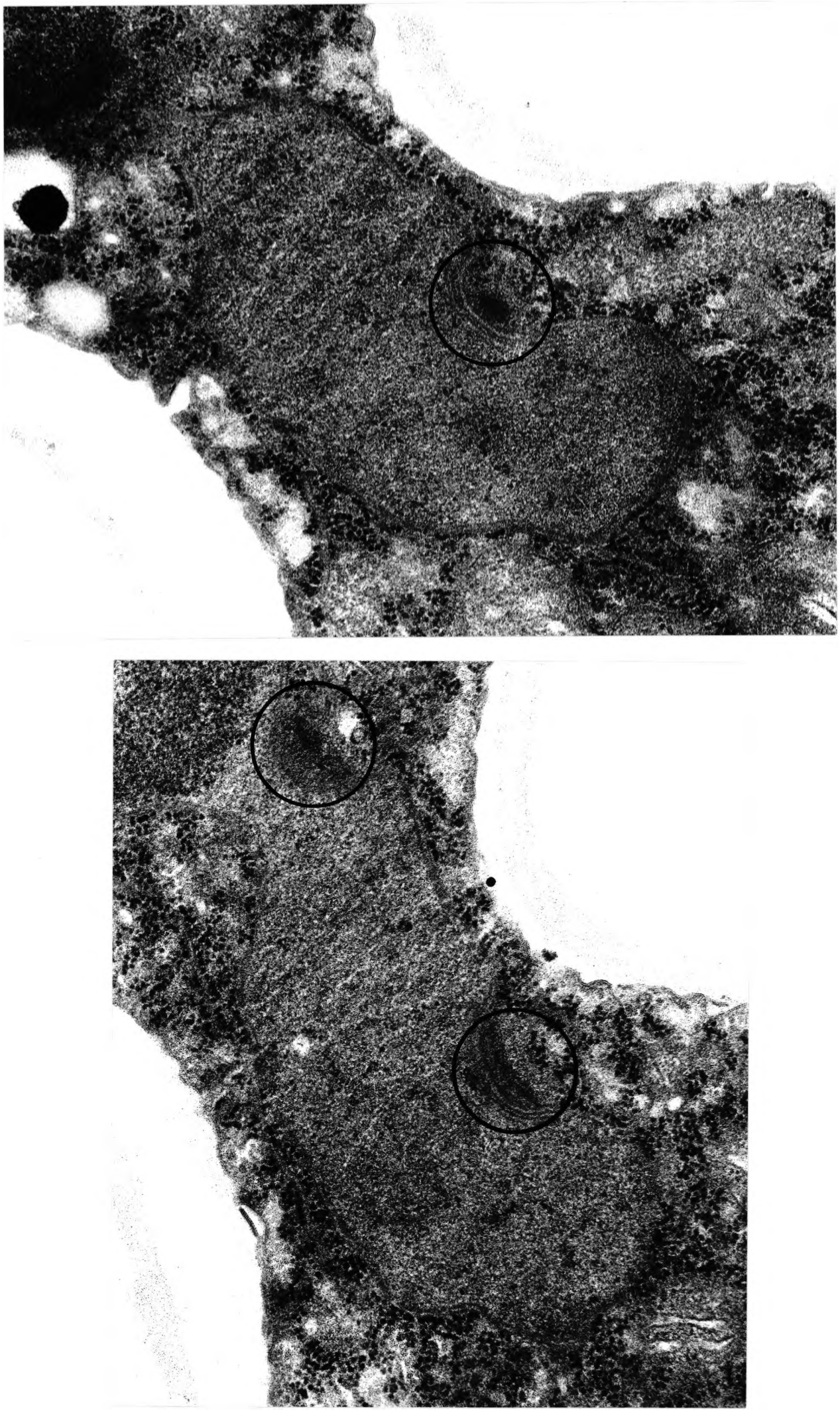
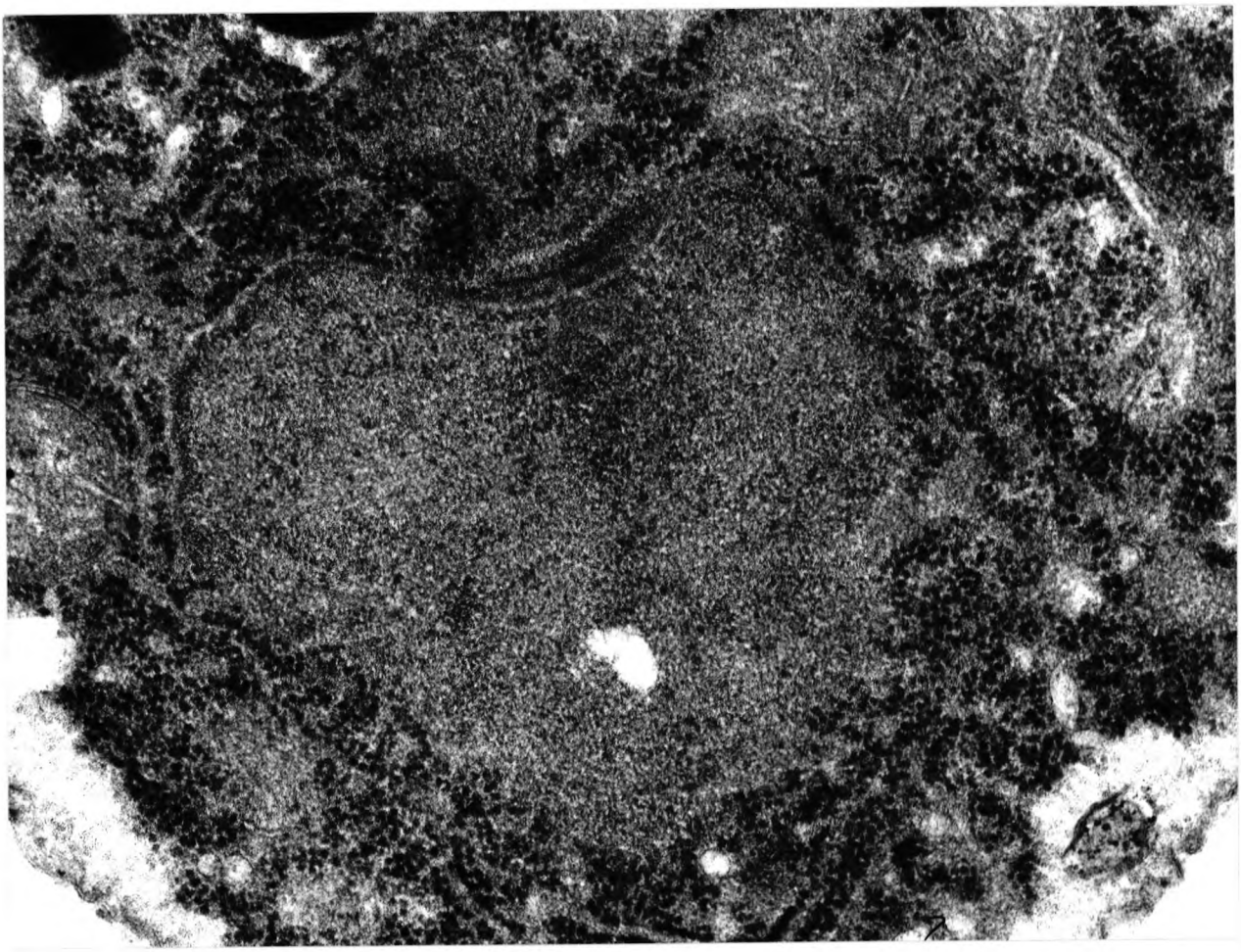


Fig. 52: Nucleus of a griseofulvin-treated nucleus, showing a spindle pole body (x 64,000)

Fig. 53: Nucleus of a griseofulvin-treated cell, showing a transverse section of the spindle pole body with radiating microtubules (arrowed) (x 57,000)



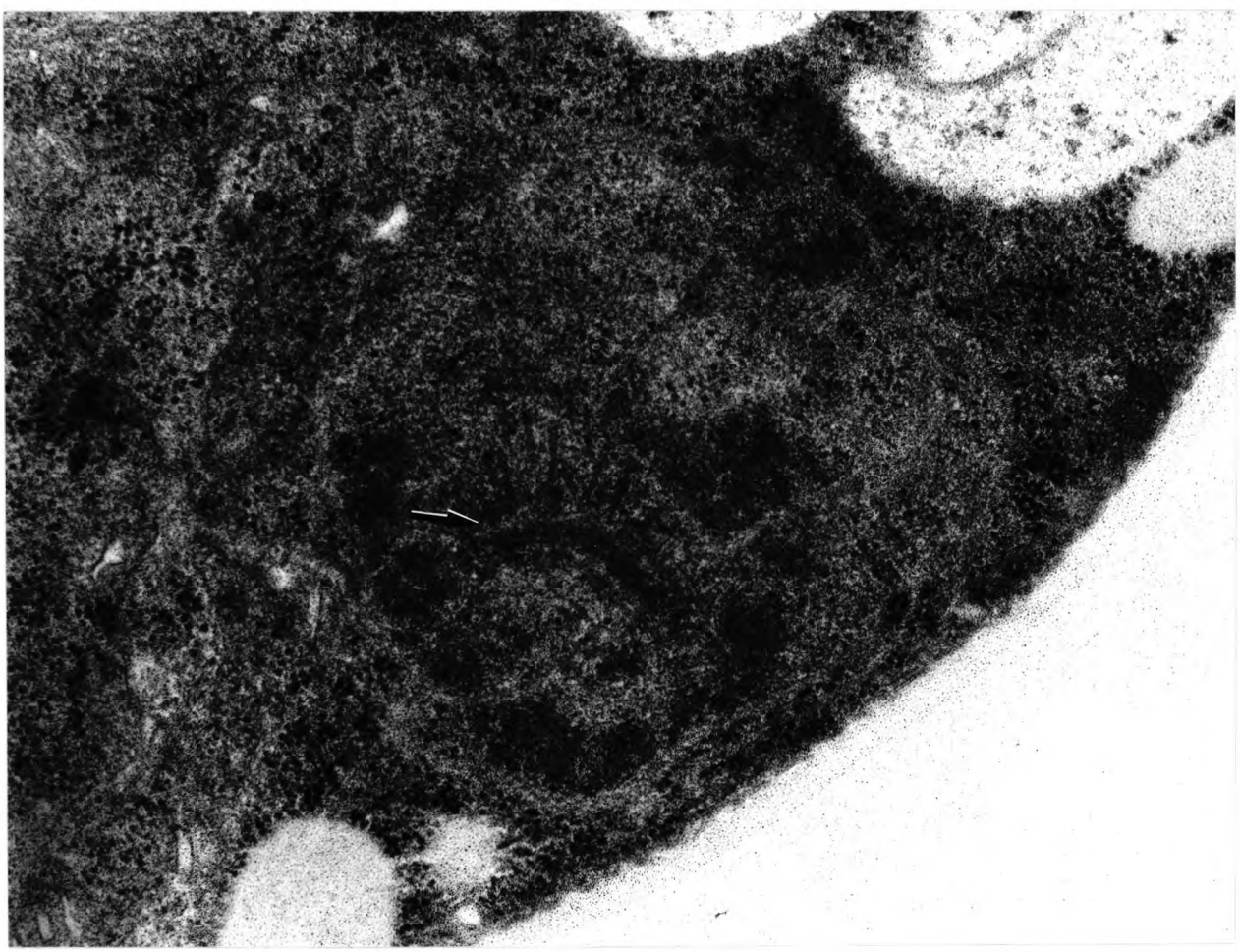
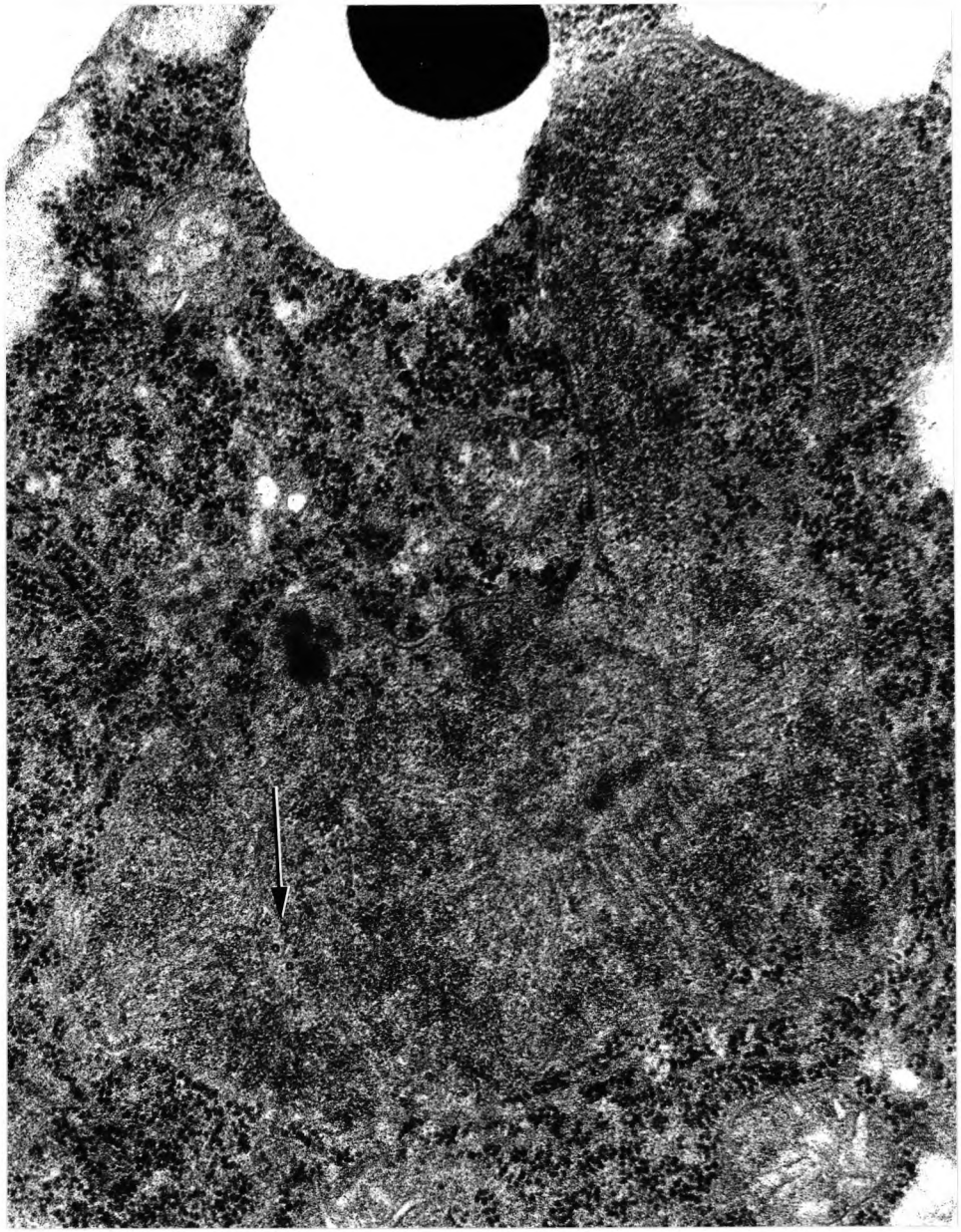


Fig. 54: Nucleus of a griseofulvin-treated cell, showing the lobed appearance of the nucleus. Spindle pole bodies are seen in glancing section (arrowed), with radiating microtubules (x 40,000)

Fig. 55: As above; microtubules can be seen in transverse section (arrowed) (x 56,000)



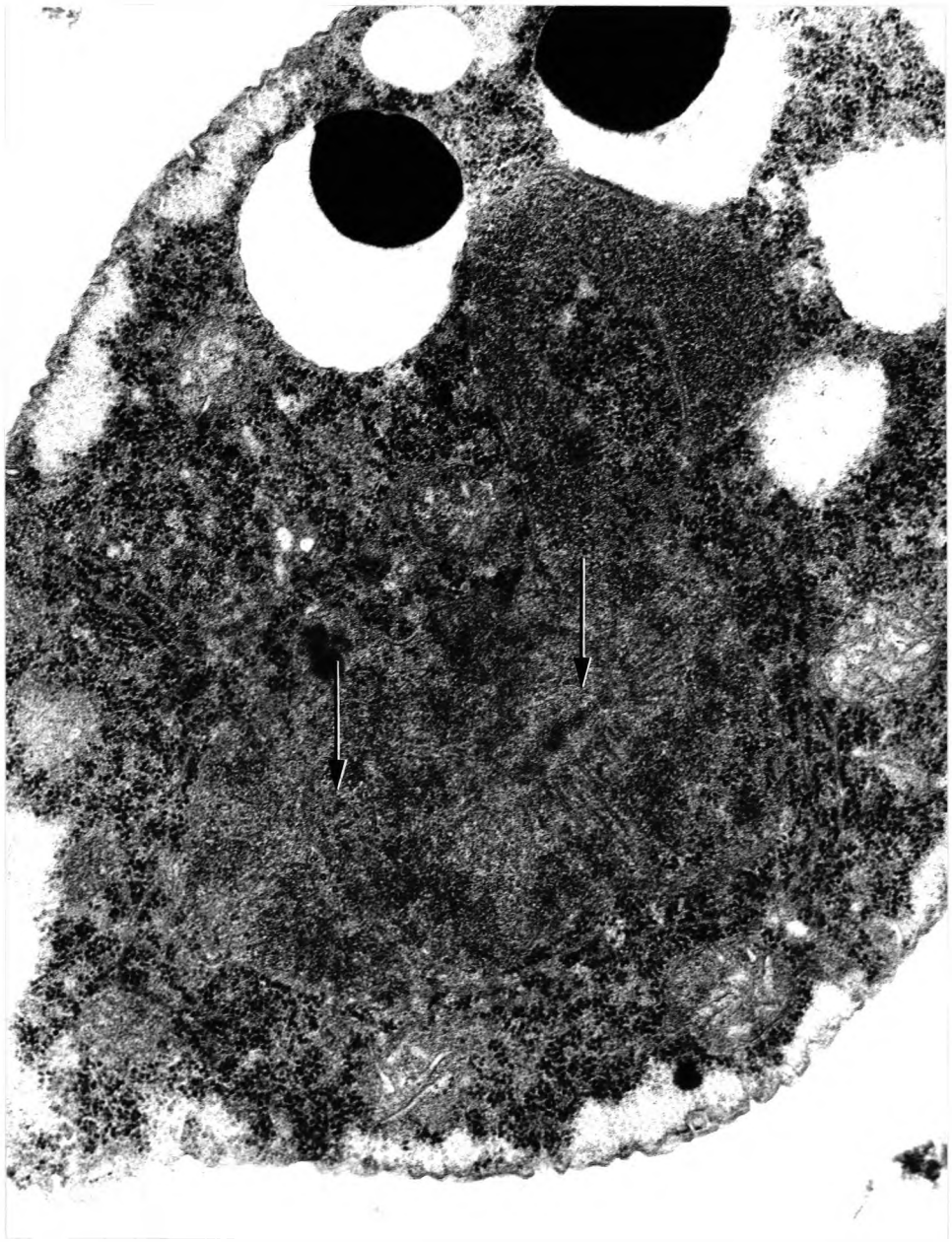
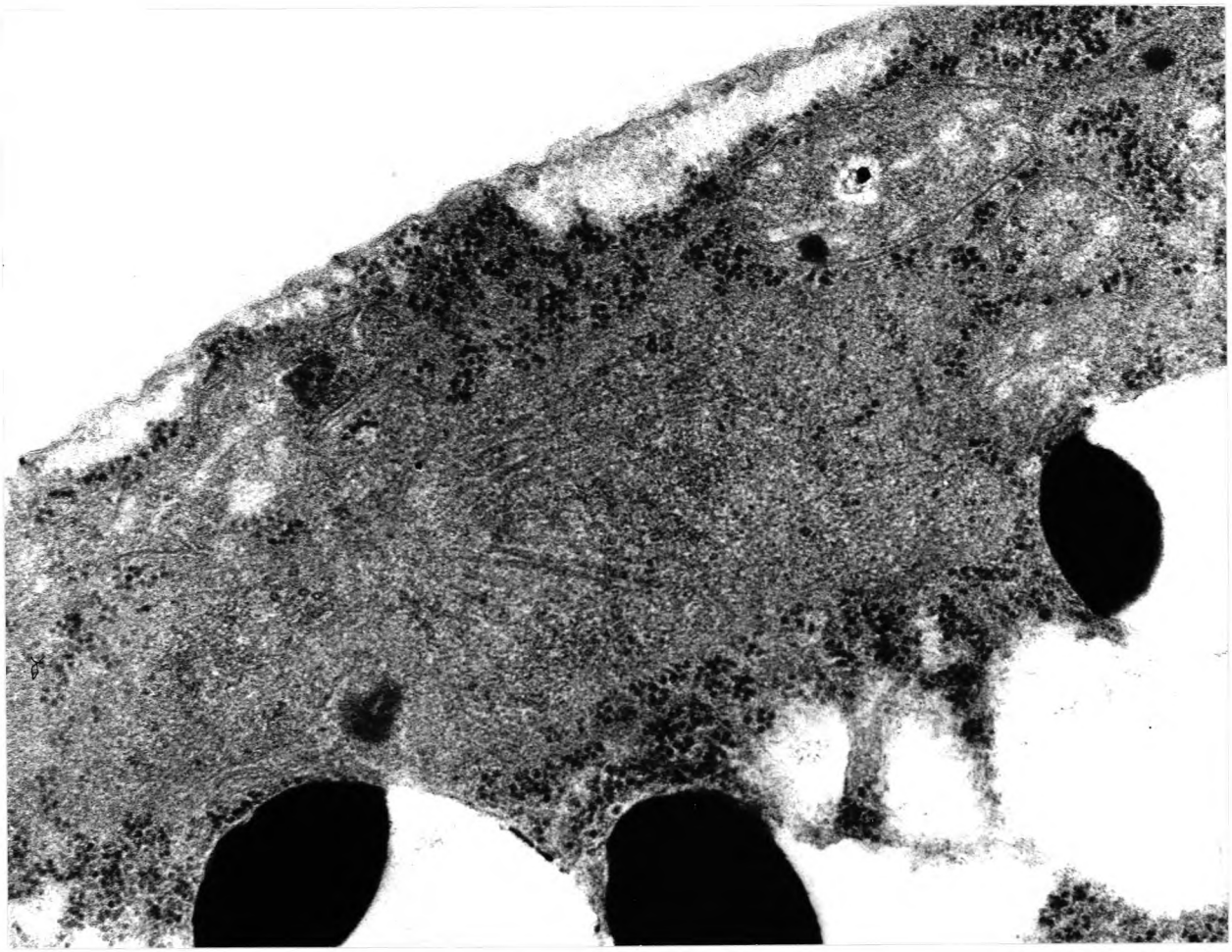


Fig. 56: Nucleus of a griseofulvin-treated cell, showing radiating microtubules in longitudinal and transverse section (x 63,000)

Fig. 57: Nucleus of a griseofulvin-treated cell, showing a spindle pole body with associated microtubules (arrowed), surrounded by chromatin aggregates (x 51,000)



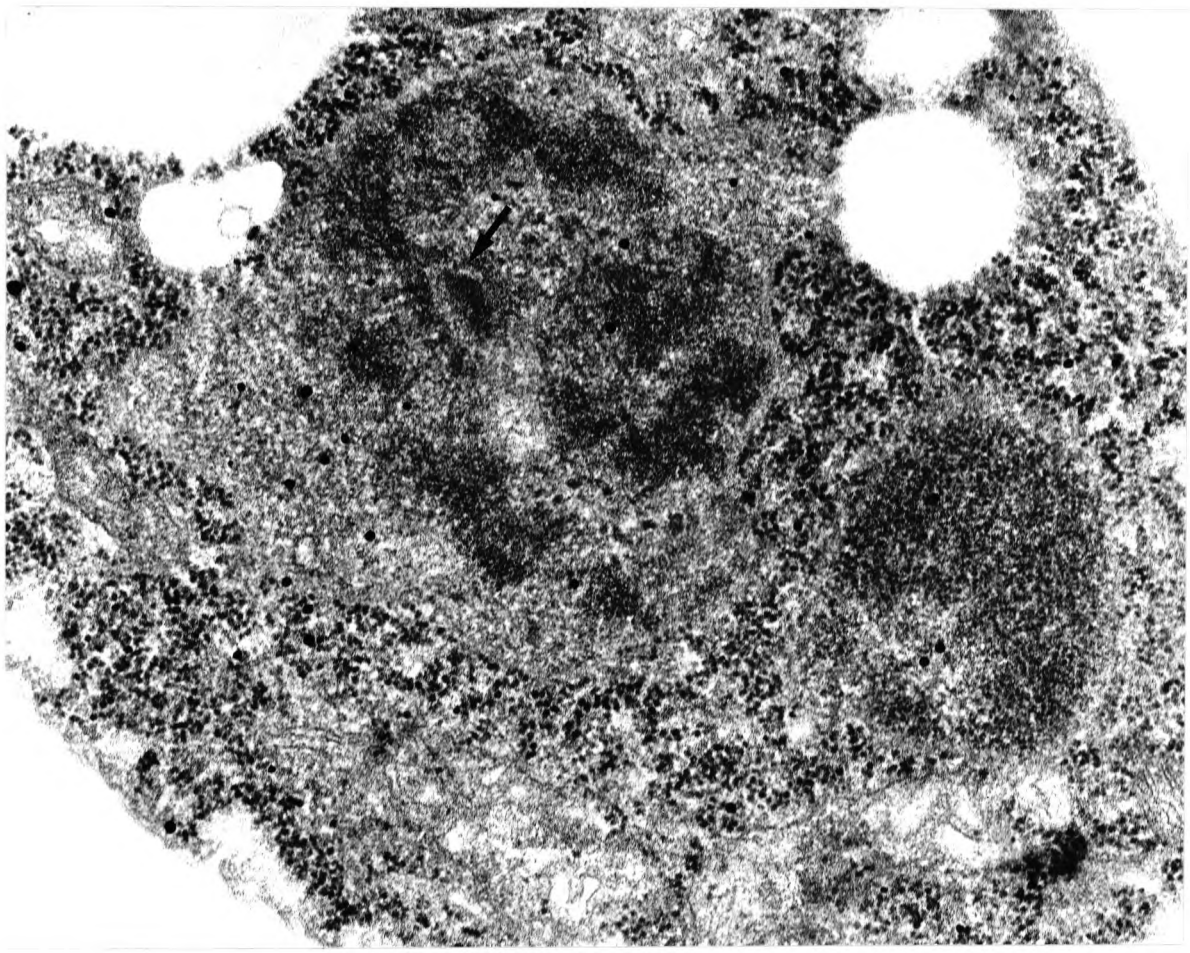
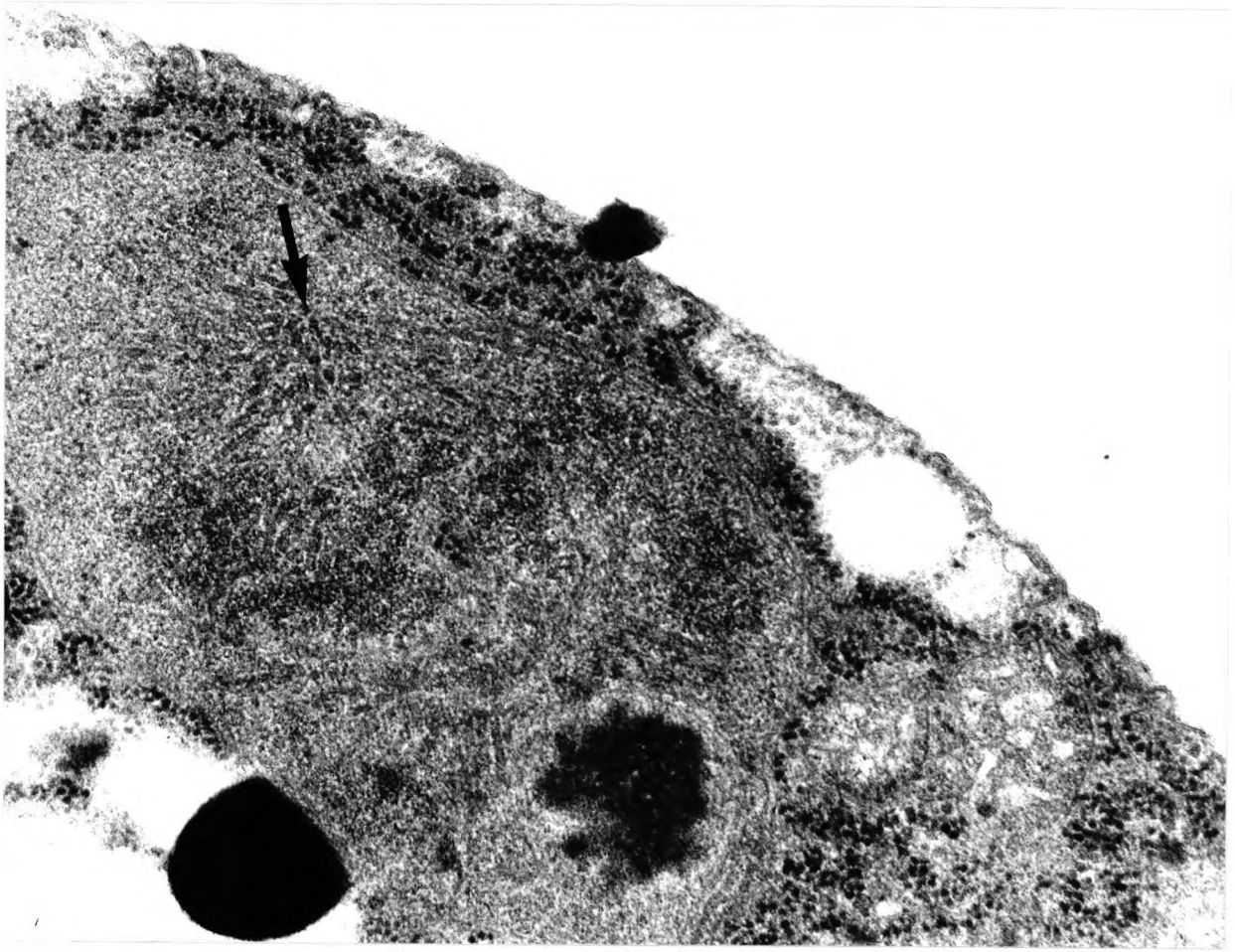


Fig. 58: Griseofulvin-treated nucleus, containing assembled microtubules (arrowed) (x 62,000)

Fig. 59: As above (x 58,000)



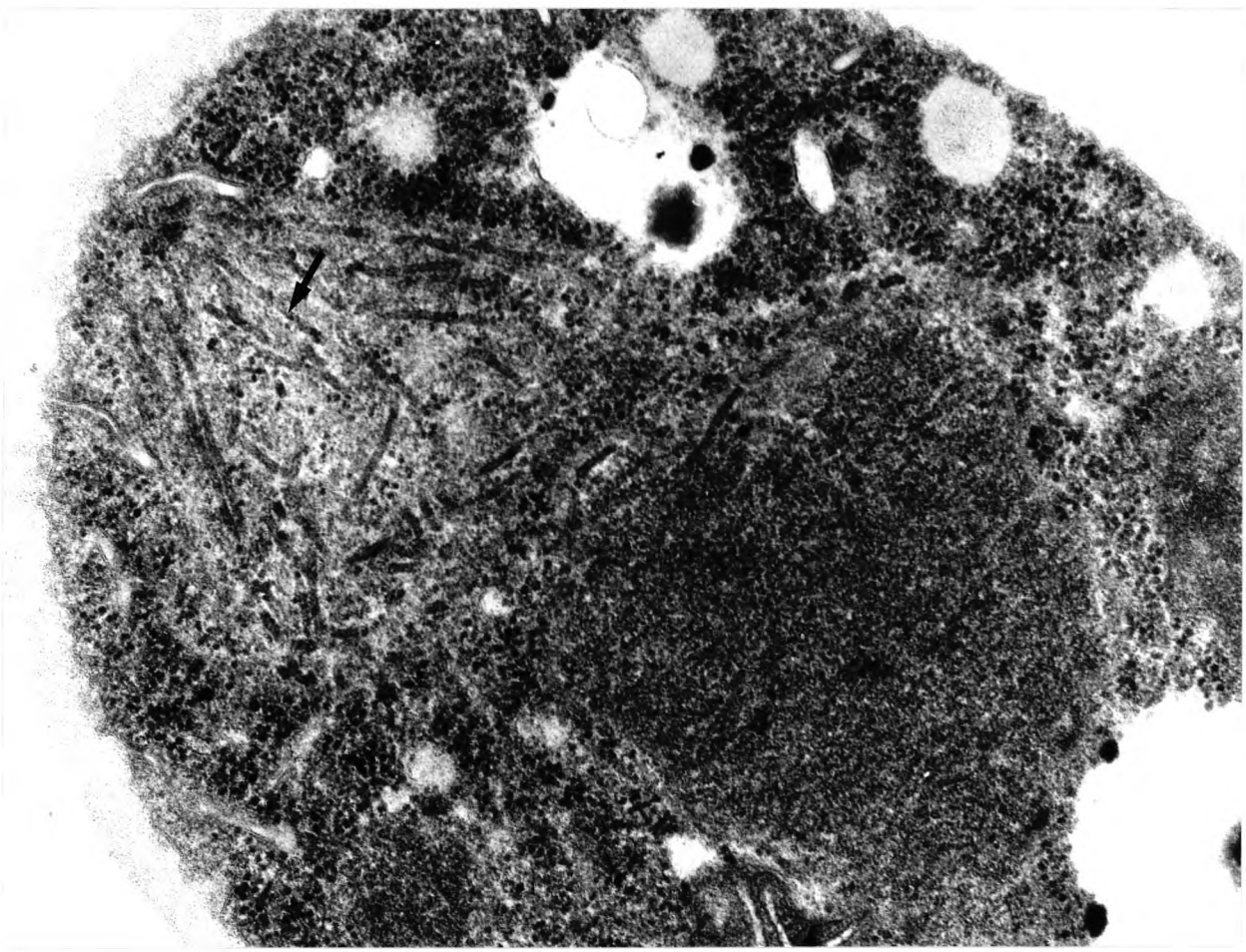
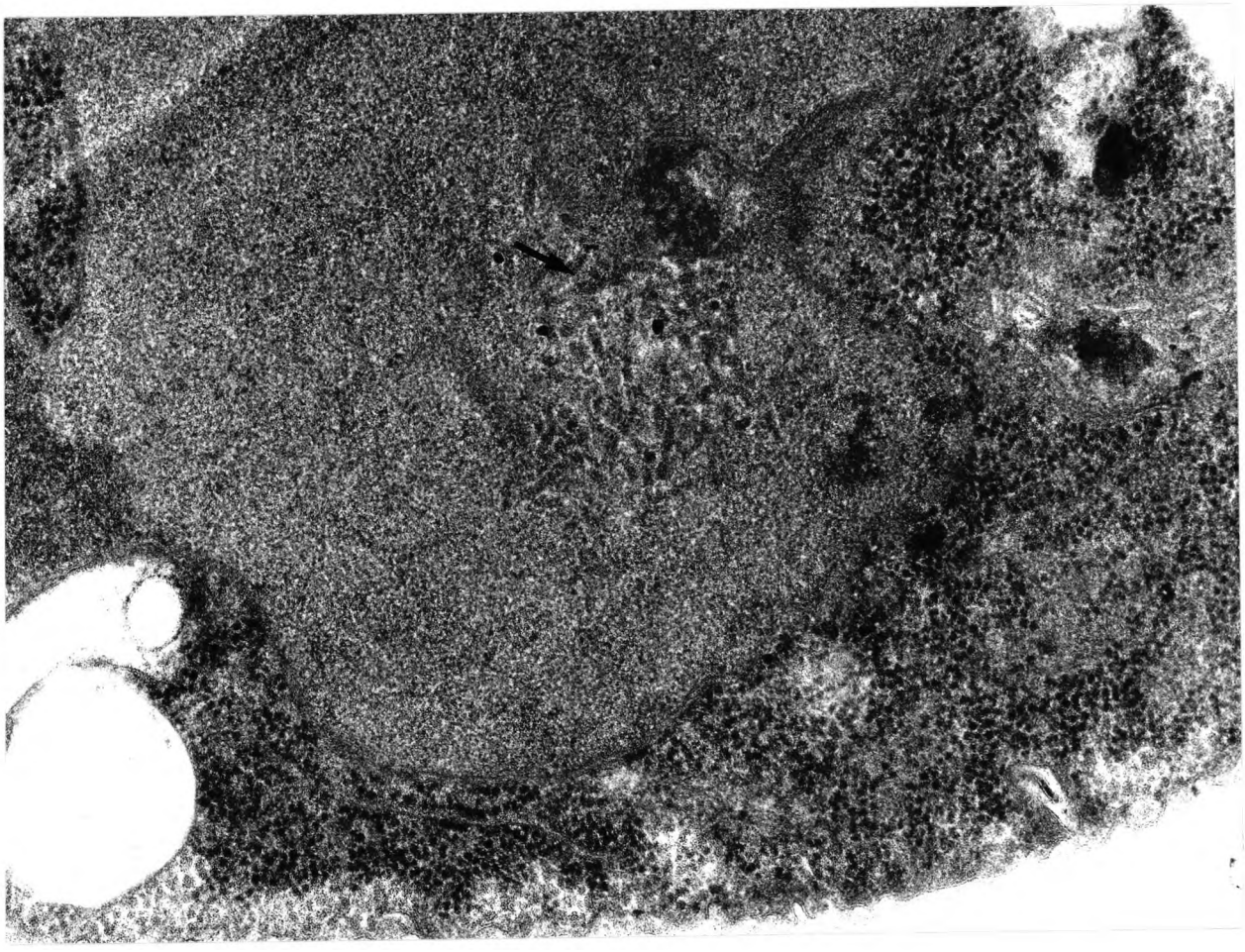


Fig. 60: Griseofulvin-treated nucleus, showing aggregates of assembled microtubules (arrowed) (x 68,000)

Fig. 61: As above (x 54,000)



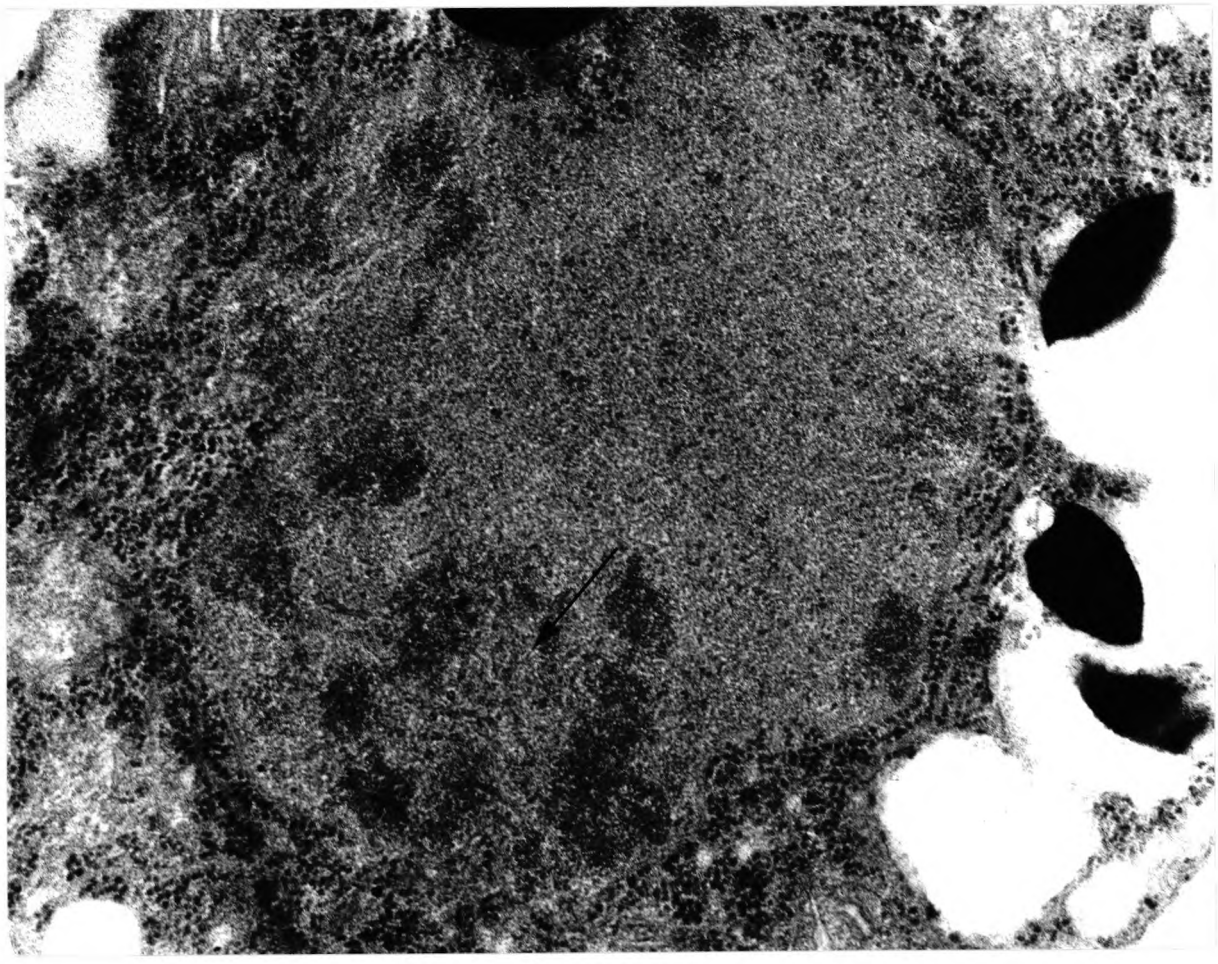
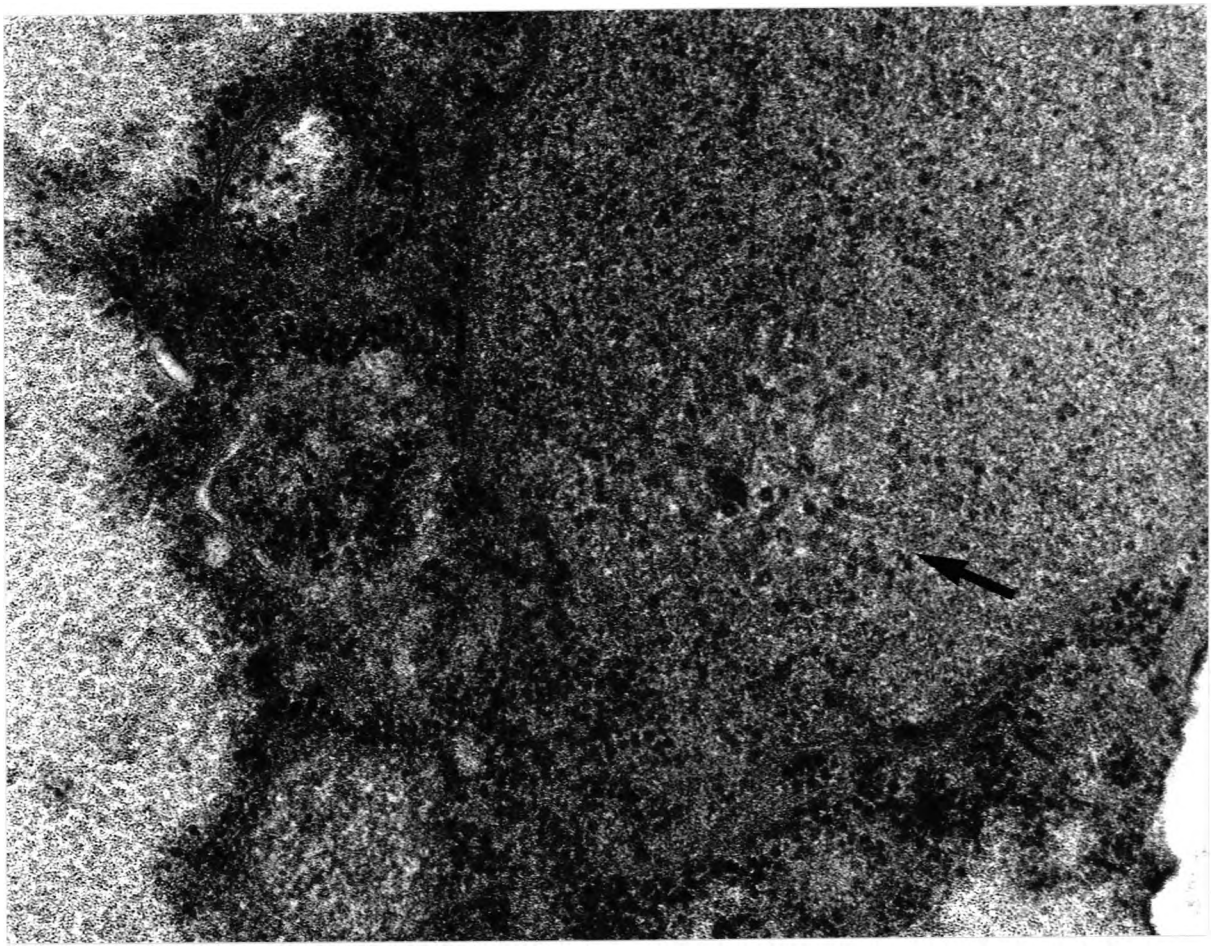


Fig. 62: Griseofulvin-treated nucleus, showing aggregates of assembled microtubules (arrowed) (x 88,000)

Fig. 63: As above (x 80,000)



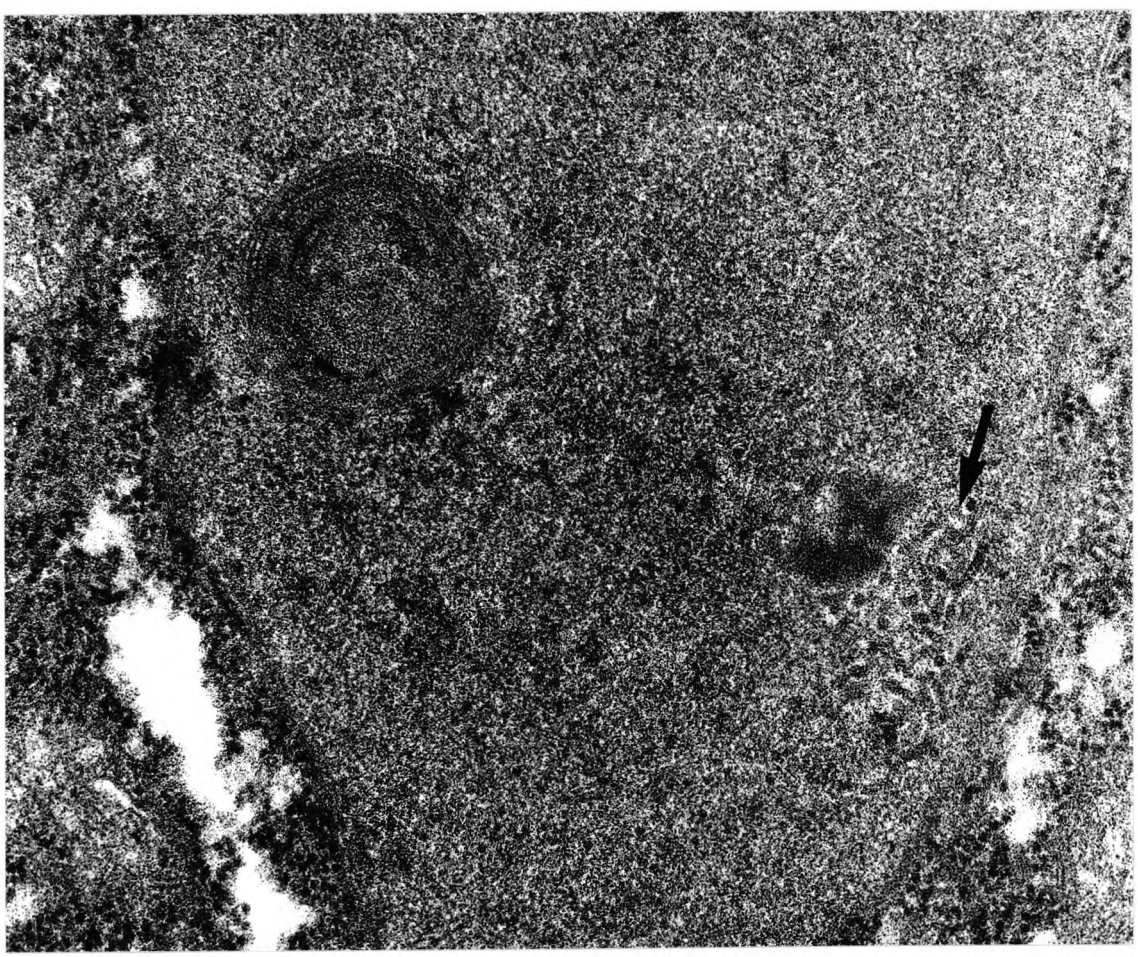
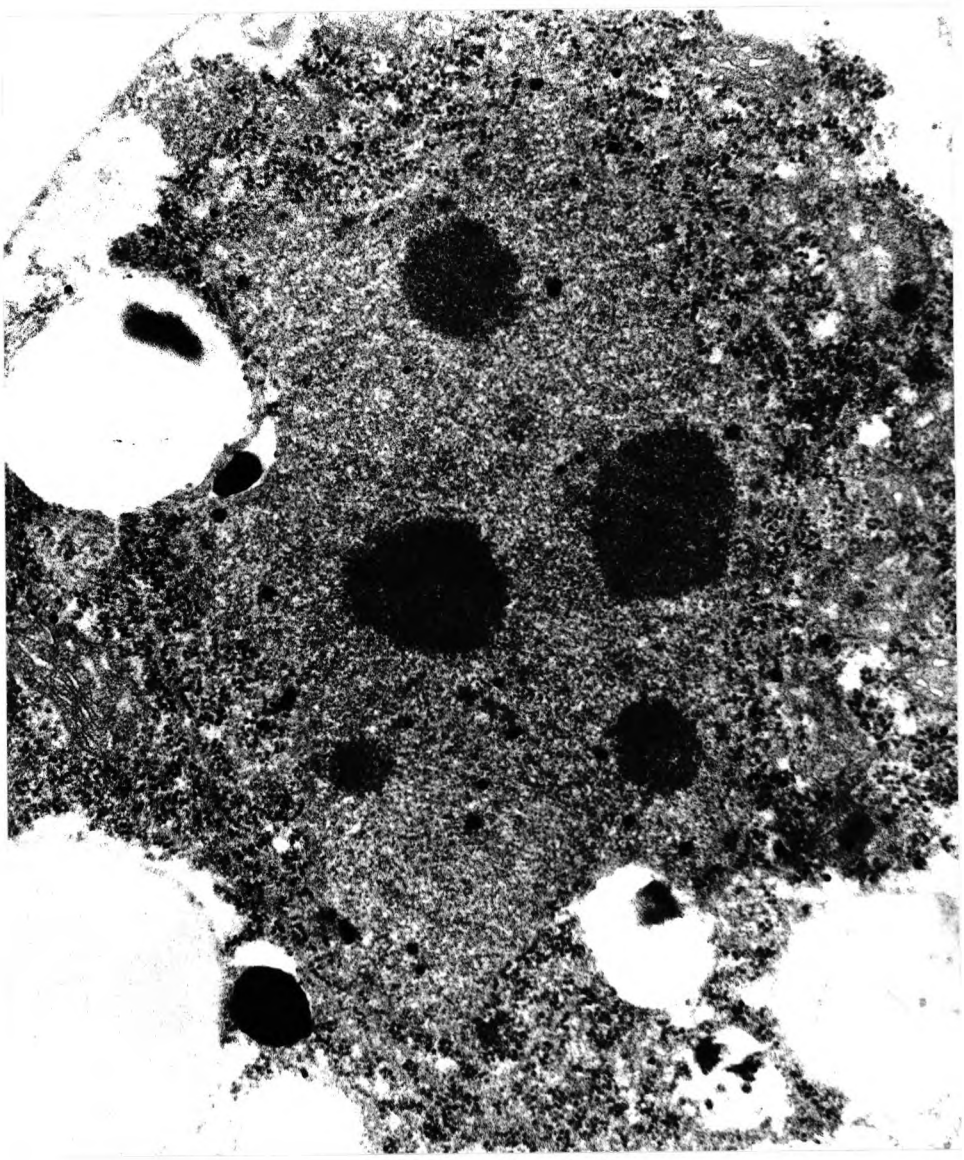


Fig. 64: Griseofulvin-treated nucleus, showing multiple nucleoli (x 40,000)



bodies. This relationship is shown in transverse (Fig. 53) and glancing sections (Figs. 54 & 55) of the pole body. In addition, numerous microtubules can be seen assembled throughout the nucleus (Figs. 58 & 59), and in many cases these are arranged in a disorganised manner, frequently appearing as aggregates of seemingly morphologically normal microtubules (Figs. 60-63).

Further evidence that genome replication has occurred in the absence of nuclear division in the presence of griseofulvin is provided by the appearance of multiple nucleoli (Fig. 64).

The evidence suggests that griseofulvin inhibits growth and cell division in this organism by causing a disorganisation but not disassembly of nuclear microtubules.

Uptake of griseofulvin

It is evident that there is a difference between Protomyces inundatus and Saccharomyces cerevisiae in their sensitivity to griseofulvin, therefore to determine the contribution of selective permeability to this difference, the uptake of griseofulvin by these two organisms was examined.

Tritiated drug bound to cells was assayed by filtration of cells onto filter-discs upon which they were washed, and subsequently counted. A radioactive griseofulvin concentration of 50μM in 0.5% DMF was used throughout the study, a concentration which inhibited the growth of Protomyces but not of Saccharomyces.

As shown in Figs. 65-67, curves for the uptake of griseofulvin by Protomyces show a rapid initial phase which is complete within 10-60 secs. In some cases there is a subsequent decrease in cell-bound radioactivity, however it reaches a plateau at approx. 0.2-0.5 n.moles/mg (dry weight) of griseofulvin accumulated. Temperature appears to affect uptake in that

Fig. 65 & 66: Uptake of [^{3}H] -griseofulvin by washed cell suspensions of Protomyces inundatus, at 25^{o}C, assayed using a filter-disc sampling procedure

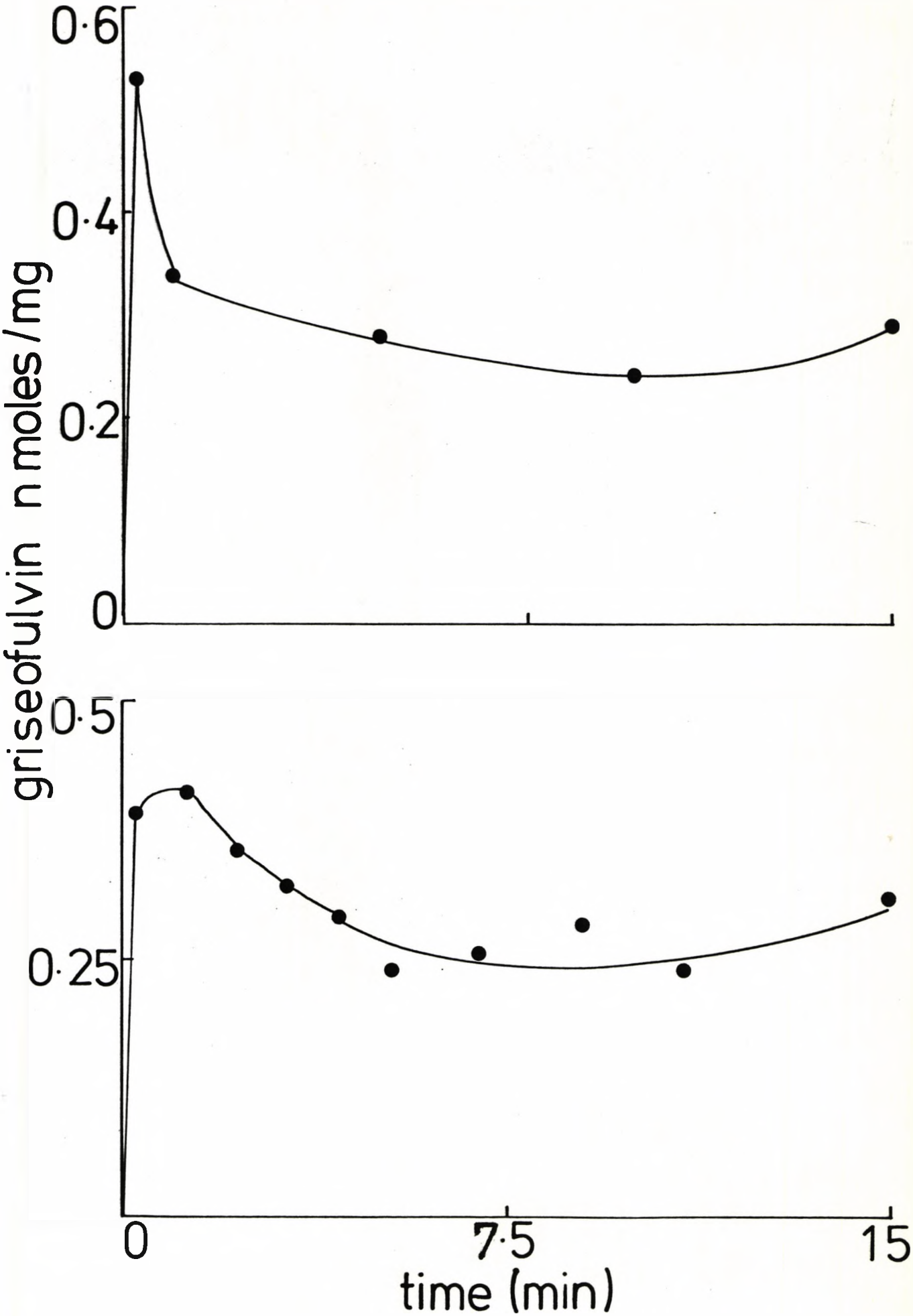
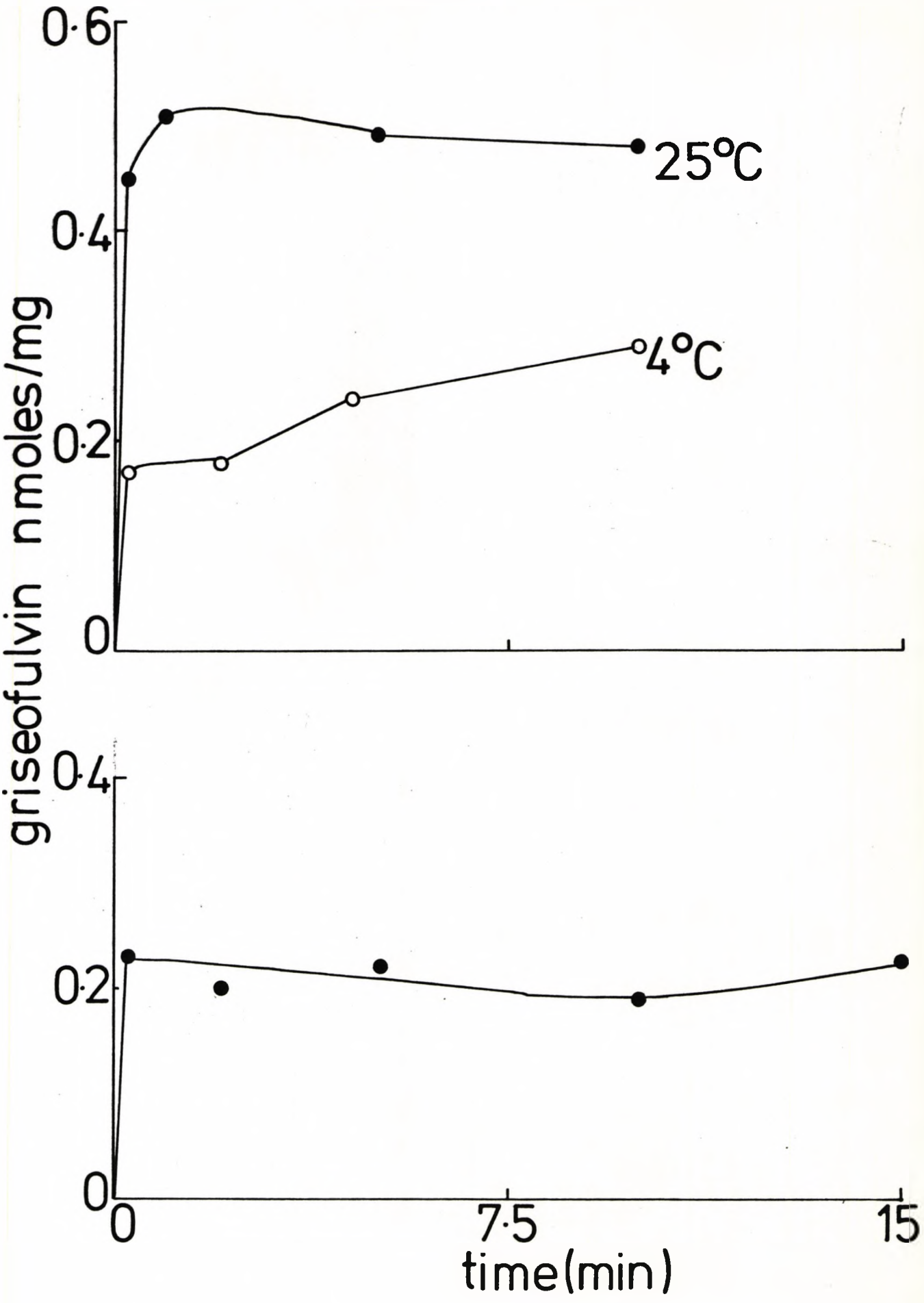

0·6
0·4
0·2
0
griseofulvin n moles/mg
0·5
0·25
0
7·5
15
time (min)

Fig. 67: Uptake of [^{3}H]-griseofulvin by washed cell suspensions of Protomyces inundatus using a filter-disc assay: effects of temperature

Fig. 68: Uptake of [^{3}H]-griseofulvin by washed cell suspensions of Saccharomyces cerevisiae at 25^{o}C using a filter-disc assay


0·6
0·4
0·2
0
25°C
4°C
griseofulvin n moles/mg
0·4
0·2
0
0
7·5
15
time(min)

initial accumulation is less at 4°C compared with 25°C, however there is a continued slower phase of accumulation over the 10 min. studied.

Saccharomyces cerevisiae also takes up the drug in a similar manner i.e. with a rapid initial uptake, reaching a plateau value of 0.1-0.2 n.moles/mg dry weight (Figs. 68 & 69). The initial peak and decline of uptake as shown by Protomyces (Fig. 65) is not shown by Saccharomyces. Stationary phase cells of Saccharomyces appear to take up more griseofulvin than logarithmic phase cells i.e. approx. 0.5 n.moles/mg (Fig. 70). This may be the result of increased permeability of stationary phase cells. Temperature seems to have no effect upon griseofulvin uptake in the case of either logarithmic or stationary phase cells (Figs. 69 & 70).

Similar results were obtained for Saccharomyces using a different assay procedure in which cells were separated from labelled medium by centrifugation through a layer of silicone (Fig. 71).

The uptake of ^{3}H-labelled colchicine into Protomyces and Saccharomyces was examined. Both yeast appear to take up this drug to a similar extent, approx. 0.5-0.6 n.moles/mg are accumulated in the cells. Kinetics of colchicine uptake are similar to those of griseofulvin uptake into these organisms (data not shown).

The ability of Saccharomyces to accumulate compounds other than anti-mitotic substances is illustrated by the uptake of ^{14}C-labelled 2-amino-iso-butyric acid (Fig. 72). This compound is an amino acid analogue which is not metabolised by animal cells, and has been used to measure transport and intracellular accumulation of amino acids without the complication of subsequent utilisation (LeCam & Freychet, 1977). As shown in Fig. 72, Saccharomyces takes up much larger quantities of this analogue compared with griseofulvin. Uptake appears to be linear over the time-scale

Fig. 69: Uptake of griseofulvin by washed cell suspensions of Saccharomyces cerevisiae using a filter-disc assay: effects of temperature

Fig. 70: Uptake of griseofulvin by washed stationary phase cells of Saccharomyces cerevisiae, using a filter-disc assay procedure

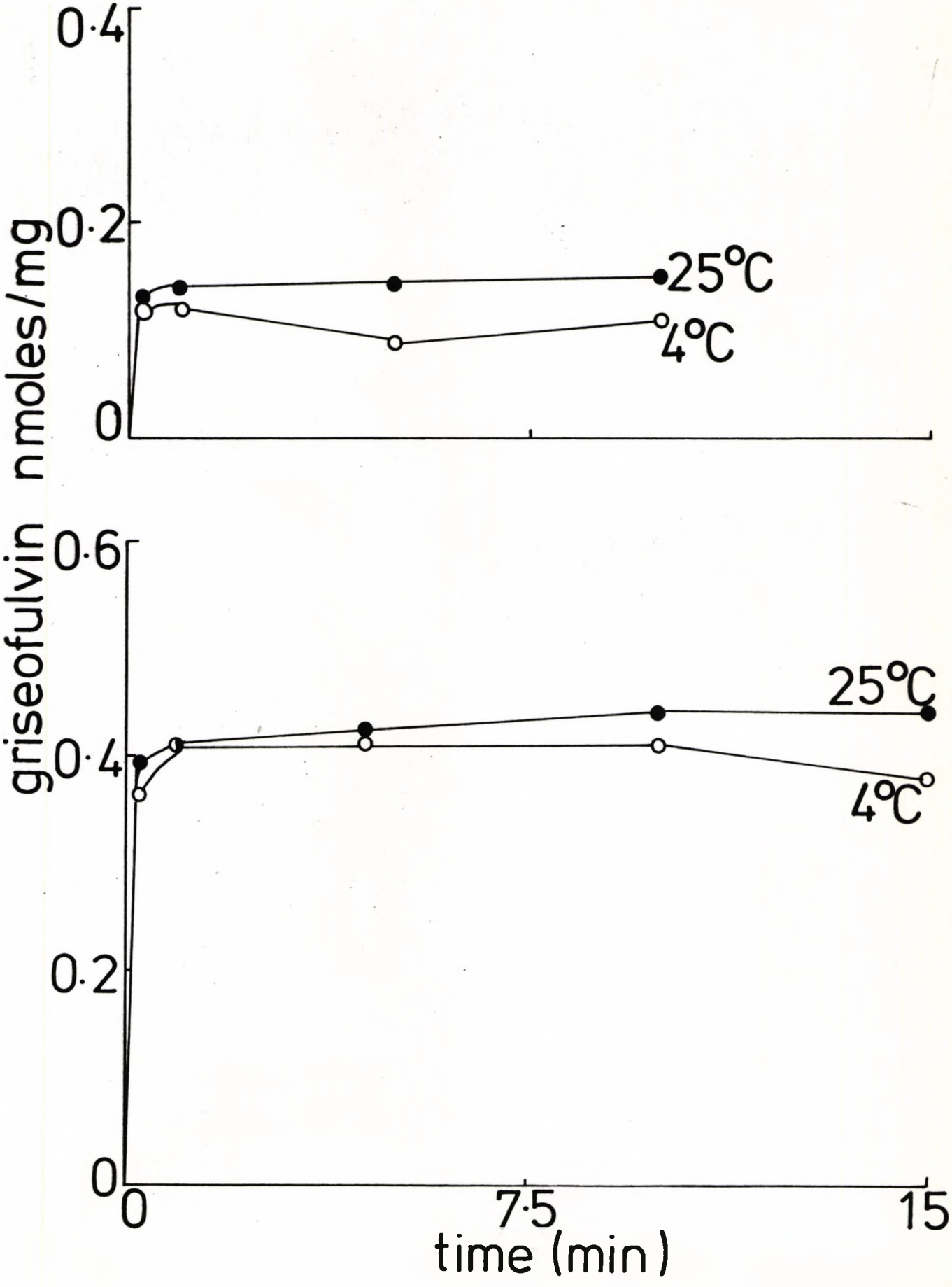
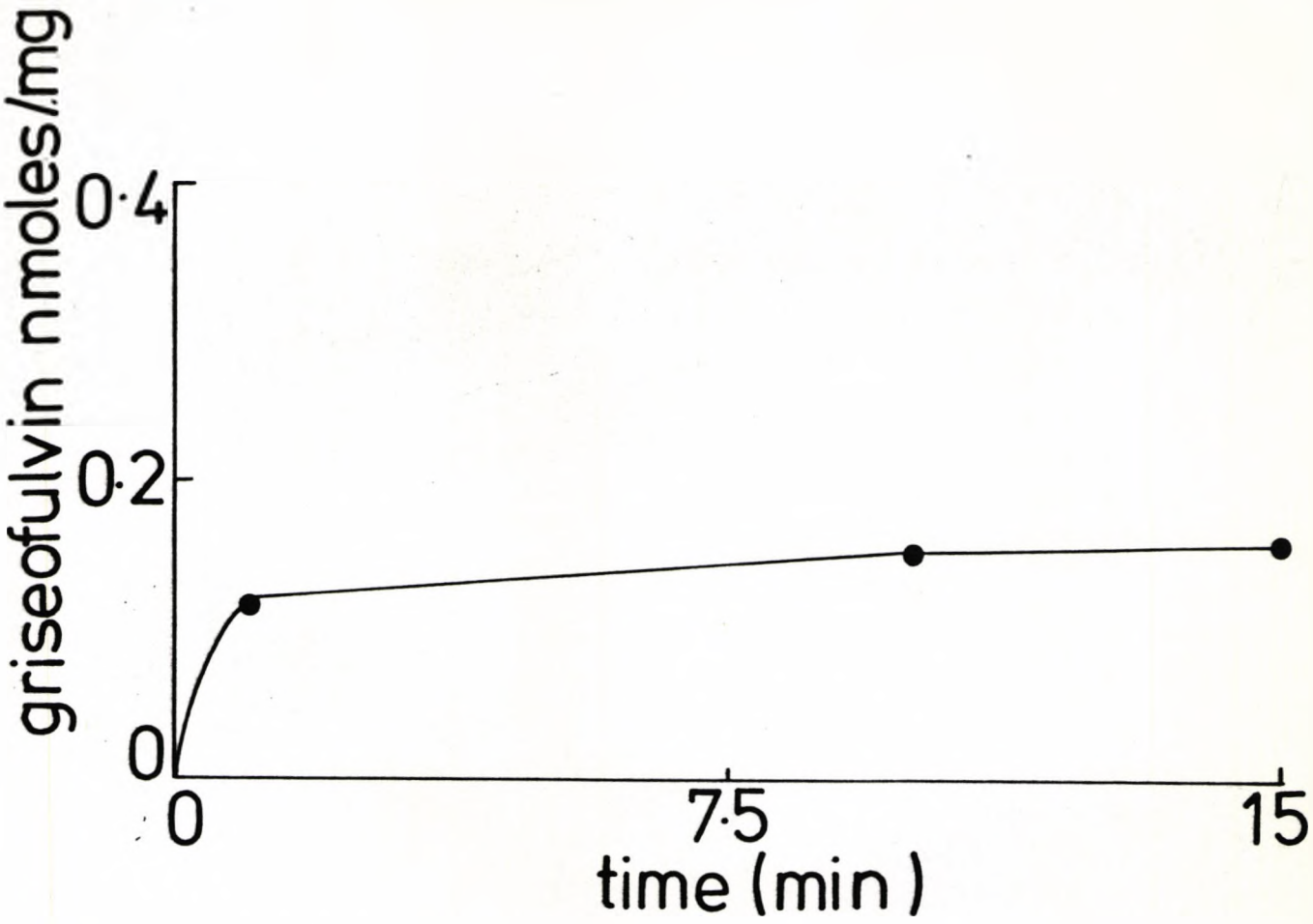
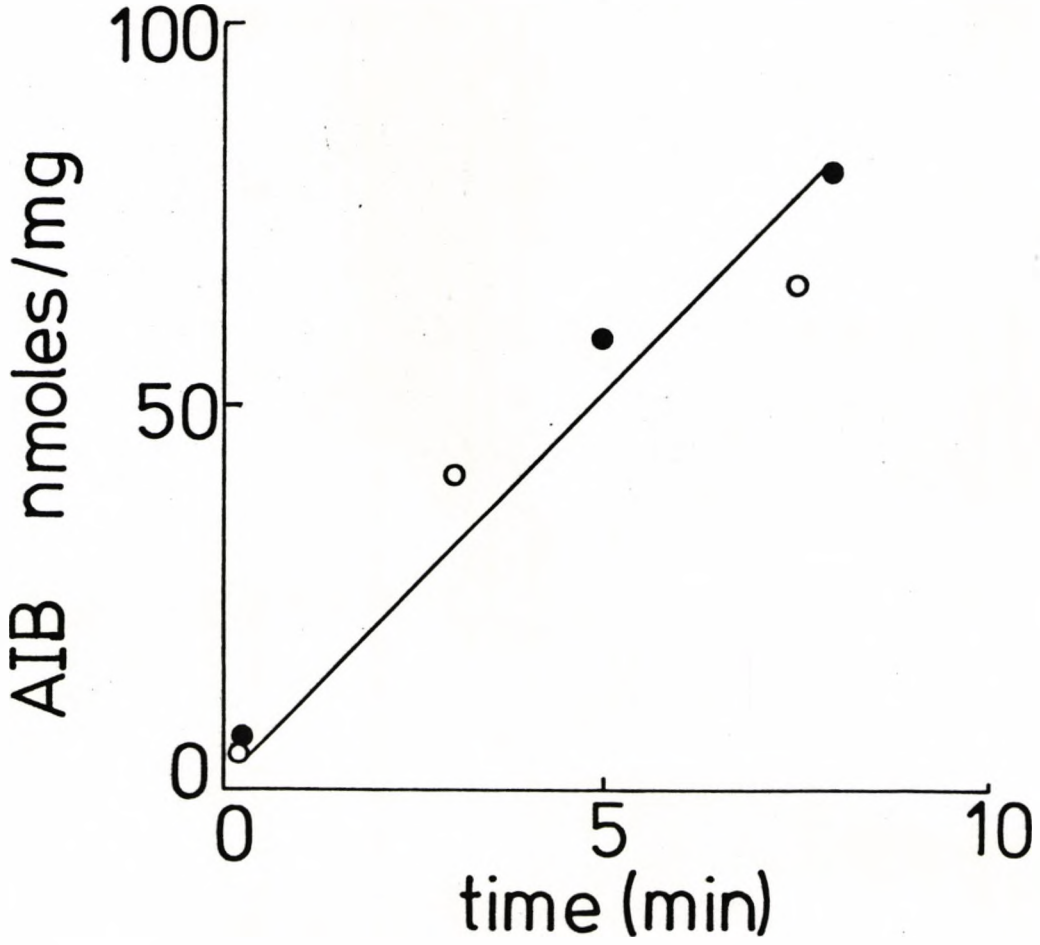

0·4
0·2
0
25°C
4°C
griseofulvin nmoles/mg
0·6
0·4
0·2
0
25°C
4°C
0
7·5
15
time (min)

Fig. 71: Uptake of griseofulvin by logarithmic phase cells of Saccharomyces cerevisiae, at 25°C, using a centrifugation assay procedure

Fig. 72: Uptake of ^{14}C-labelled 2-amino-iso-butyric acid by washed cell suspensions of Saccharomyces cerevisiae from a 0.5mM solution, at 25°C

(●) & (○), duplicate experiments


griseofulvin nmoles/mg
0·4
0·2
0
0
7·5
15
time (min)


AIB nmoles/mg
100
50
0
0
5
10
time (min)

studied. N-acetyl glucosamine was also taken up by Saccharomyces cerevisiae (Fig. 73).

These experiments demonstrate that cells prepared in the manner described, and used in these, and drug uptake experiments, are viable cells capable of normal amino acid uptake. In addition the filter disc assay is shown to be a suitable assay for the study of uptake of radio-labelled substances.

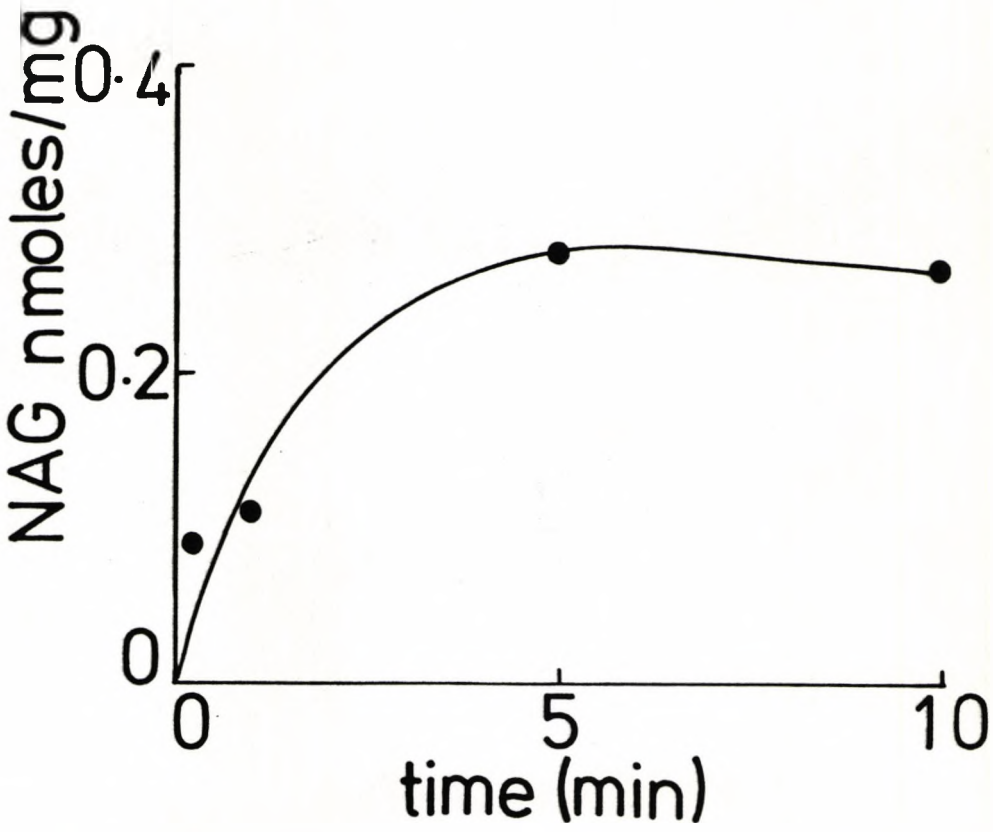
Previously published work suggests that uptake of griseofulvin into sensitive fungi takes place over a long time period (El Nakeeb & Lampen, 1965b; c), therefore uptake into Protomyces and Saccharomyces was examined under similar conditions in an attempt to reproduce these results with these fungi.

In the case of the griseofulvin sensitive yeast Protomyces no uptake, over and above the 0.2 n.moles/mg initially accumulated, took place over a 4-6 hour period. However, after approx. 4 hours incubation with ^{3}H-labelled griseofulvin in non-growing conditions (i.e. washed cell suspensions in buffer minus vitamins and exogenous energy source) cells of Protomyces began to take up increasing quantities of radioactivity (Figs. 74-76). The rate and extent of this 'secondary phase' of drug accumulation was variable c.f. Figs. 74-76. No such secondary accumulation occurred with Saccharomyces cerevisiae (Fig. 74).

This secondary (long term) griseofulvin uptake, or binding, correlates with griseofulvin sensitivity of the two yeast species, however, because of the variability of this uptake in different experiments compared with the initial or short time-course uptake, it is possible that this secondary phase of griseofulvin binding is the result, and not the cause of differences in drug sensitivity between the two yeast. Griseofulvin may have effects upon the Protomyces cells during a prolonged incubation in non-growing

Fig. 73: Uptake of 1.0mM ^{3}H-labelled N-acetyl glucosamine by washed cell suspensions of Saccharomyces cerevisiae, at 25°C, using a filter-disc assay

Fig. 74: Uptake of [^{3}H]-griseofulvin by washed cell suspensions of Protomyces inundatus (●) and Saccharomyces cerevisiae (▲), at 25°C, over an 8 hour time period. Filter-disc assay used.


NAG n moles/mg
0·4
0·2
0
0
5
10
time (min)

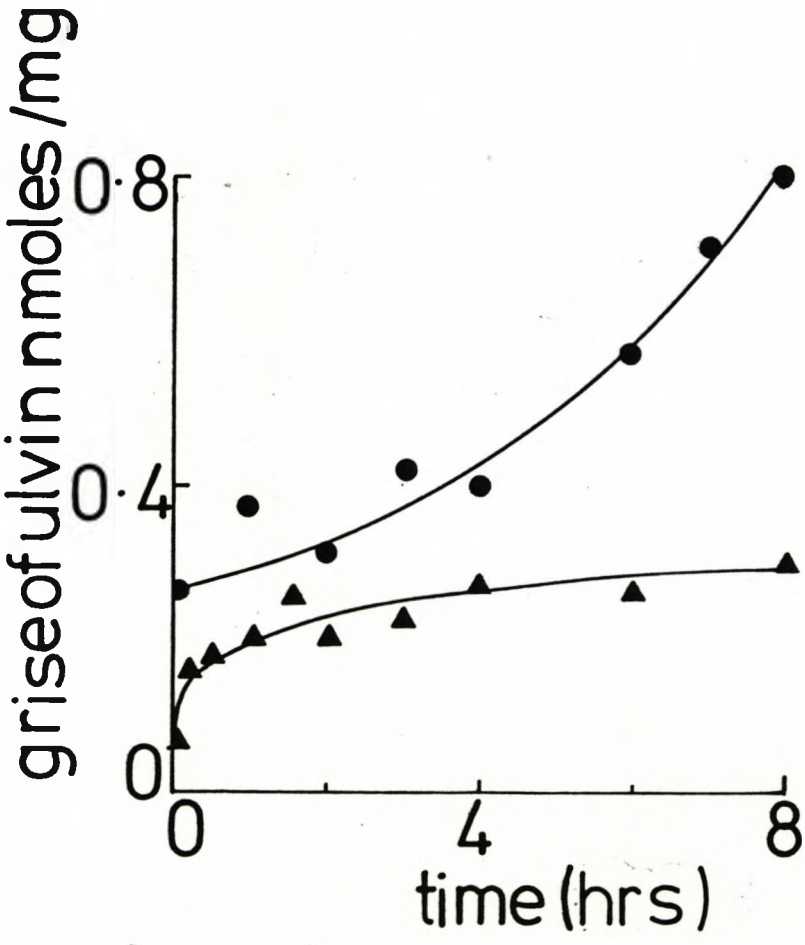
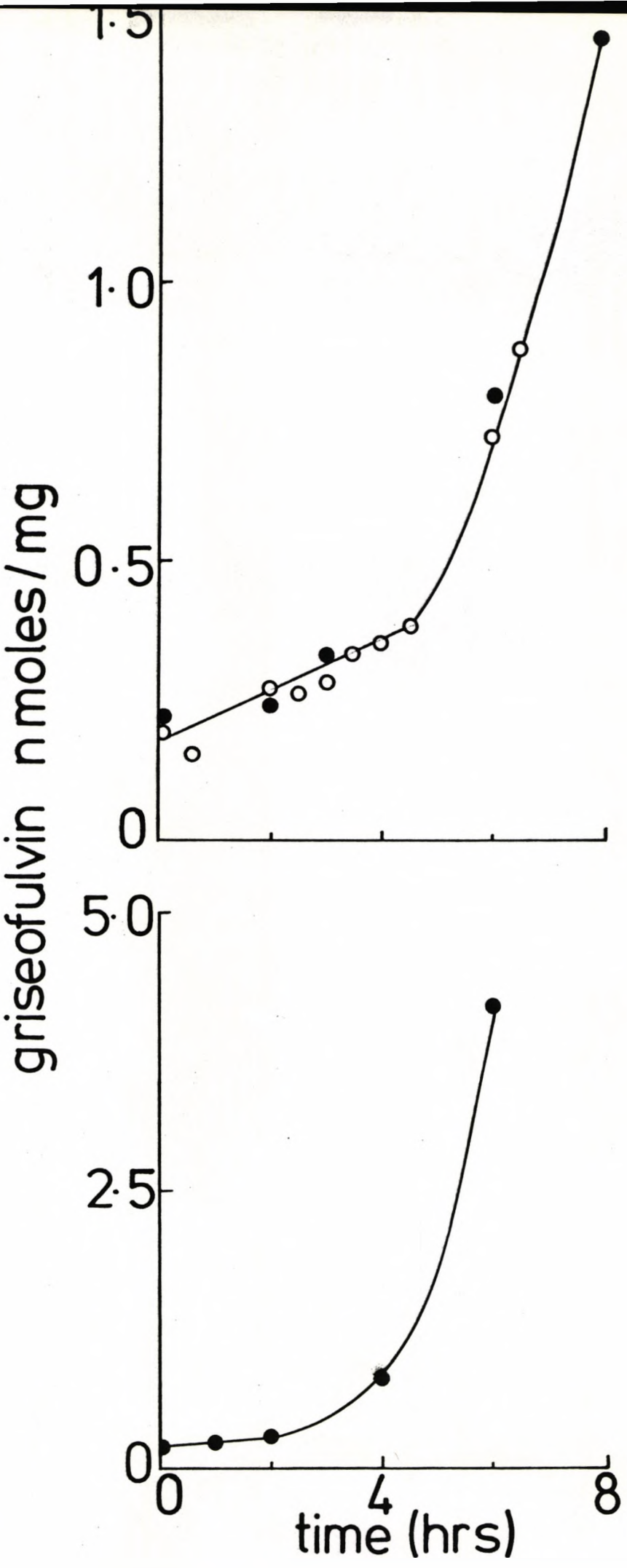

griseofulvin n moles/mg
0·8
0·4
0
0
4
8
time (hrs)

Fig. 75 & 76: Uptake of griseofulvin by washed cell suspensions of Protomyces inundatus at 25°C, over an 8 hour time period using a filter-disc assay. (○) & (●) duplicate experiments


griseofulvin n moles/mg
1·5
1·0
0·5
0
5·0
2·5
0
0
4
8
time (hrs)

conditions which lead to the breakdown of cell integrity and hence an increased permeability to the drug. Saccharomyces cerevisiae, being insensitive to griseofulvin, does not demonstrate such an increase in permeability.

It appears therefore that both Protomyces inundatus and Saccharomyces cerevisiae take up griseofulvin over very short periods of time. There is a small quantitative difference in the amount of drug taken up by these two yeast, but it is unlikely that such a small difference is the basis of the insensitivity of Saccharomyces to the drug.

Isolation of microtubule proteins from Saccharomyces cerevisiae - initial problems and solutions

Microtubule protein is thought to constitute a very small percentage of the total soluble proteins of Saccharomyces cerevisiae (Haber et. al., 1972), compared with mammalian brain where 15-20% of the soluble protein is tubulin (Borisy & Taylor, 1967). In order to purify microtubule proteins from Saccharomyces by a temperature-sensitive assembly-disassembly procedure as used with brain tissue, the yeast extract must therefore be concentrated to a high enough total protein concentration to raise the tubulin above its critical concentration (Co) for assembly.

Attempts to assemble Saccharomyces microtubules in vitro from extracts, using the preparative procedure developed for brain, were unsuccessful even when the protein concentration of the extract was raised by vacuum dialysis to 70-80 mg/ml. No microtubules were visible in the electron microscope, neither was there any enrichment of microtubule proteins in the pellets analysed by SDS-PAGE (data not shown).

Attempts were therefore made to discover the reason(s) for the failure of the above preparation procedure, and it was found that extracts of

Saccharomyces cerevisiae strain cdc 13 inhibit the assembly of brain microtubules in vitro as assayed by turbidimetry. Typical inhibition kinetics are shown in Figs. 77 & 78. The cdc 13 extracts in the presence and absence of 1mM PMSF inhibit both the rate and extent of microtubule assembly, and in addition extend the lag phase preceding the start of assembly.

At high extract concentrations the increase in turbidity at 400nm appears to be biphasic. This increase in turbidity over and above the microtubule formation also occurs on warming the extract alone at 37°C and is not apparently due to microtubule formation when inspected by electron microscopy, also no plateau value is reached within the range of the spectrophotometer. This phenomenon was probably due to protein denaturation and/or precipitation and could not be correlated with any specific conditions. However, this non-specific turbidity increase complicates the analysis of inhibitory activity by making the determination of plateau values inaccurate at high extract concentrations. By subtracting this non-specific increase in the extract alone from the curve for extract plus microtubule protein, the true microtubule assembly kinetics can be seen (Fig. 77).

Inhibitory activity of these extracts may be expressed as per cent inhibition of assembly of the control, i.e. the reduction of the total change in OD 400nm, adjusted for a 1:1 ratio of extract to microtubule protein. Using this method it is possible to compare the efficacy of various treatments in reducing the inhibition of microtubule assembly.

It is evident that cdc 13 extracts strongly inhibit brain microtubule assembly at very low concentrations, therefore at very high concentrations in the presence of small quantities of microtubule proteins - as in the initial experiments above - assembly of microtubules in vitro under these

Fig. 77: Assembly of brain microtubules *in vitro* in the presence of extracts of *Saccharomyces cerevisiae* cdc 13, assayed by turbidimetry.

A (●), control: 1.85 mg/ml brain microtubule protein

B (▲), 1.85 mg/ml brain microtubule protein plus 1.9 mg/ml extract protein

C (○), 1.9 mg/ml extract protein alone

D (Δ), curve B minus curve C

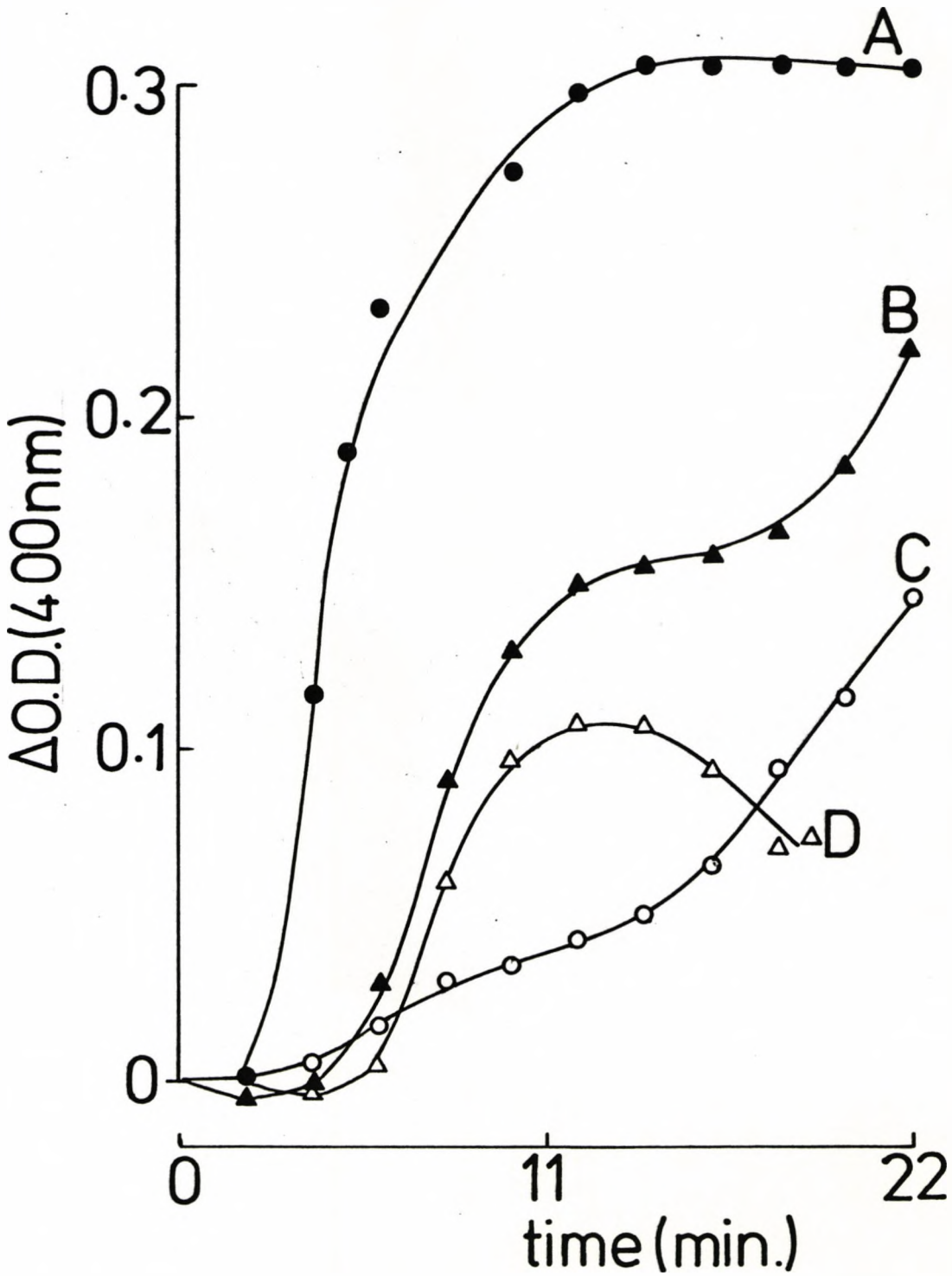
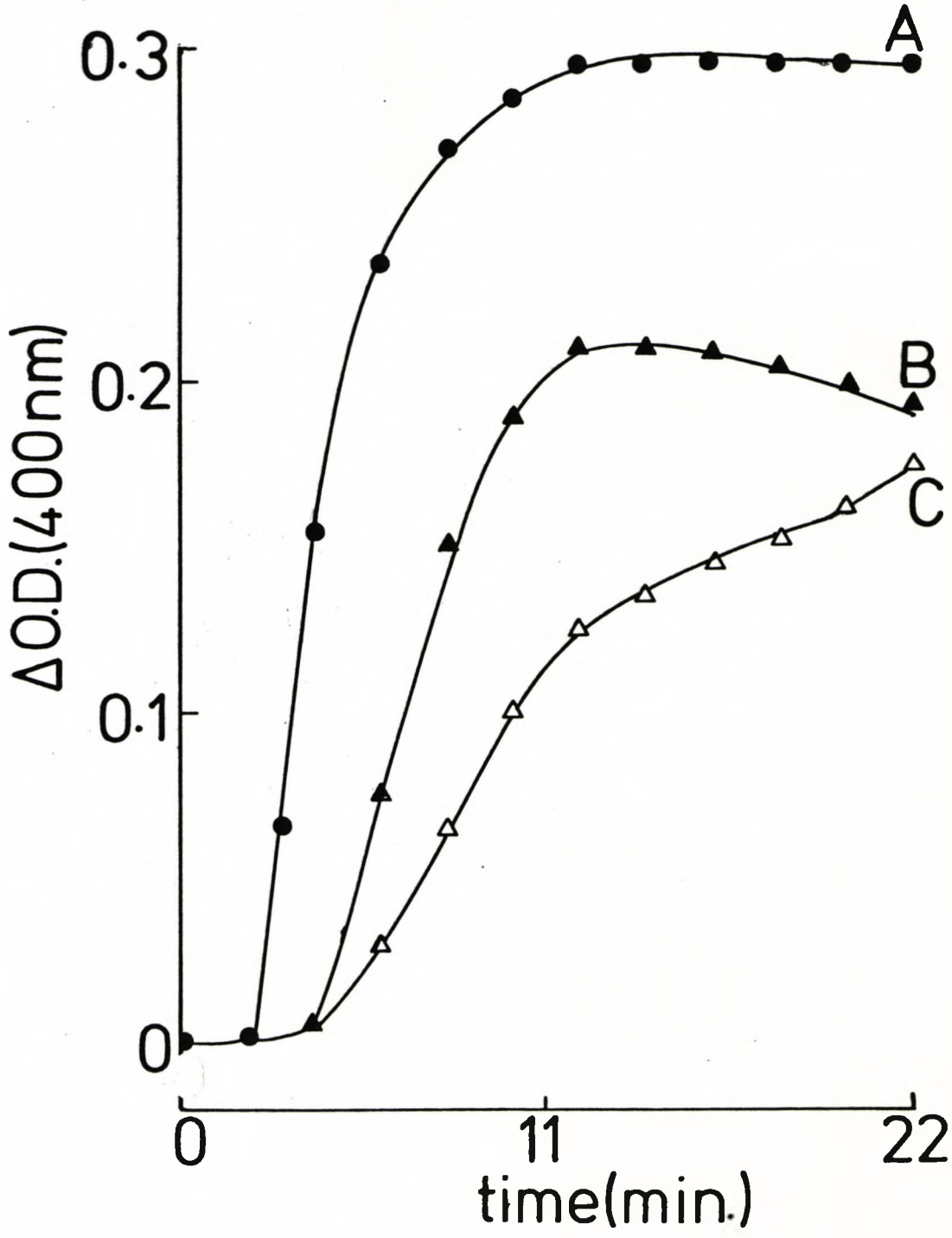

A
B
C
D
0·3
0·2
0·1
0
ΔO.D.(400nm)
0
11
22
time (min.)

Fig. 78: Assembly of brain microtubule protein *in vitro* in the presence of extracts of *cdc* 13 prepared in the presence of 1mM PMSF, 1% (v/v) DMF, assayed by turbidimetry

A (●), control: 1.85 mg/ml brain microtubule protein

B (▲), 1.85 mg/ml brain microtubule protein plus 2.1 mg/ml extract + PMSF

C (Δ), 1.85 mg/ml brain microtubule protein plus 3.2 mg/ml extract + PMSF


0·3
0·2
0·1
0
ΔO.D.(400nm)
A
B
C
0
11
22
time(min.)

conditions would be precluded, hence the traditional microtubule purification procedure is inapplicable in this case.

An alternative method for assembling Saccharomyces microtubule proteins in vitro is to enrich the yeast extract in microtubule proteins from brain, which can then be taken through multiple cycles of assembly-disassembly to encourage the co-polymerisation of Saccharomyces microtubule proteins. This procedure would not constitute a purification of yeast microtubules as such, but would serve as an indication of the protein components of yeast microtubules. For this method, or indeed any attempts to assemble Saccharomyces cerevisiae microtubules as a homologous system, to be successful the inhibition of microtubule assembly by components of the yeast extract must be abolished or substantially reduced.

Protease activity

From table 7 it can be seen that the preparation of Saccharomyces cerevisiae cdc 13 extract in the presence of 1mM PMSF reduced but did not abolish the inhibitory activity of the extracts. This suggests that protease activity is not solely responsible, or that PMSF-insensitive protease activities are also present in the system.

Protease activity in Saccharomyces extracts was assayed using brain microtubule protein as a substrate for the enzymes, incubated together under polymerising conditions. The effect of the digestion on brain protein was examined by SDS-PAGE as described in Materials and Methods. Fig. 79 shows the results of the assay of protease activity in cdc 13 extracts. Incubation of extract with brain microtubule protein results in the disappearance of HMW-MAPs from the top of the 6% acrylamide gel after as little as 5 min at 37^{o}C. A variety of standard protease inhibitors including PMSF had no effect on the digestion of HMW-MAPs (Fig. 80).

Table 7 Inhibition of brain microtubule assembly *in vitro* by extracts of *cdc* 13

Treatment	% inhibition
No treatment	48.6 (3)
Extract made in the presence of 1mM PMSF, 1% v/v DMF	23.3 (7)

N.B. Neither DMF nor PMSF at the above concentrations affect microtubule assembly *in vitro*. Figures are mean values, the number of observations made appear in parentheses.

Fig. 79: Assay of protease activity in Saccharomyces cerevisiae cdc 13 extracts. SDS-PAGE of incubations of extract with 2.0 mg/ml brain microtubule protein at 37°C for 30 min

A, control i.e. microtubule protein alone 2.0 mg/ml

Subsequent assays contained microtubule protein plus:

B, 5.3 mg/ml extract

C, 2.8 mg/ml extract

D, 1.1 mg/ml extract

E, 5.3 mg/ml extract + 1mM PMSF (1% DMF)

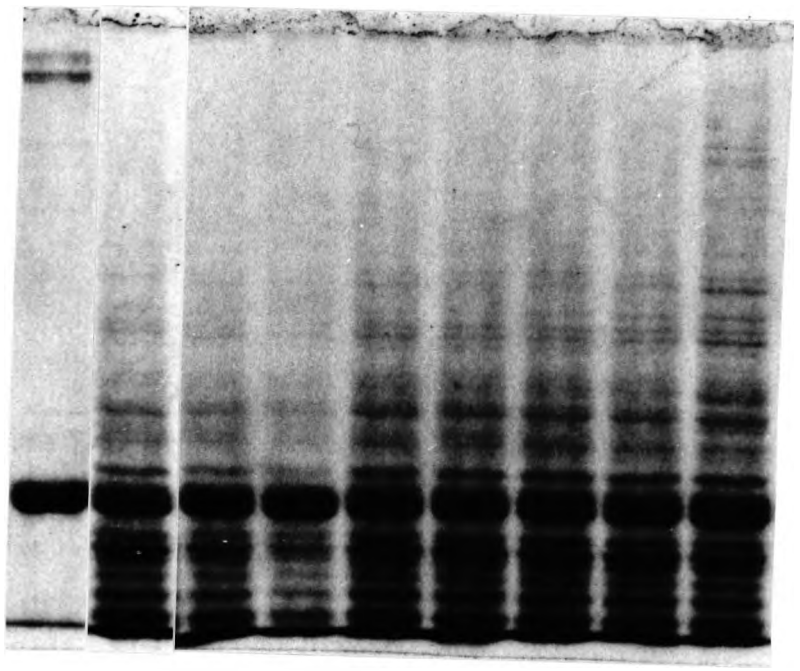
F, 5.3 mg/ml extract + 50µg/ml pepstatin

G, 5.3 mg/ml extract + 1mM PMSF + 50µg/ml pepstatin cocktail (1% (v/v) DMF)

H, 5.3 mg/ml extract prepared in the presence of PMSF/ pepstatin cocktail

I, 5.3 mg/ml extract + 200µM TLCK

Gels were 6% acrylamide



A B C D E F G H I

Fig. 80: Assay of protease activity in Saccharomyces cerevisiae cdc 13 extracts. SDS-PAGE of incubations of extract with 2.1 mg/ml brain microtubule protein at 37°C for 30 min. Extract was at 1 mg/ml throughout.

A, control i.e. brain microtubule protein alone.

Subsequent assays contained microtubule protein plus:

B, extract + 1mM PMSF in 1% (v/v) DMF

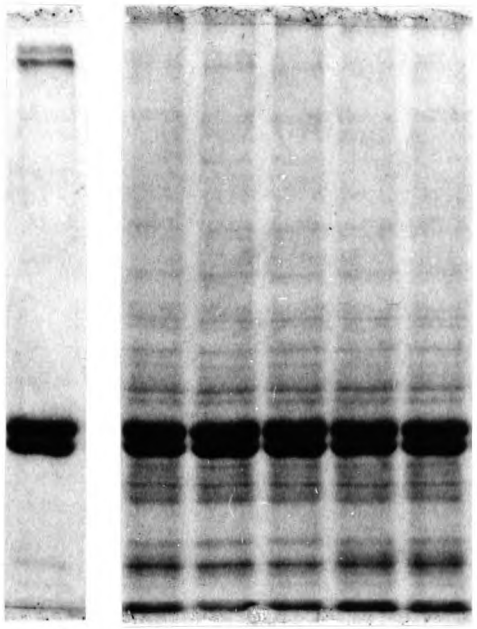
C, extract + 200μM TLCK

D, extract + 20μg/ml chymostatin

E, extract + 50μg/ml pepstatin

F, extract + 1% (v/v) DMF

Gels were 6% acrylamide


A B C D E F

Saccharomyces cerevisiae is reported to contain three major types of protease activity (Pringle, 1975), of which types A and B may be inhibited by PMSF, and type C is sensitive to pepstatin. However a cocktail of these two inhibitors failed to prevent the digestion of the MAPs from brain even though some reduction of autolytic digestion of extract proteins was noticeable.

The digestion of the HMW-MAPs from brain microtubule preparations may, in part, explain the assembly inhibition kinetics shown in Figs. 77 & 78, where both rate and extent of assembly are inhibited in the presence of extracts. HMW-MAPs are necessary for initiation but not for elongation of microtubules (Murphy et. al., 1977) but affect both the rate and extent of assembly in vitro. The increase in lag period before assembly begins in the presence of extracts also suggests the inhibition of an initiating activity (Roobol et. al., 1976).

Extracts of a Saccharomyces cerevisiae strain 20B-12 deficient in proteases A,B and C do not cause the disappearance of brain HMW-MAPs when incubated with brain microtubule protein, even during prolonged incubations in polymerising conditions as seen in Fig. 81. However, extracts of this strain do still inhibit microtubule assembly in vitro to a greater extent than cdc 13 extracts treated with PMSF, Fig. 82 and table 8. Discounting the difficulties in making accurate estimates of inhibition for reasons already mentioned, it is possible that the target of the PMSF-sensitive inhibitory activity may not be the HMW-MAPs themselves but a protein such as the tau protein which is known to be active in stimulating microtubule assembly. Even though the HMW-MAPs are unaffected by the 'protease-deficient' extract, the tau protein may be attacked. The SDS-PAGE assay is very sensitive in terms of the cleavage of the HMW-MAP but it is not

Fig. 81: Assay of protease activity in Saccharomyces cerevisiae 20B-12 extracts. SDS-PAGE of incubations of 0.7 mg/ml brain microtubule protein with extract at 37°C for 30 min.

A, control i.e. microtubule protein alone

Subsequent assays contained microtubule protein plus:

B, 0.7 mg/ml extract

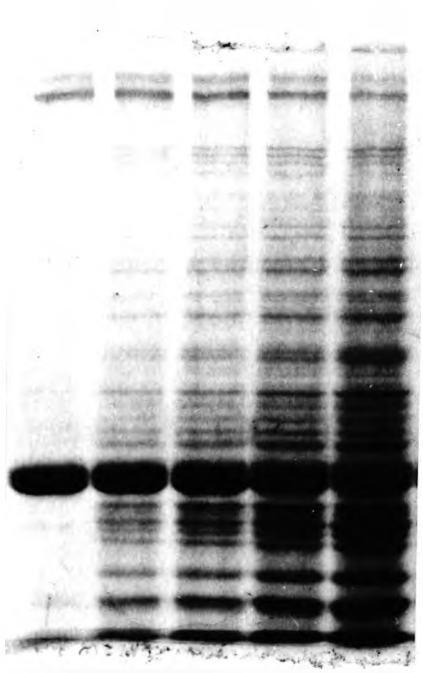
C, 1.4 mg/ml extract

D, 2.7 mg/ml extract

E, 6.9 mg/ml extract

Ratio of extract:microtubule protein ranged from 1.0-10.

Gels were 6% acrylamide



A B C D E

Fig. 82: Brain microtubule assembly *in vitro* in the presence of extracts of *Saccharomyces cerevisiae* strain 20B-12 (protease deficient)

A(●), control: 1.14 mg/ml brain microtubule protein

B(○), 1.14 mg/ml brain microtubule protein plus 0.73 mg/ml extract

C(▲), 1.14 mg/ml microtubule protein plus 1.1 mg/ml extract

D(△), 1.14 mg/ml microtubule protein plus 1.5 mg/ml extract

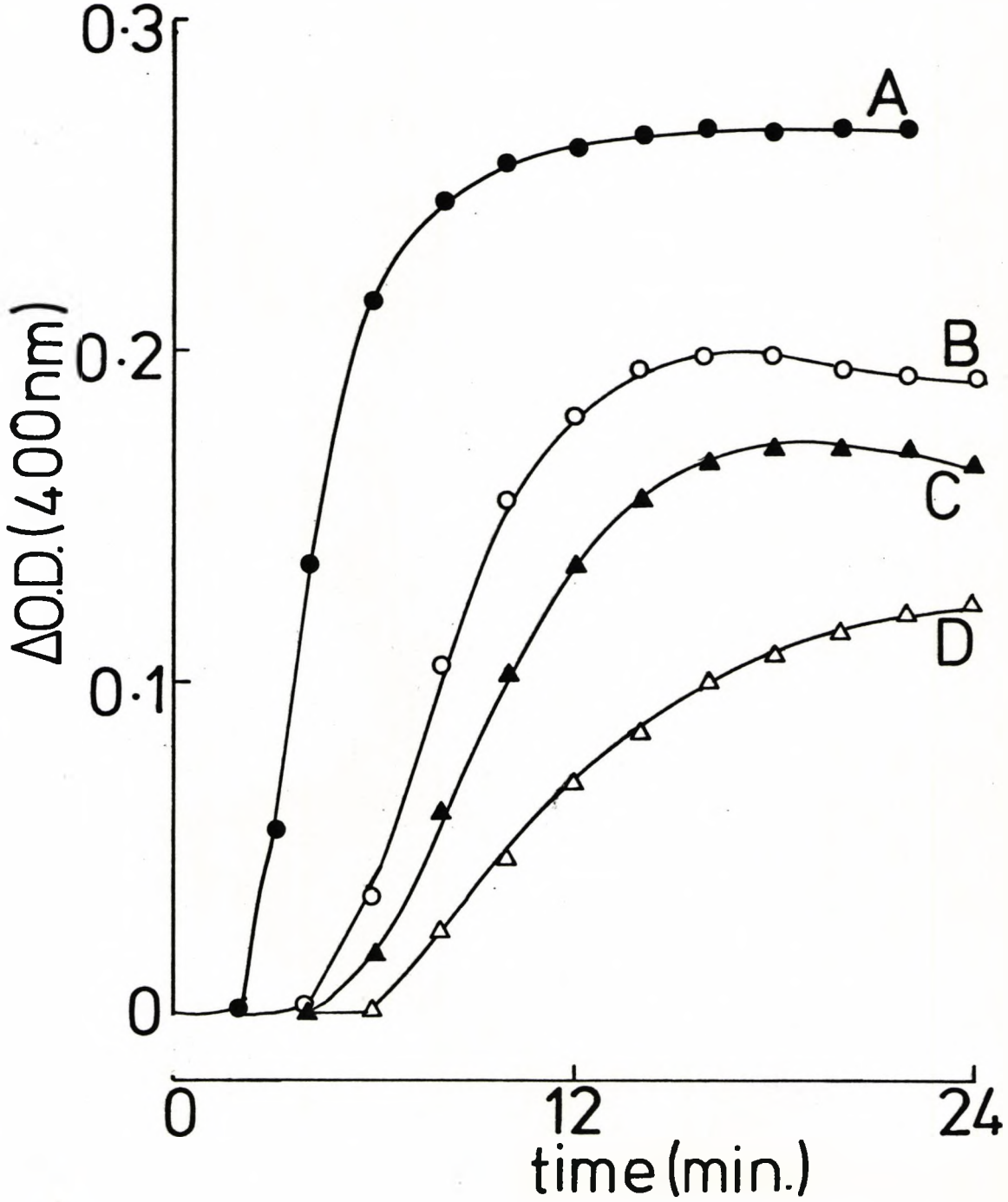

0·3
0·2
0·1
0
ΔO.D.(400nm)
A
B
C
D
0
12
24
time (min.)

Table 8 Inhibition of brain microtubule assembly *in vitro* by extracts of 20B-12 protease deficient strain of *Saccharomyces cerevisiae*

Treatment	% inhibition
No treatment	42.1 (8)
Dialysed extract	41.6 (3)
RNase/DNase treatment	18.4 (5)
Assayed plus 4M glycerol	4.7 (3)

N.B. Figures are mean values, the number of observations appear in parentheses.

possible to distinguish proteolytic digestion of a peptide such as tau which is usually obscured, in our gel system, by the α-tubulin band.

Inhibition of assembly by extracts of 20B-12 appears to be linear with respect to extract protein concentration at a constant brain microtubule protein concentration, as shown in Fig. 83. The ratio of extract to brain protein in the experiment was between 0.4 and 1.9. Presumably the linearity of the relationship extends until extracts are 100% inhibitory.

The effects of various treatments of 20B-12 extract on the inhibition of microtubule assembly are shown in table 8. Dialysis of the extract does not reduce the inhibitory activity, which is therefore not due to a small molecule or solute. RNA is known to be an inhibitor of microtubule assembly in vitro (Bryan et. al., 1975) and DNA appears to bind MAPs (Corces et. al., 1978). A pre-incubation of extract at 30°C for 15 min. with RNase (20μg/ml) and DNase (10μg/ml) reduces the inhibition substantially (Fig. 84).

Glycerol promotes microtubule assembly, and consequently the addition of 4M glycerol to the system reduces the inhibition to a low level. However, as shown in Fig. 85, in the presence of 4M glycerol the change in optical density (ΔOD) of the control is reduced to approx. one half that in the absence of glycerol, even though the mass of assembled material is equivalent. The lag phase is also virtually abolished. This reduction in ΔOD(400nm) means that assays of inhibition are less sensitive in the presence of glycerol, therefore all measurements of inhibitory activity and the effects of various treatments on the inhibition were made in the absence of glycerol.

GTP-ase activity of Saccharomyces cerevisiae extracts

Saccharomyces cerevisiae cdc 13 extracts were found to contain a GTP hydrolytic activity when extracts were incubated with 1mM GTP, the

Fig. 83: Inhibition of brain microtubule assembly by extracts of Saccharomyces cerevisiae 20B-12 at various concentrations. Microtubule protein was at 2.3 mg/ml throughout. Inhibition was calculated as the % reduction of the control ΔO.D.(400nm).

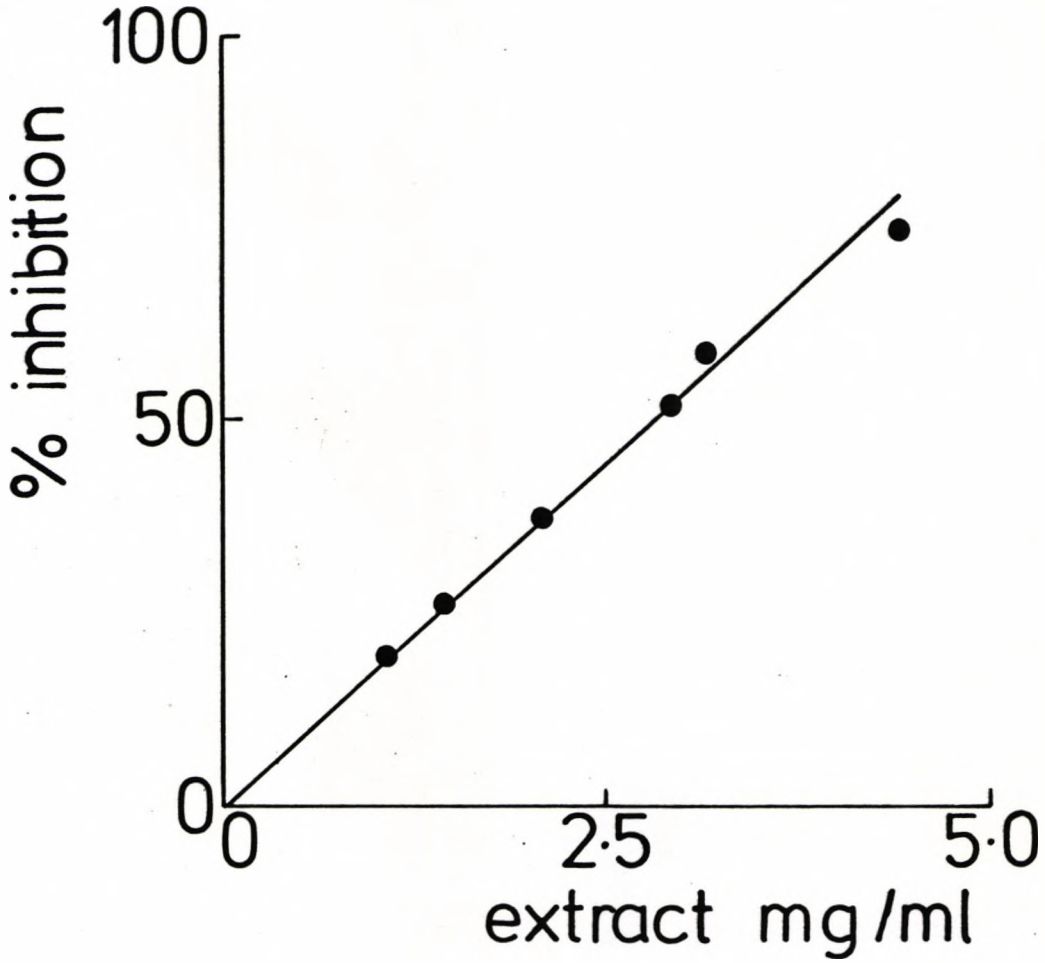

100
50
0
% inhibition
0
2·5
5·0
extract mg/ml

Fig. 84: Brain microtubule assembly *in vitro* in the presence of *Saccharomyces cerevisiae* 20B-12 extracts.

A(●), control: microtubule protein 1.14 mg/ml

B(○), microtubule protein + 1.09 mg/ml extract

C(▲), microtubule protein + 1.09 mg/ml extract pre-treated with RNase (20 µg/ml) and DNase (10 µg/ml)

D(△), microtubule protein + 1.0 mg/ml extract + GTP generating system of NDP-kinase and 2mM ATP

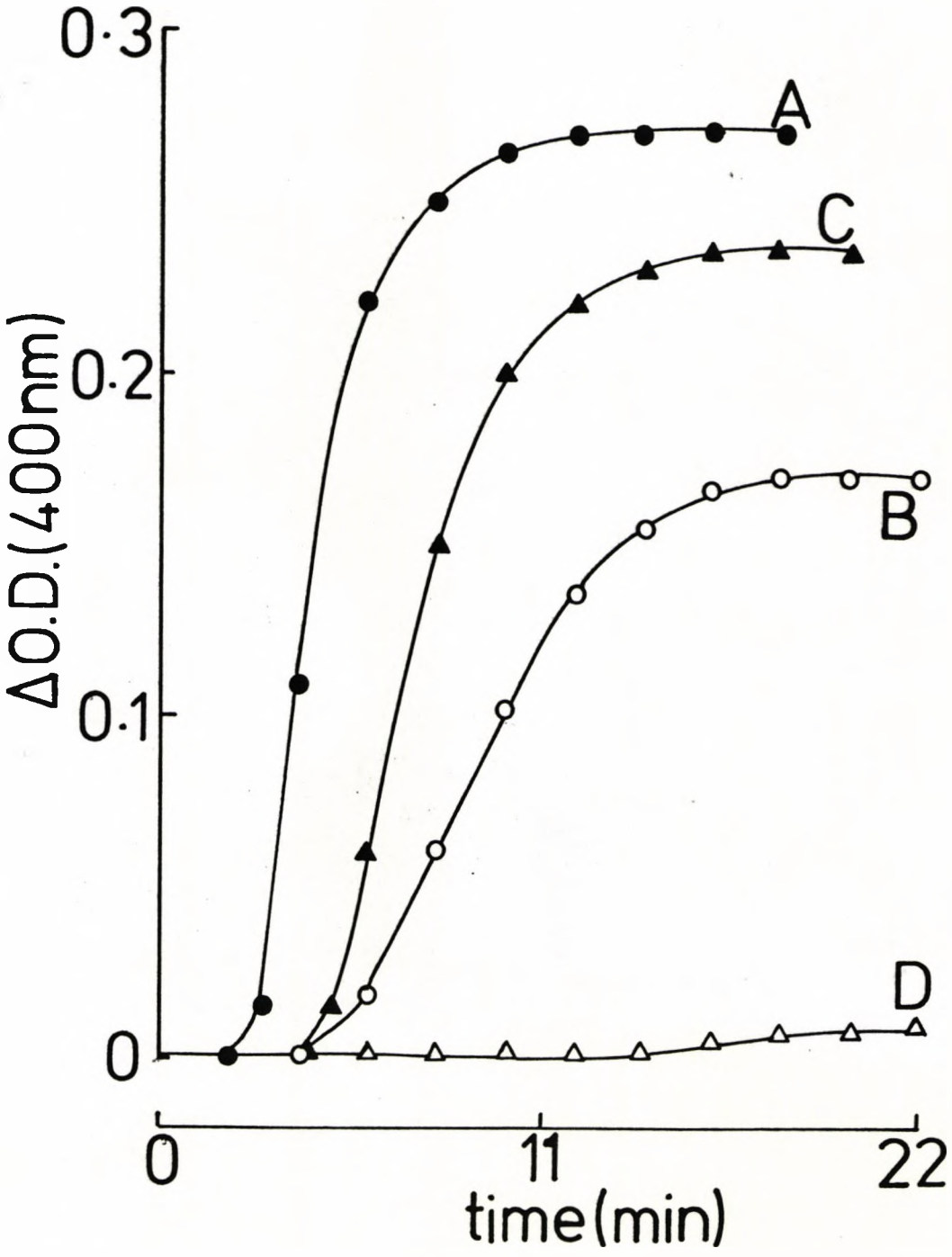
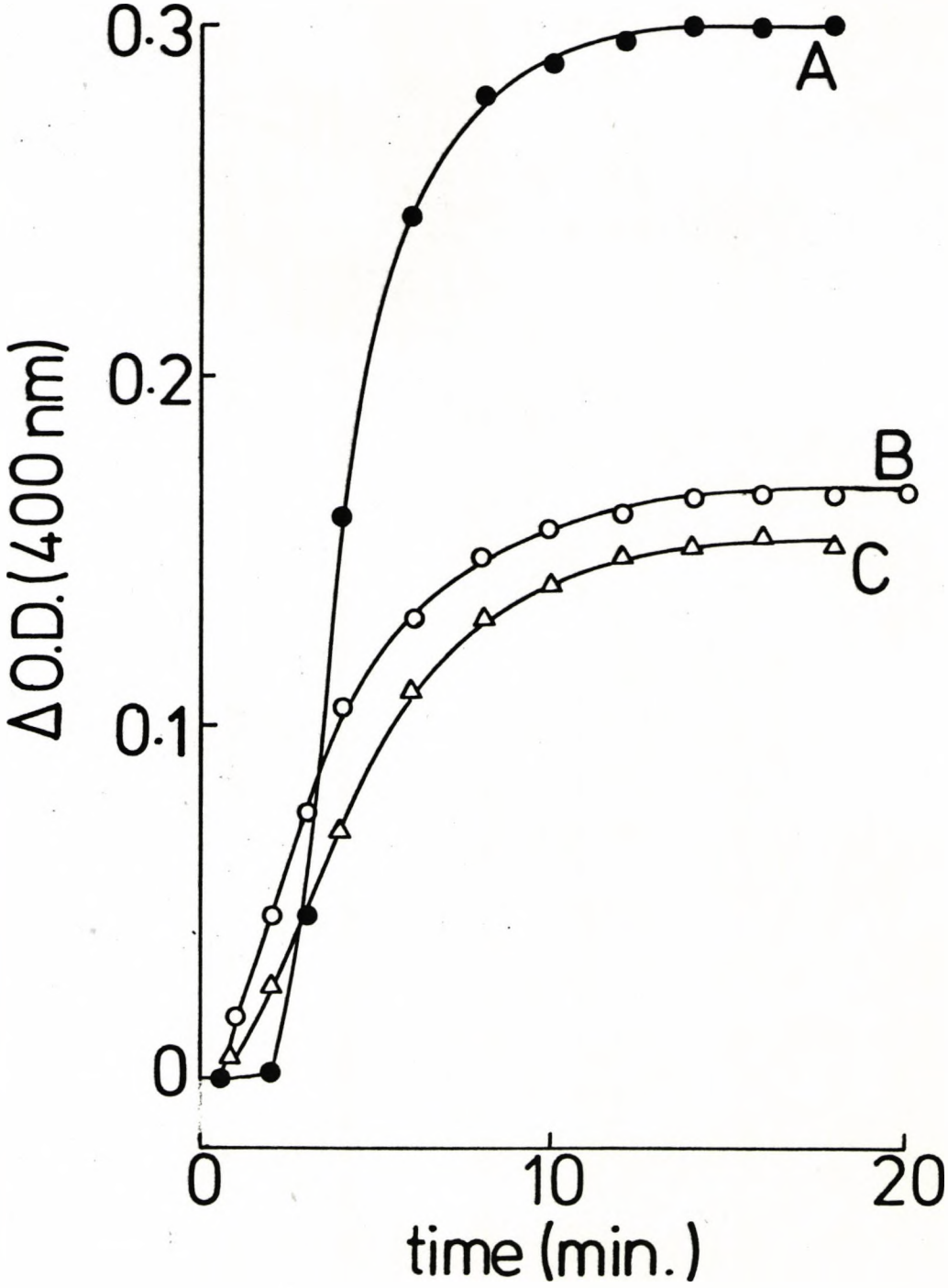

0·3
0·2
0·1
0
ΔO.D.(400nm)
A
C
B
D
0
11
22
time(min)

Fig. 85: Brain microtubule assembly *in vitro* in the presence of 4M glycerol and *Saccharomyces cerevisiae* 20B-12 extract.

A(●), control: 2.65 mg/ml microtubule protein

B(○), 2.65 mg/ml microtubule protein + 4M glycerol

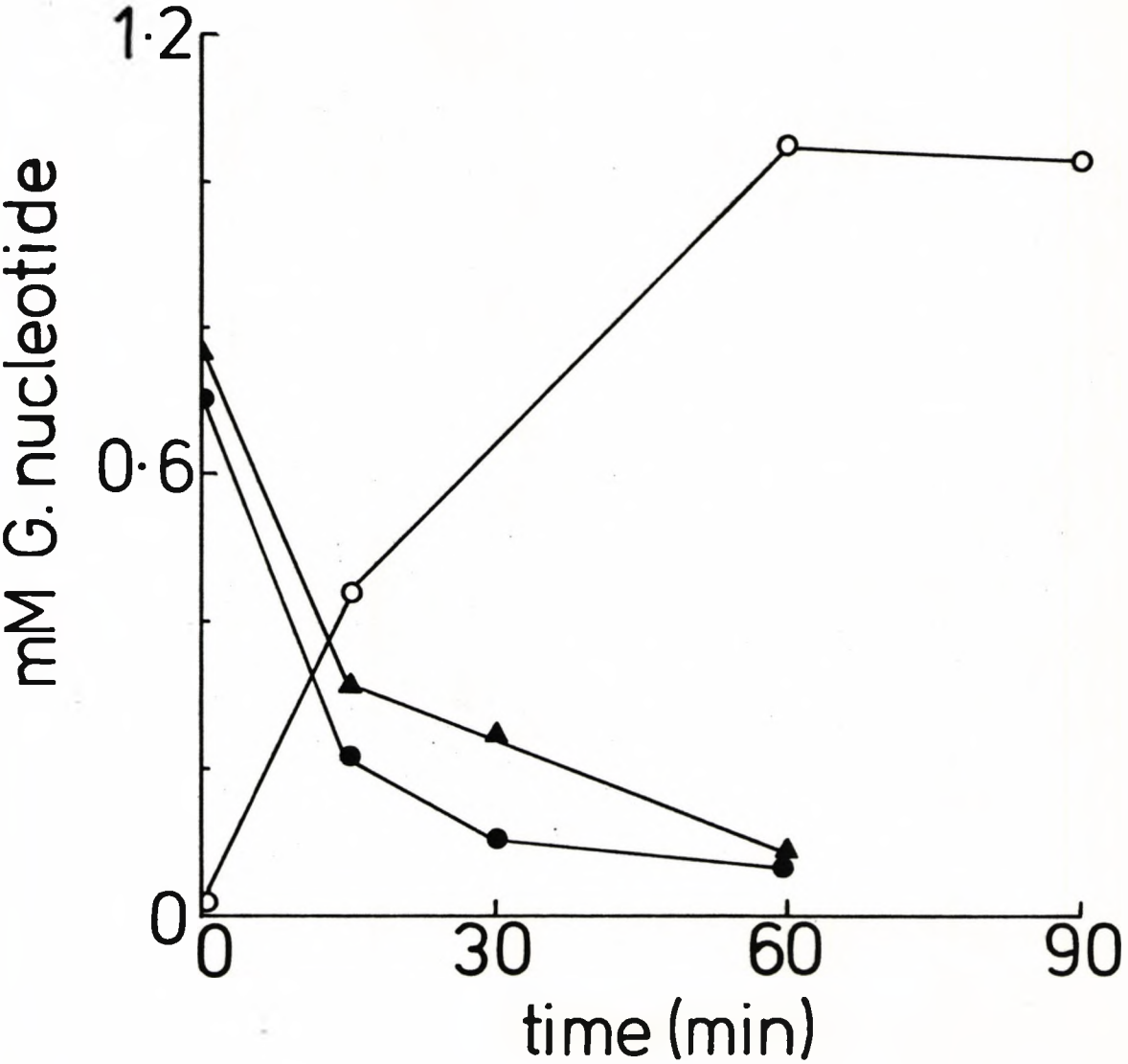
C(△), microtubule protein + 3.64 mg/ml extract + 4M glycerol


0·3
0·2
0·1
0
ΔO.D.(400 nm)
A
B
C
0
10
20
time (min.)

GTP level fell, as shown in Fig. 86. There was a corresponding rise in the level of GDP. Brain microtubule assembly may be supported by low levels of GTP i.e. down to 0.05-0.1mM, however under these conditions the plateau O.D. value is unstable and tends to decrease slowly with time at 37^{o}C due to the fact that microtubule assembly in vitro is a dynamic equilibrium. This decrease in plateau O.D. can be seen in Figs. 81 & 82 in samples assembled in the presence of cdc 13 extract, and it is an obvious disadvantage in a heterologous assembly system, in that the yield of co-polymer would be decreased during a prolonged incubation and centrifugation. Various regeneration systems for the maintainance of GTP levels were considered, however in the presence of yeast extracts these appeared to inhibit brain microtubule assembly (Fig. 84).

In order to identify yeast microtubule proteins by co-polymerisation with brain microtubules it is important that the optimum conditions for assembly are provided. In a heterologous system containing Saccharomyces proteins and brain microtubule protein there are evidently many factors which contribute to the inhibition of microtubule assembly in vitro. One of the more important obstacles to co-polymerisation is the protease activity which must be reduced if meaningful results are to be obtained. Digestion of the brain microtubule proteins and, perhaps more importantly, the yeast proteins must be abolished. To this end the protease deficient 20B-12 strain of Saccharomyces cerevisiae was used for co-polymerisation experiments. In addition a cocktail of protease inhibitors was included in the mixture to minimise any specific protease activity which may still remain - even at a low level e.g. specific attack of brain tau proteins. RNase and DNase treatment was included, to remove inhibitory activity associated with nucleic acids, also glycerol was used at 4M to reduce inhibition further and to help to increase the yield. The problem of the

Fig. 86: The effect on GTP and GDP levels of incubating cdc 13 extracts with 1mM GTP. The concentration of guanine nucleotide (GTP or GDP) was assayed after deproteinisation of the sample.
GTP level, (▲) after incubation with 1.04 mg/ml extract; (●) after incubation with 2.08 mg/ml extract
GDP level (O) after incubation with 2.08 mg/ml extract


1·2
0·6
0
mM G. nucleotide
0
30
60
90
time (min)

GTP-hydrolytic activity remained, as no regenerating system was found to be suitable due to the inhibitory effects on assembly of all the systems tested. However this activity seems to act solely in reducing the yield of polymerised microtubules and is not likely to qualitatively affect the proteins in a co-polymer. No attempt was made therefore to alieviate the GTP-ase activity in co-polymerisation mixtures.

Co-polymerisation of Saccharomyces cerevisiae and brain microtubule proteins

Extracts of Saccharomyces cerevisiae 20B-12 labelled with ^{35}S by growing cells in medium containing ^{35}S-labelled sulphate for one generation were used for co-polymerisation experiments. The labelled extract was enriched with either whole microtubule protein (i.e. containing HMW-MAPs) or microtubule protein depleted in HMW-MAPs by polymerisation in DMSO. Although no HMW-MAPs are visible on SDS gels, this MAP-depleted protein is still capable of polymerisation in vitro with or without the presence of glycerol in the assembly buffer.

Co-polymerisation was performed in the presence of 4M glycerol, also RNase and DNase were included in the assembly mixture i.e. conditions designed to minimise inhibition of assembly by yeast extract as determined previously. MAP-depleted protein was used in order to expose the maximum MAP-binding sites of tubulin to the yeast extract. The ratio of extract protein to brain protein in co-polymerisation mixtures was approx. 3:1.

Fig. 87 shows autoradiograms of SDS-PAGE of the first and second co-polymer pellets (HP_1 and HP_2 respectively) from experiments using MAP-depleted brain protein. The HP_1 contains a large number of yeast proteins, most of which are lost during the second cycle of assembly-disassembly. The major labelled yeast proteins remaining associated with the brain microtubules at the end of the two cycles of assembly have approximate molecular

Fig. 87: Co-polymerisation of ^{35}S-labelled Saccharomyces cerevisiae proteins with MAP-depleted brain microtubule protein.

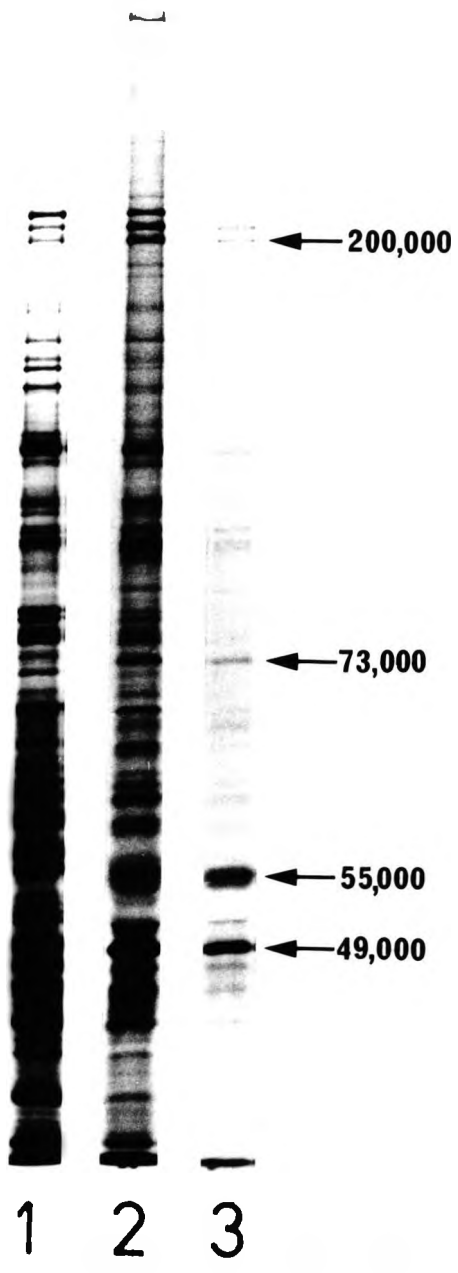
Autoradiograms of SDS-PAGE on 6% acrylamide gels

1, ^{35}S-labelled extract

2, HP_1 co-polymer

3, HP_2 co-polymer

The SDS molecular weights of major proteins are indicated


200,000
73,000
55,000
49,000
1
2
3

weights of 49,000 and 55,000 daltons. In addition, there are other proteins of approx. 73,000; 110,000; 130,000; 200,000 and 230,000 daltons.

Fig. 88 shows the tubulin region of the gel in more detail. The comparison of a stained gel with its autoradiogram shows that the 55,000 dalton yeast protein migrates as a diffuse band in the same position as brain α-tubulin. This suggests that the 55,000 dalton yeast protein is homologous with brain α-tubulin.

One of the less prominent yeast proteins in this area of the gel has a mobility slightly greater than that of brain β-tubulin and an estimated mol. wt. of approx. 52,000. There is apparently no radioactive protein from Saccharomyces which co-migrates with brain β-tubulin. The other major protein in this area of the gel has a molecular weight of approx. 49,000 daltons. This pattern of co-polymerising yeast proteins remains the same through a third cycle of assembly.

Fig. 89 shows the results of similar experiments using whole microtubule protein from brain to enrich the labelled extract. The pattern of co-polymerising radioactive proteins is the same irrespective of whether whole microtubule protein or MAP-depleted protein is used.

Assuming that Saccharomyces cerevisiae microtubules have the same optimum conditions for assembly in vitro as those of brain, then yeast proteins remaining with brain proteins throughout three cycles of temperature dependent assembly-disassembly are likely candidates for Saccharomyces tubulin and MAPs.

Migration on SDS polyacrylamide gels was used to determine the molecular weights of the various proteins in the co-polymer. Unknowns, together with proteins of known molecular weight were run in adjacent tracks on a 6% acrylamide gel. Molecular weights were determined from a standard plot as shown in Fig. 90.

Fig. 88: The tubulin region on a 6% polyacrylamide gel of a HP_2 co-polymer preparation. A comparison of a stained gel (1) with its autoradiogram (2).

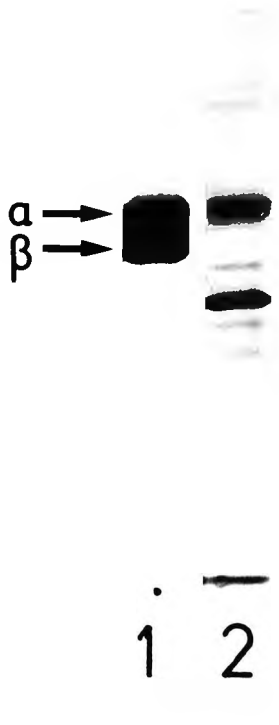

α
β
1
2

Fig. 89: Co-polymerisation of ^{35}S-labelled Saccharomyces cerevisiae proteins with whole microtubule protein; autoradiograms of SDS-PAGE on 6% acrylamide gels.

A, HP_1 co-polymer

B, HP_2 co-polymer

C, HP_3 co-polymer

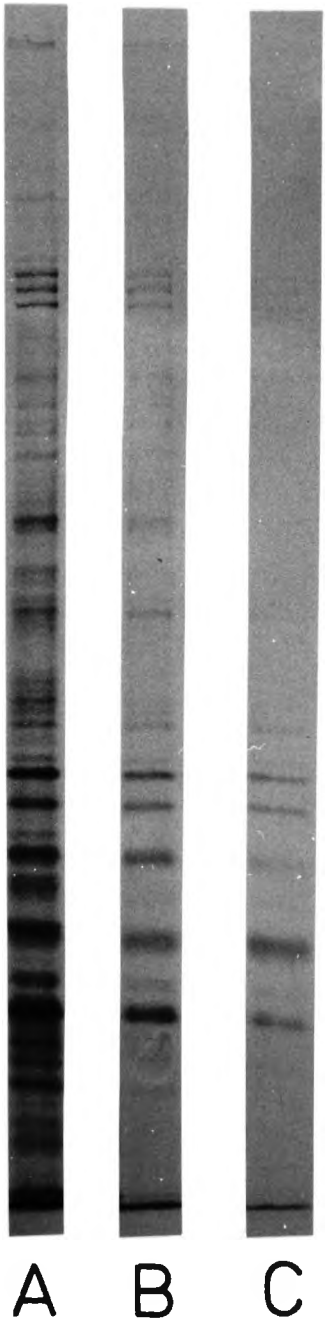

A
B
C

Fig. 90: Standard curve for the estimation of molecular weight by SDS-PAGE on 6% gels

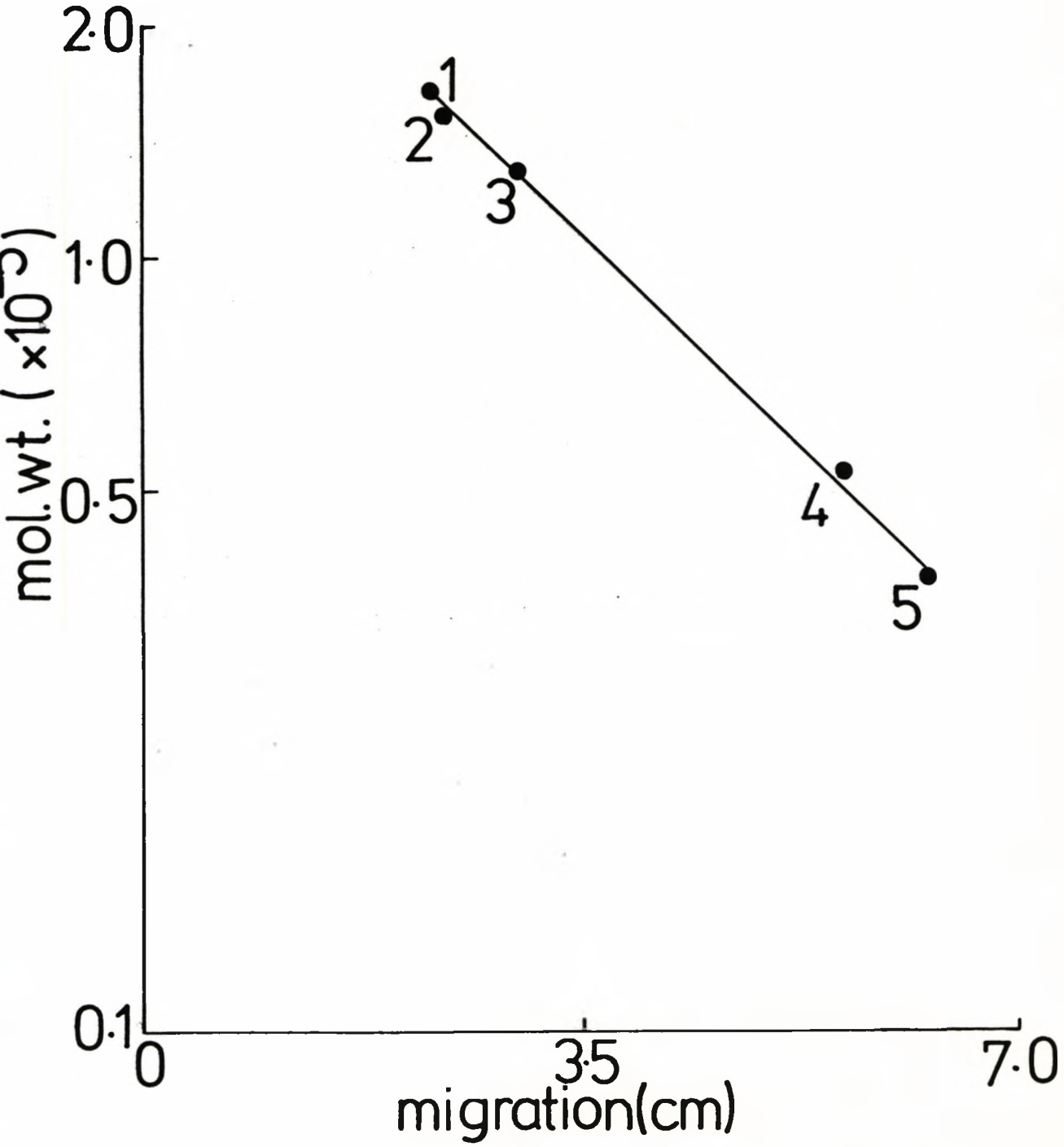
Standard proteins used were:

1 & 2, β' & β subunits of RNA polymerase mol. wt. 165,000 and 155,000 respectively

3, β-galactosidase; mol. wt. 130,000

4, glutamate dehydrogenase; mol. wt. 53,000

5, α-subunit of RNA polymerase; mol. wt. 39,000


2·0
1·0
0·5
0·1
mol. wt. (×10⁻⁵)
1
2
3
4
5
0
3·5
7·0
migration(cm)

Losses of microtubule protein throughout the co-polymerisation procedure were monitored. The recovery of MAP-depleted brain protein after three cycles of assembly-disassembly was 52% for a control and 38% for a co-polymer, in an experiment where the extract to microtubule protein ratio in the co-polymerisation mixture was 1:1. In an experiment where this ratio was 2:1 the co-polymer yield was only 15%. No correction was made for the proportion of Saccharomyces cerevisiae proteins in the co-polymer, therefore all protein in the HP_3 co-polymer pellets was assumed to be MAP-depleted brain protein. Actual yields of brain protein will be slightly less than the values stated for co-polymer preparations. There is a 50% recovery of starting material in the control after three cycles of assembly-disassembly. The per cent recovery is less in the presence of yeast extract and appears to depend upon the relative amount of extract and brain protein present. It is unlikely that protease activity is responsible for this reduction in recovery in the co-polymer as compared with the control, however it is possible that the GTP-ase activity present in the extract may cause a decrease in yield. As seen from Fig. 82, the turbidity plateau value tends to fall in the presence of high concentrations of extract at $37^{o}C$.

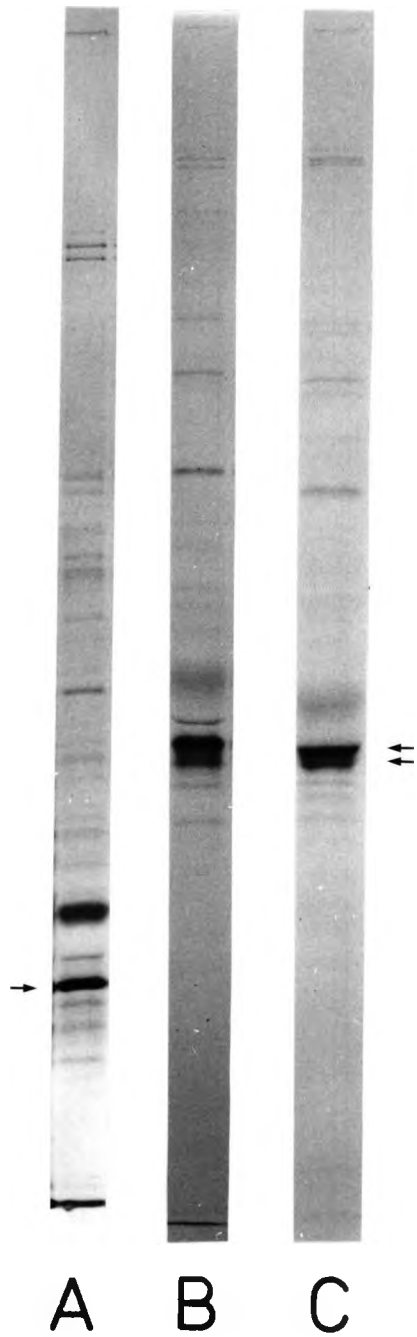
Fig. 91 shows the effect of subjecting co-polymer preparations to SDS-PAGE under different electrophoretic conditions. When co-polymer samples are electrophoresed on 7.5% acrylamide gels as opposed to 6% gels the 49,000 mol. wt. protein band appears to split or spread, appearing as possibly three bands on an autoradiogram. If this band on the gel did consist of a group of proteins with closely similar molecular weights they may appear as a single band on a lower per cent gel where proteins at the lower end of the size range become compressed, compared with their migration behaviour on a less porous gel. Increasing the percentage does

Fig. 91: Autoradiograms of SDS-PAGE of labelled Saccharomyces cerevisiae HP_2 co-polymer preparations. Electrophoresis was performed under various conditions;

A, 6% acrylamide, pH 8.3 electrode buffer

B, 7.5% acrylamide, pH 8.3 electrode buffer

C, 7.5% acrylamide, pH 9.2 electrode buffer


A
B
C

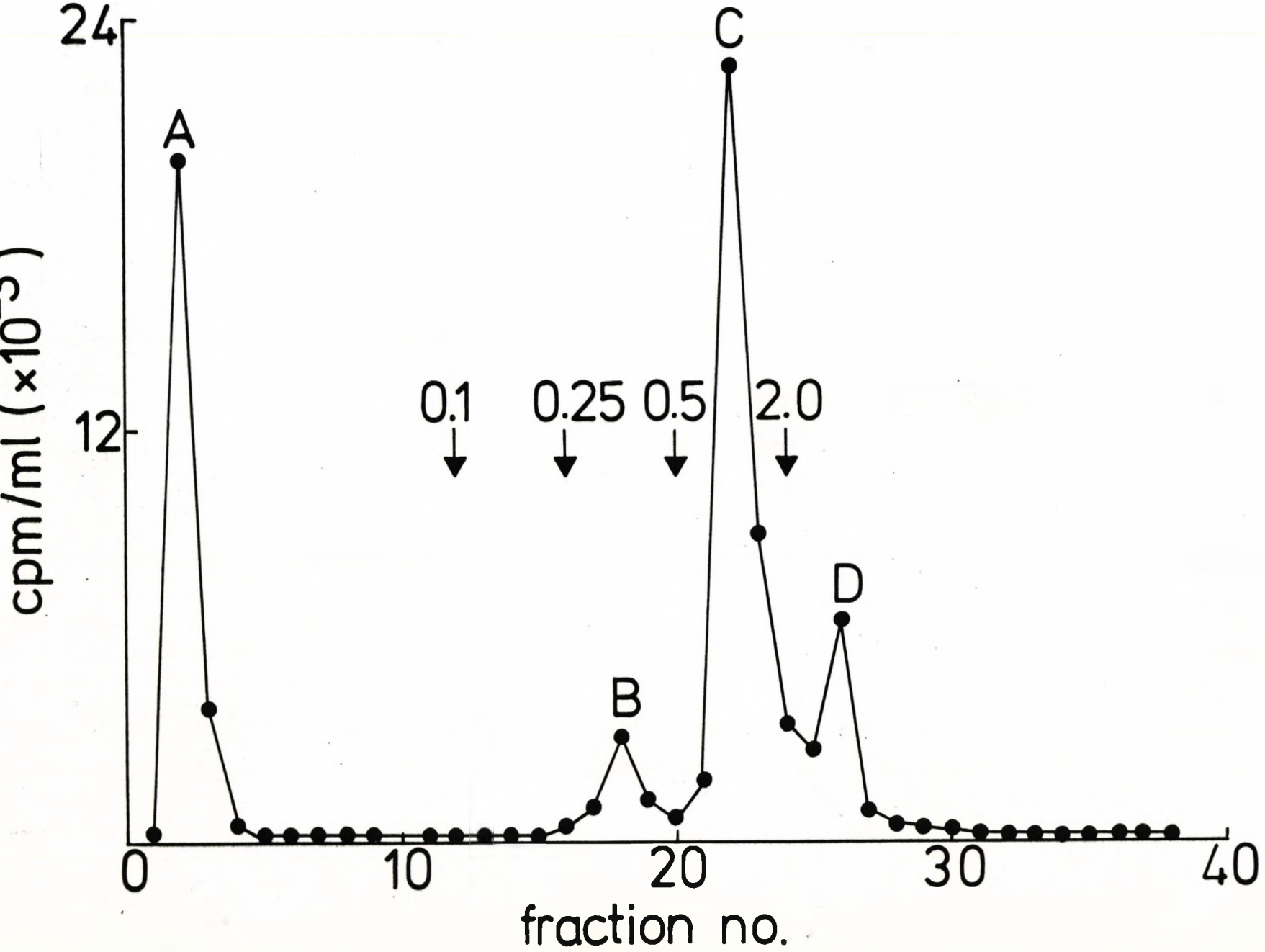
not however alter the general pattern of the 55,000 and 52,000 dalton protein bands. The use of an electrode buffer of pH 9.2 does alter the pattern of the proteins in the tubulin region of the gel in that the 52,000 dalton protein band is no longer visible, and there are two strongly radioactive bands below the tubulin region. It is possible therefore to radically alter the migrating behaviour of proteins during SDS-PAGE by altering the conditions even slightly, and proteins that appear as a single species under certain conditions may be revealed as a mixture when the conditions are changed.

Phosphocellulose chromatography of the Saccharomyces cerevisiae co-polymer

Phosphocellulose chromatography has been used to prepare brain tubulin free from MAPs. Tubulin is an acidic protein and does not bind to phosphocellulose whilst the MAPs, being in general neutral to basic molecules, bind readily and may be eluted with salt. Fractionation by phosphocellulose column-chromatography is therefore a useful method for identifying the various proteins in a co-polymer as tubulin-like or MAP-like components.

A HP_3 co-polymer of MAP-depleted brain protein and ^{35}S-labelled yeast proteins was fractionated using phosphocellulose. 2.4mg of HP_3 co-polymer protein in 1.6mls were applied to a 3 x 1cm phosphocellulose column at $4^{o}C$. Flow rate was 20ml/hour. Total radioactivity loaded was 270,000 cpm. Fig. 92 shows the elution of radioactivity from the column. Peak A is the unbound material eluting at the void volume of the column. The column was then washed with 5 column-volumes of buffer prior to addition of the salt washes. Bound material was eluted using 10ml volumes each of 0.1M, 0.25M, 0.5M and 2.0M KCl in column buffer. No radioactive material was eluted with 0.1M salt but peaks of radioactivity were obtained on

Fig. 92: Separation of radioactive yeast proteins in a HP_3 co-polymer by phosphocellulose chromatography. Arrows denote the addition of 10 ml batches of buffer containing 0.1M, 0.25M, 0.5M and 2.0M KCl.


24
12
cpm/ml (×10⁻³)
A
B
C
D
0.1
0.25
0.5
2.0
0
10
20
30
40
fraction no.

washing with 0.25M, 0.5M and 2M KCl - peaks B, C and D respectively. Peak C contained the most radioactivity.

The pattern of radioactive yeast proteins in each peak from the column compared with the starting material is shown in Fig. 93. Peak A is the unbound fraction which contains the brain tubulin and also two radioactive yeast proteins. These are the protein which co-migrates with the brain ∝-tubulin, and the 52,000 dalton protein. The behaviour of these two yeast proteins during cycles of assembly-disassembly and phosphocellulose chromatography suggests that they are Saccharomyces cerevisiae tubulins. Peaks B and C contain the associated proteins which have remained bound to the phosphocellulose. Peak C contains the majority of these proteins which were eluted with 0.5M KCl. They include the 49,000; 73,000; 110,000 and 130,000 dalton proteins.

Assembly kinetics of the co-polymer

Co-polymerisation experiments were performed using unlabelled Saccharomyces cerevisiae extract supplemented with HMW-MAP-depleted microtubule protein and the co-polymer formed was tested for its ability to assemble in vitro assayed by turbidimetry.

Extract protein : microtubule protein ratio in these experiments was 6:1, and the protein was taken through two cycles of assembly-disassembly to form a HP_2 co-polymer and control pellets. Fig. 94 shows SDS-PAGE of HP_2 co-polymer and control preparations stained with Coomassie brilliant blue. The 49,000 mol. wt. protein band is visible below the stained tubulin bands in the co-polymer HP_2. Tubulin only is visible in the control HP_2.

HP_2 co-polymer and control pellets were resuspended in cold PEMG buffer, depolymerised on ice and their assembly assayed in the spectrophotomete

Fig. 93: Autoradiograms of 6% SDS-gels showing ^{35}S-labelled yeast proteins in a HP_3 copolymer, and in peaks A-D from the phosphocellulose column.

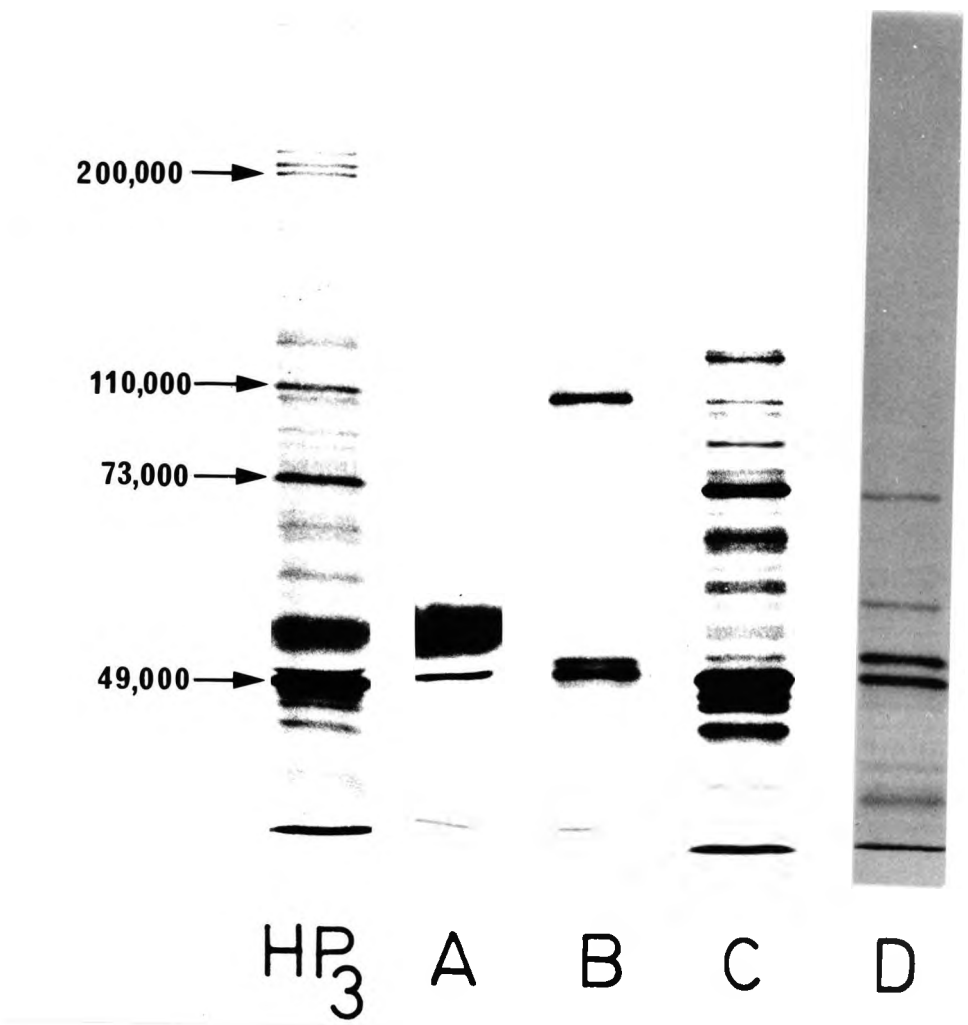
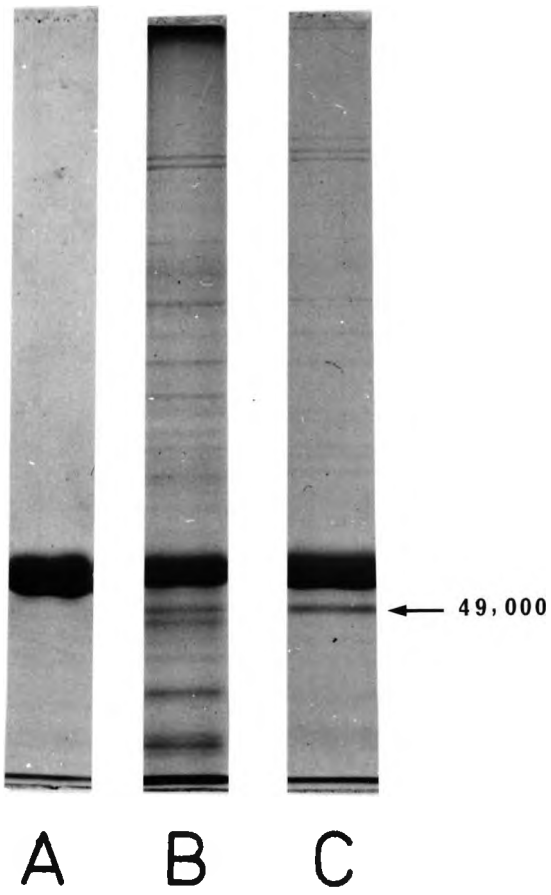

200,000
110,000
73,000
49,000
HP3
A
B
C
D

Fig. 94: SDS-PAGE (6% gels) of un-labelled co-polymer preparations. Gels were stained with Coomassie brilliant blue.

A, control, i.e. HMW MAP-depleted brain microtubule protein assembled under the same conditions as the co-polymer

B, HP_1 co-polymer

C, HP_2 co-polymer


49,000
A
B
C

Although no HMW-MAPs are visible on SDS gels of the MAP-depleted protein (control) it is still capable of assembly *in vitro* with, or without the presence of glycerol.

The critical concentration (Co) is the concentration below which protein will not assembly into microtubules. This parameter may be measured by plotting the extent of assembly - assayed by turbidimetry and expressed as change in O.D.400nm (ΔOD400) - against the total protein concentration. Extrapolation to zero assembly gives an intercept with the x-axis which is an estimate of Co. The Co for whole microtubule protein is approx. 0.2mg/ml (Fig. 95 & 96). For MAP-depleted protein the value is approx. 1 mg/ml in the absence of glycerol (Fig. 95). Assayed in the presence of glycerol the Co is approx. 0.6 mg/ml (Fig. 96). The MAP-depleted protein taken through two cycles of assembly-disassembly in parallel with the co-polymer has a Co value of approx. 2 mg/ml in the absence of glycerol, whereas for MAP-depleted protein prepared by polymerisation in DMSO only (i.e. without further cycles in the presence of glycerol) this value is approx. 1 mg/ml.

Critical concentration determinations were performed in a similar manner using the HP_2 co-polymer and MAP-depleted microtubule protein taken through cycles of assembly-disassembly as a control in parallel with the co-polymer. The results are shown in Fig. 97. The co-polymer appears to have a lower Co value than the control, the total extent of assembly and the initial rate of assembly are also greater in the co-polymer than in the control. These data suggest that the co-polymer contains components derived from *Saccharomyces* which stimulate microtubule assembly *in vitro*. Brain MAPs are known to stimulate brain microtubule assembly *in vitro* by increasing both the rate and extent of microtubule formation. The yeast

Fig. 95: Microtubule assembly (ΔOD 400nm) against protein concentration for (●) whole microtubule protein and (▲) MAP-depleted microtubule protein, in the absence of glycerol

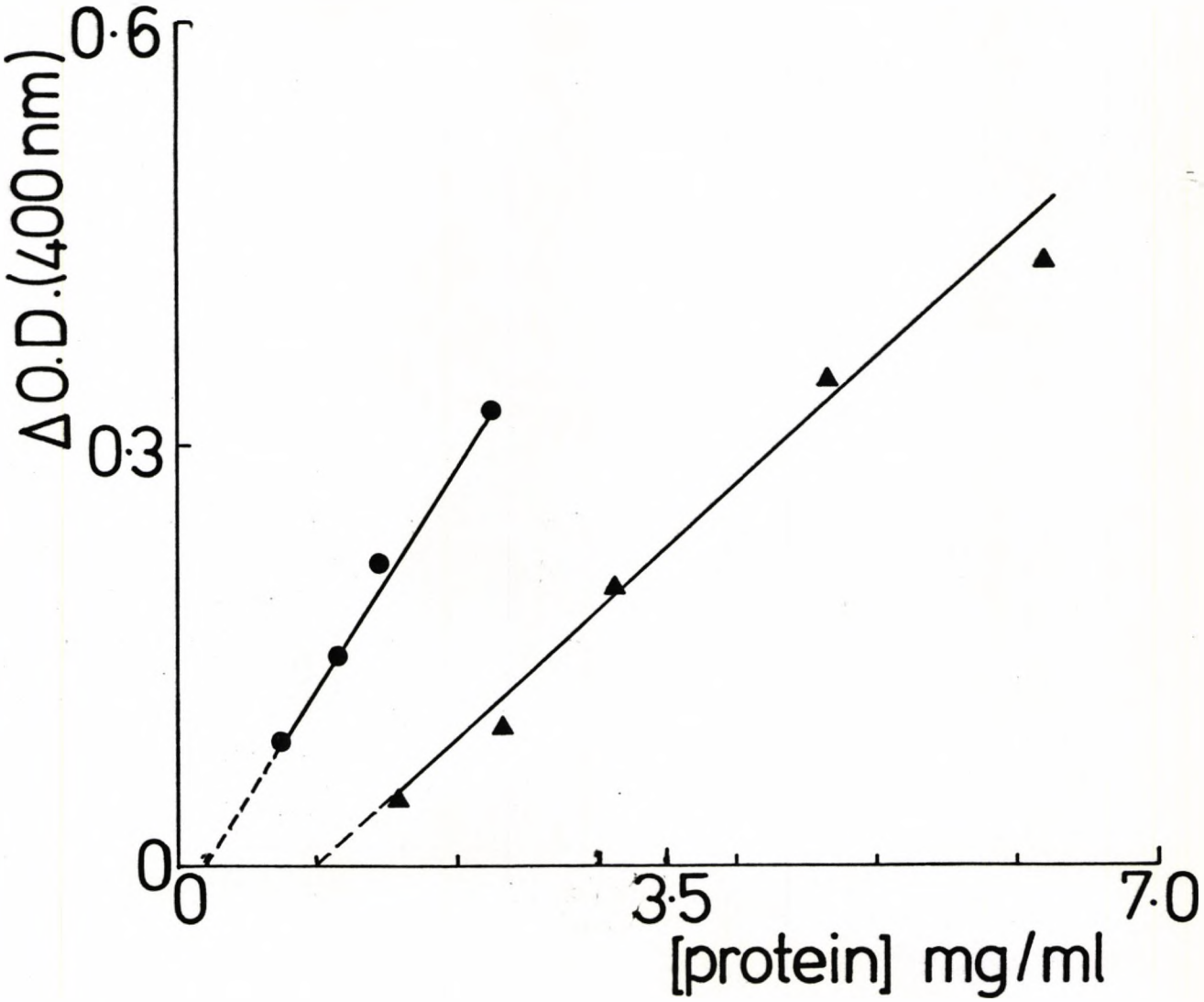

0·6
0·3
0
ΔO.D.(400nm)
0
3·5
7·0
[protein] mg/ml

Fig. 96: Microtubule assembly (ΔOD 400nm) against protein concentration for (●) whole microtubule protein in the absence of glycerol, and (▲) MAP-depleted protein in the presence of 4M glycerol.

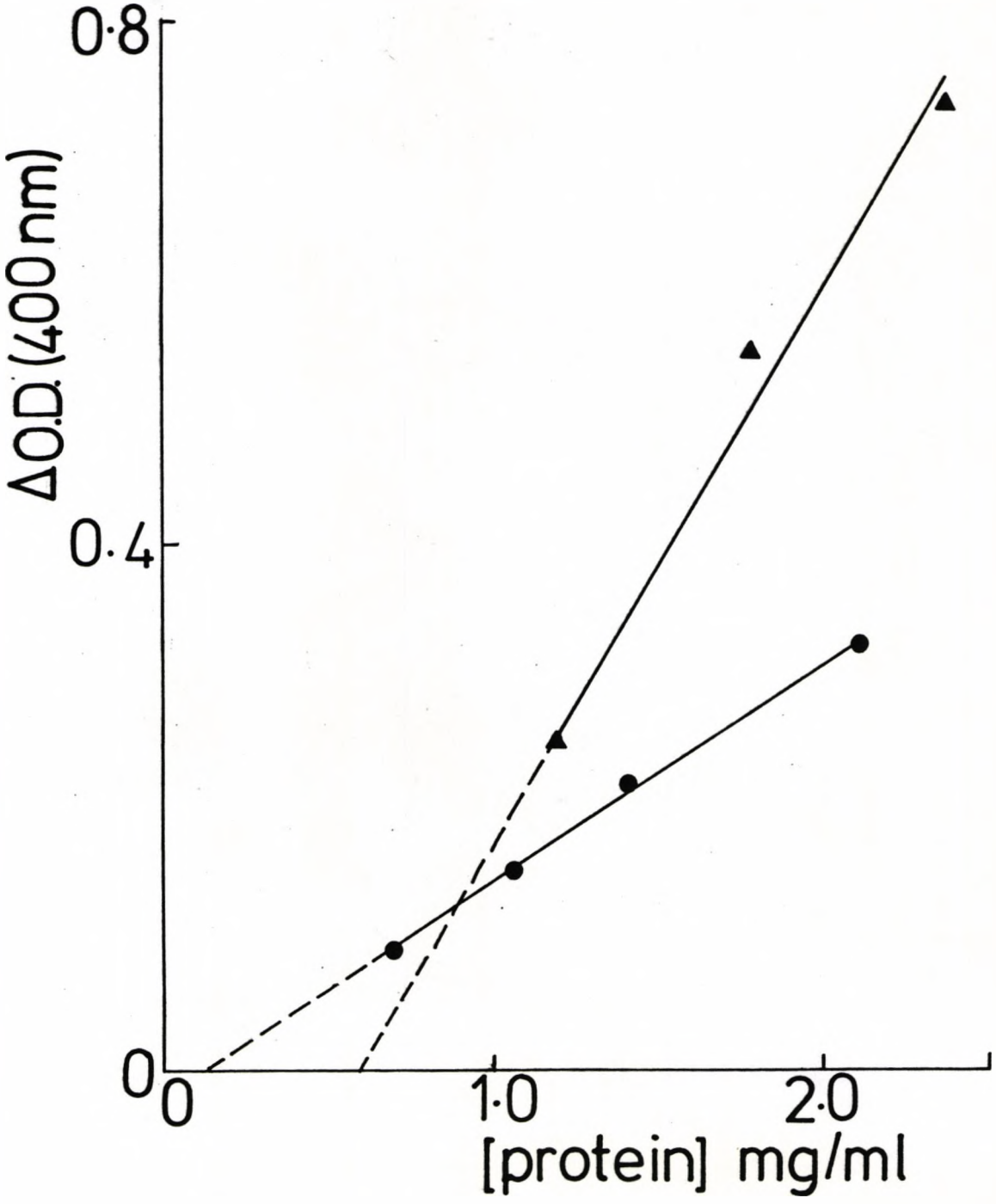

0·8
0·4
0
ΔO.D. (400 nm)
0
1·0
2·0
[protein] mg/ml

Fig. 97: Assembly of a HP_2 co-polymer preparation (▲) compared with a control of MAP-depleted microtubule protein (●), in the absence of glycerol, assayed by turbidimetry. ΔOD (400 nm) is plotted against protein concentration. Extrapolation to the x-axis gives an estimate of C_o.

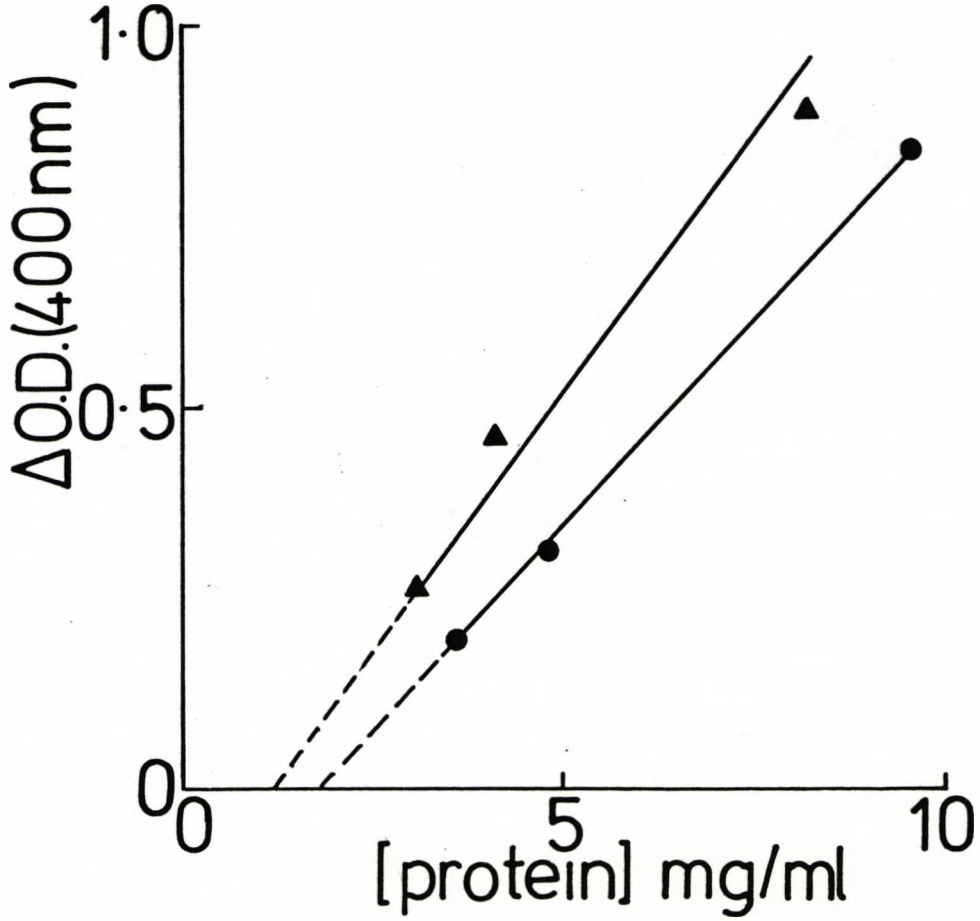

1·0
0·5
0
ΔO.D.(400nm)
0
5
10
[protein] mg/ml

Table 9 Assembly data for co-polymer and control protein

Sample/[protein] mg/ml	Initial rate ΔOD(400nm)/min
CONTROL	
3.58	0.006
4.78	0.019
9.55	0.085
COPOLYMER	
3.28	0.012
4.4	0.035
8.75	0.088

proteins associated with the brain microtubules may therefore contain one or more species of yeast MAP.

Electron microscopy of co-polymer preparations

Co-polymer and control microtubules were examined by electron microscopy. Figs. 99 & 100 show microtubules formed at 37°C in control (MAP-depleted) and co-polymer samples respectively, demonstrating that the co-polymer microtubules are similar to those of the control and also to brain microtubules formed from whole microtubule protein (Fig. 98).

Figs. 102 & 103 show the cold depolymerisation products of control and HP_2 co-polymer preparations respectively. The co-polymer preparation contains ring-shaped aggregates in the cold, whereas none are visible in the MAP-depleted control in which only rod-shaped structures are seen. Whole microtubule protein contains characteristic ring/spiral structures in the cold (Fig. 101) and these disappear when the MAPs are removed by phosphocellulose chromatography. Therefore it appears that the MAPs are responsible for the formation of rings in brain microtubule preparations in vitro; the presence of rings in the co-polymer preparations is a further indication of the presence of components from Saccharomyces which have some MAP-like characteristics.

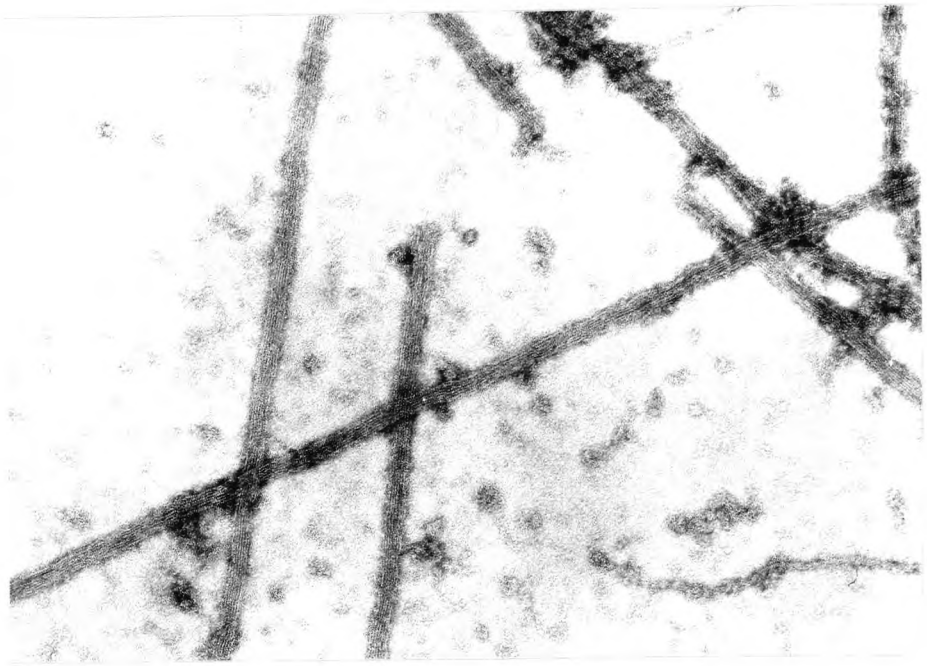
Initial attempts to purify Saccharomyces cerevisiae MAPs

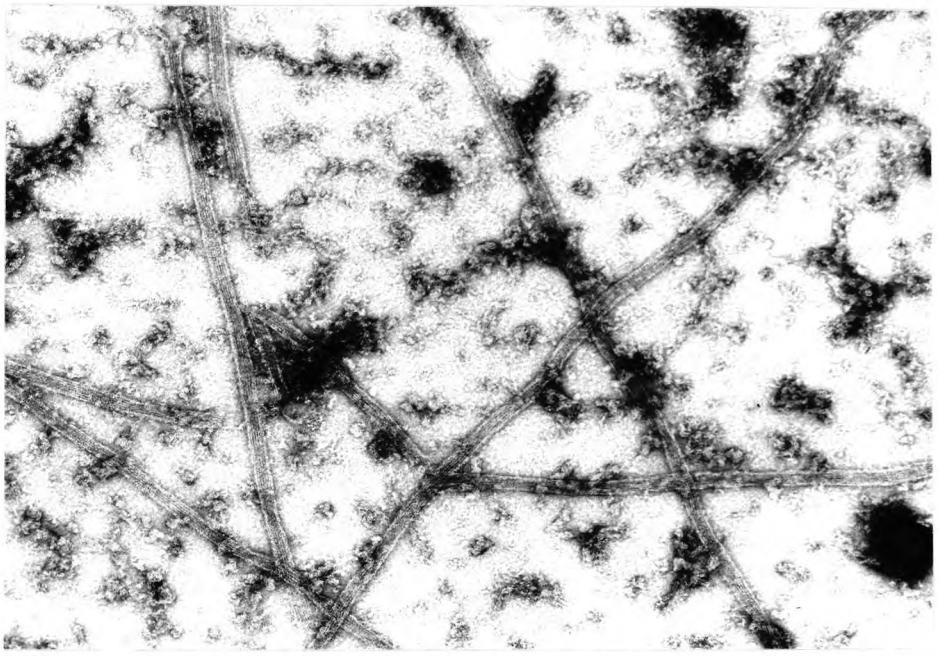
Initial attempts to purify MAPs from Saccharomyces cerevisiae were based on the selection, firstly of yeast proteins which bind to phosphocellulose and elute with an appropriate salt concentration, and subsequently of the proteins from these fractions which are capable of binding to tubulin to form microtubules.

Fig. 98: Electron micrograph of negatively stained brain microtubules formed at 37°C *in vitro*

Fig. 99: Microtubules formed at 37°C *in vitro* from HP_2 co-polymer preparations

Fig. 100: Microtubules formed *in vitro* from HP_2 MAP-depleted control preparations





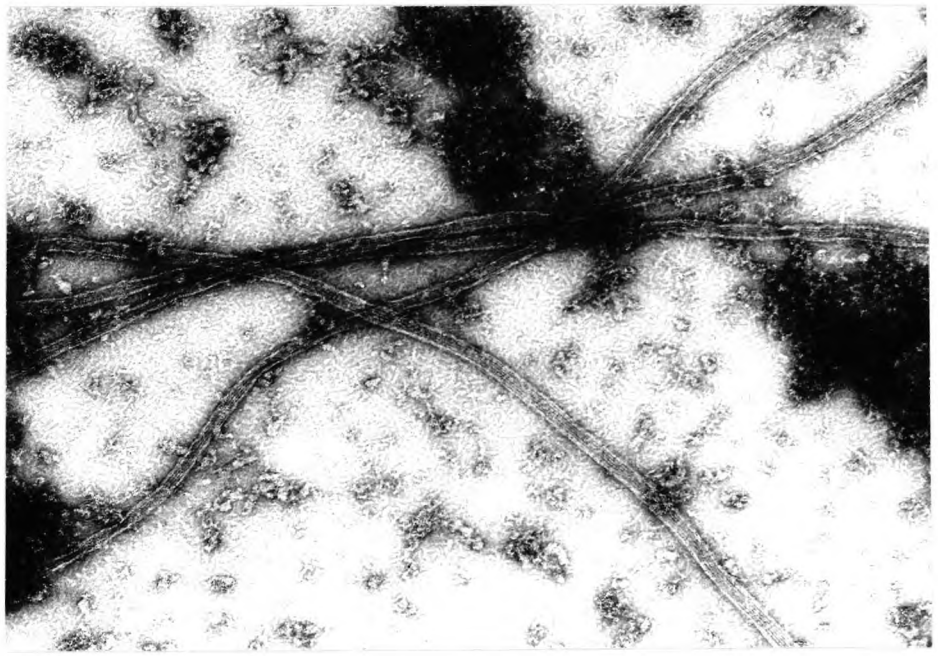
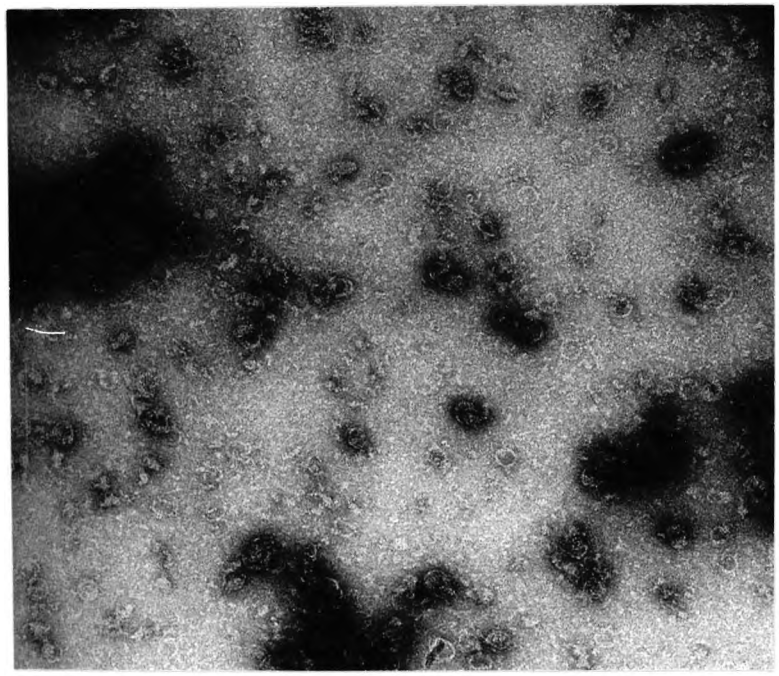
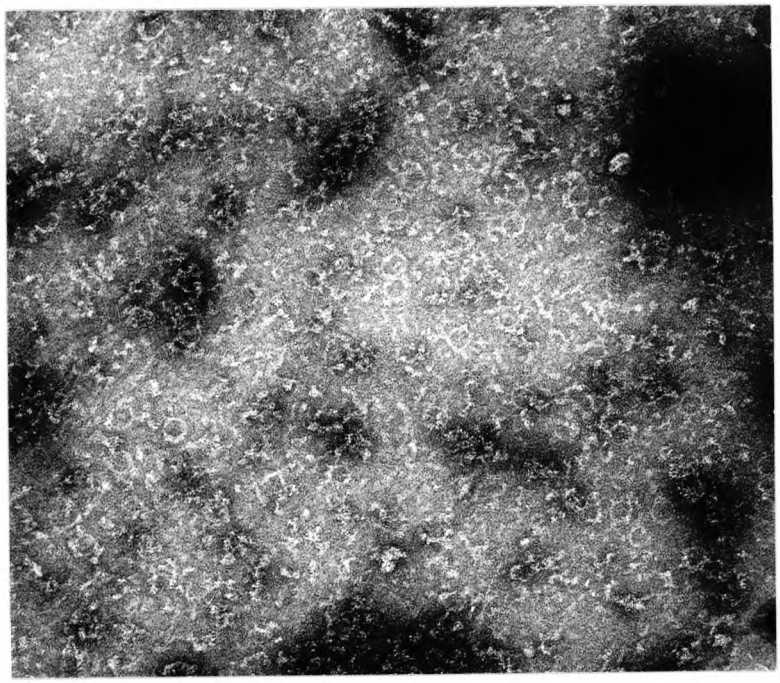


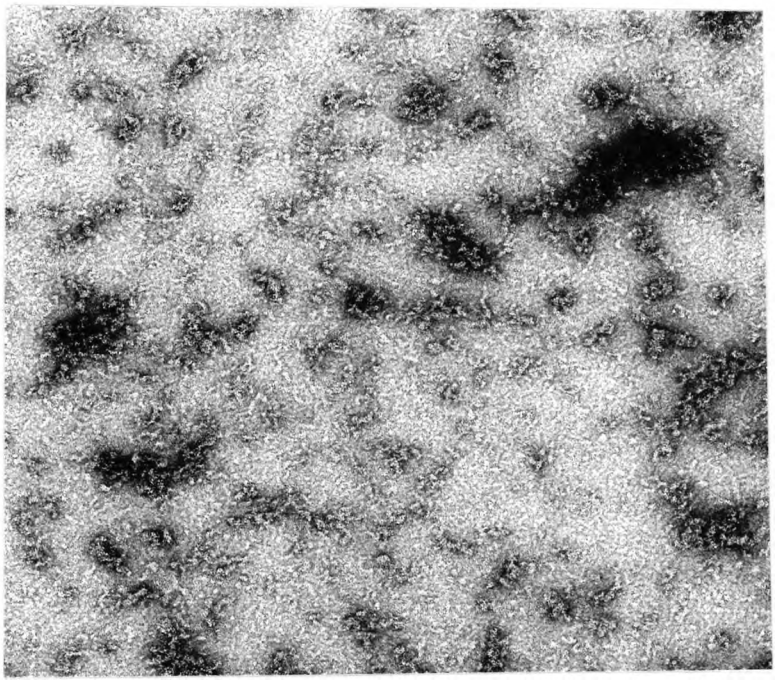
Fig. 101: Electron micrographs of the cold depolymerisation products of brain microtubule protein, showing rings

Fig. 102: Cold depolymerised HP_2 co-polymer preparation showing rings

Fig. 103: Cold depolymerised HP_2 control (MAP-depleted microtubule protein) preparations







Unlabelled Saccharomyces extract was fractionated by chromatography on phosphocellulose as described in Materials and Methods. SDS-PAGE of the proteins in each fraction is shown in Fig. 104. Each fraction contains a number of proteins, however the fractions eluting with 0.25M and 0.5M KCl contained most of the associated proteins seen in the co-polymer preparations. The 0.5M KCl fraction contained a 49,000 mol. wt. protein (Fig. 104).

The fractions were dialysed, mixed with tubulin and the microtubules collected as described. The resulting pellets were resuspended in PEMG buffer, depolymerised at 4°C, cleared by centrifugation and their assembly assayed by turbidimetry in the presence of 4M glycerol. Results of the assay are shown in Fig. 105 in the form of a critical concentration plot of extent of assembly against protein concentration. This shows that the Co values for the control (tubulin alone) and the tubulin assembled in the presence of the 0.25M KCl fraction from the column are similar i.e. approx. 0.6 mg/ml. However, the tubulin assembled with the 0.5M KCl fraction had a much lower Co of approx. 0.2 mg/ml, and both the initial rate and the extent of microtubule assembly are much greater compared with the control.

Fig. 104 shows SDS-PAGE of the fractions from the phosphocellulose column and the pellets obtained from the polymerisation of brain tubulin with these fractions. The 0.5M KCl fraction contained mainly 49,000, 42,000 and 130,000 mol. wt. proteins. In the pellet of this fraction assembled with brain tubulin (the 0.5M fraction co-polymer) only the 49,000 mol. wt. protein is visible on stained gels. The co-polymer of the 0.25M fraction with brain tubulin contains a protein with slightly greater mobility than the 49,000 dalton band, but no other proteins are visible on a stained gel.

Proteins from the 0.5M KCl fraction of Saccharomyces cerevisiae extract are capable of stimulating the assembly of brain tubulin into

Fig. 104: SDS-PAGE of fractions from the phosphocellulose chromatography of yeast extract, and the pellets obtained by assembly of brain tubulin with these fractions.

A, tubulin prepared by phosphocellulose chromatography

B, extract

C, fraction eluting from the phosphocellulose column with 0.25M KCl

D, co-polymer of brain tubulin with C

E, fraction eluting from the column with 0.5M KCl

F, co-polymer of brain tubulin with E

G, HP_2 co-polymer with MAP-depleted microtubule protein

Gels were stained with Coomassie brilliant blue.

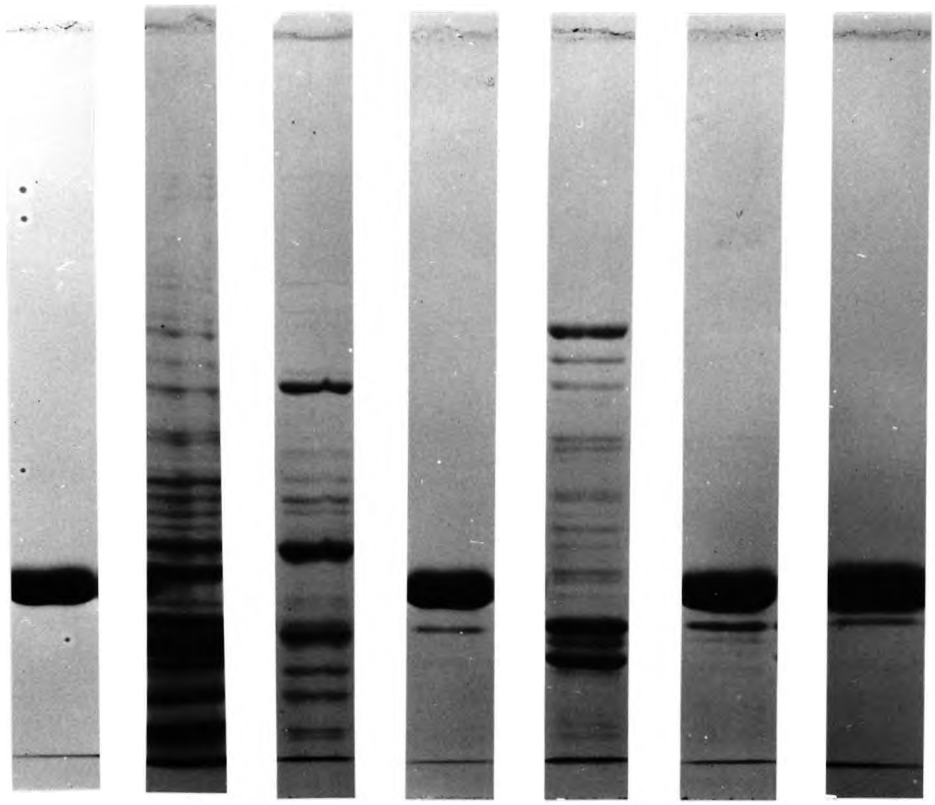
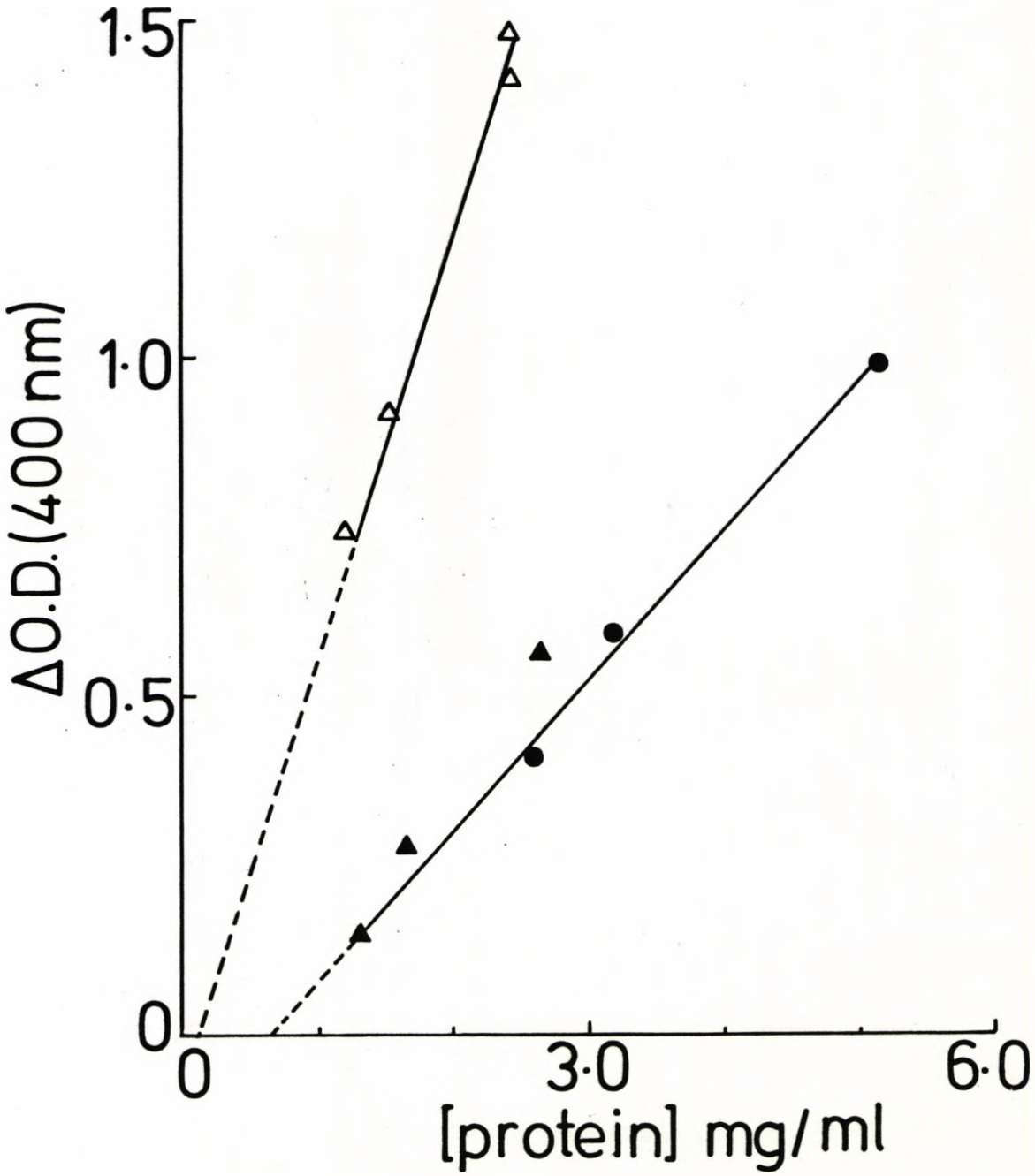

A
B
C
D
E
F
G

Fig. 105: Assembly, expressed as ΔOD (400nm) against protein concentration (mg/ml) of tubulin co-polymerised with fractions from the phosphocellulose chromatography of Saccharomyces cerevisiae extract.

(●), control; tubulin prepared by phosphocellulose chromatography

(▲), tubulin co-polymerised with proteins from a 0.25M KCl fraction of yeast extract

(△), tubulin co-polymerised with proteins from a 0.5M KCl fraction of yeast extract


1·5
1·0
0·5
0
ΔO.D.(400nm)
0
3·0
6·0
[protein] mg/ml

microtubules with an increase in initial rate and total extent of assembly compared with a control. It appears therefore that this fraction contains MAP-like proteins from Saccharomyces and that it may be possible to isolate them in this manner in quantities large enough to enable further characterisation.

The identity of the 49,000 dalton protein in the 0.5M fraction as the protein appearing in the radio-labelled co-polymer preparations is confirmed by co-electrophoresis of samples of both preparations. Results of this experiment are shown in Fig. 106. Autoradiograms of the co-electrophoresis show that the radioactive 49,000 dalton protein in co-polymers migrates with the protein in the 0.5M fraction from the phosphocellulose column which stimulates tubulin assembly.

Co-polymerisation of brain microtubules with labelled Protomyces inundatus extract

Co-polymerisation experiments were performed using ^{35}S-labelled extracts of Protomyces inundatus enriched by addition of HMW-MAP-depleted microtubule protein as described in Materials and Methods, in the same way as for Saccharomyces cerevisiae.

Samples were retained throughout three cycles of assembly-disassembly for SDS-PAGE. The pattern of radioactive Protomyces proteins associated with brain microtubules is shown in Fig. 107. As with Saccharomyces cerevisiae there are numerous Protomyces proteins present in the HP_1 co-polymer, many of which disappear in the course of the second and third cycles of assembly. Major proteins associated with the brain microtubules have molecular weights of approx. 50,000; 55,000; 135,000 and 150,000.

Fig. 108 shows a comparison of a stained gel of a Protomyces HP_3 co-polymer with its autoradiogram. As in the case of Saccharomyces

Fig. 106: Fractions from phosphocellulose chromatography of Saccharomyces cerevisiae extract eluting with 0.5M KCl co-electrophoresed on 6% SDS-polyacrylamide gels with a radioactive ^{35}S-labelled HP_3 co-polymer.

A, autoradiogram of a HP_3 copolymer

B, 0.5M salt fraction from the phosphocellulose column, stained with coomassie brilliant blue

C, stained gel of co-polymer co-electrophoresed with the 0.5M salt fraction

D, autoradiogram of track C

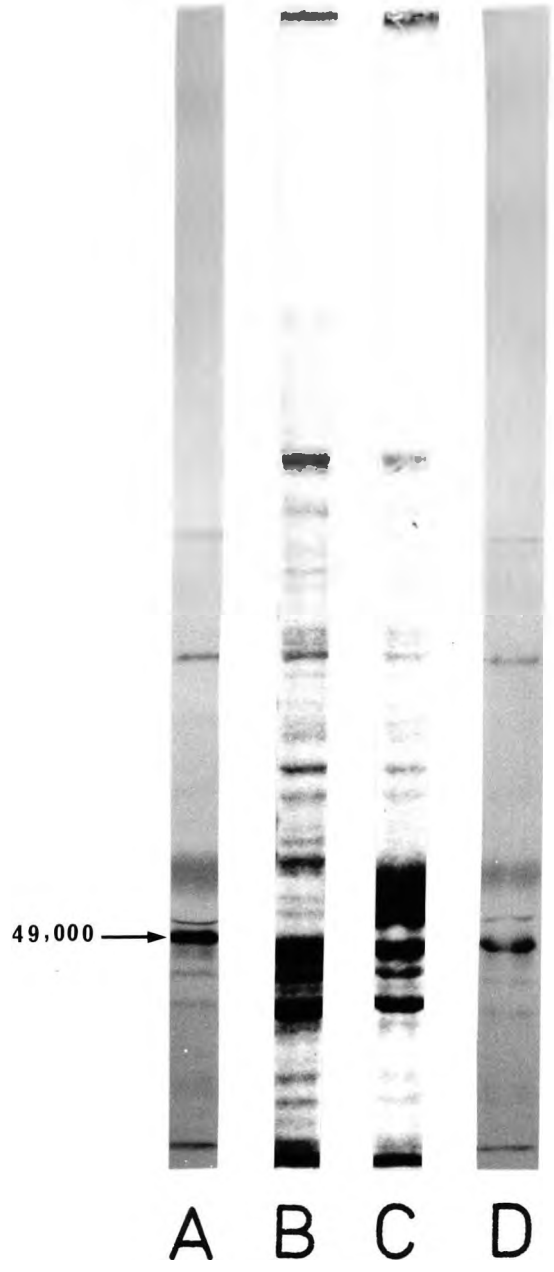

49,000
A
B
C
D

Fig. 107: Co-polymerisation of ^{35}S-labelled proteins from Protomyces inundatus with MAP-depleted brain microtubule protein. Autoradiograms of SDS-PAGE on 6% gels.

A, ^{35}S-labelled Protomyces inundatus extract

B, HP_1 co-polymer

C, HP_2 co-polymer

D, HP_3 co-polymer

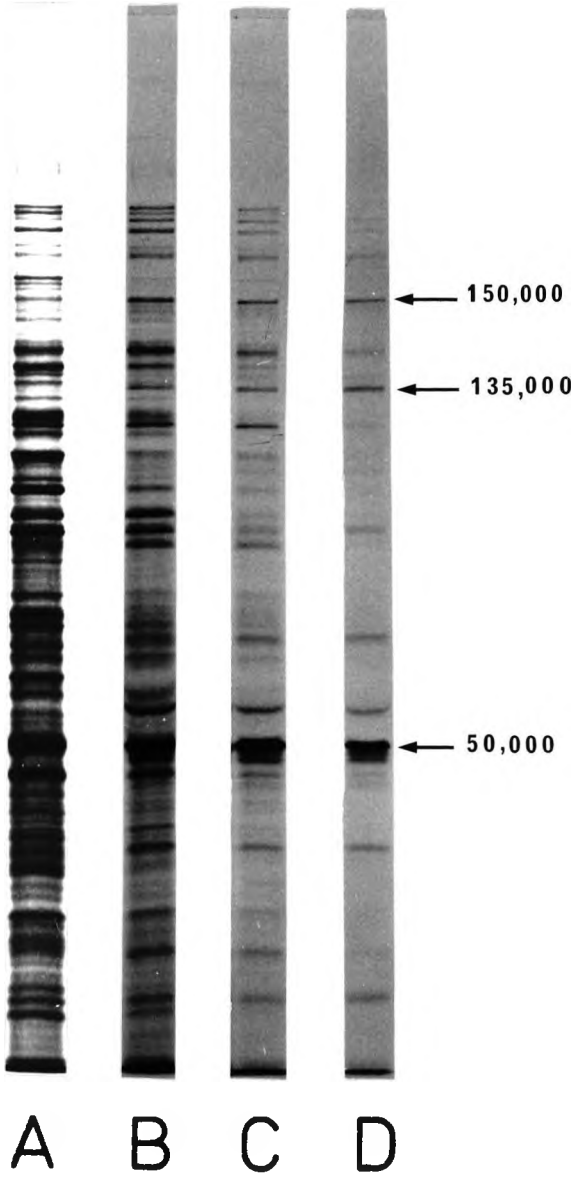

150,000
135,000
50,000
A
B
C
D

Fig. 108: The tubulin region of a 6% gel of a HP_3 co-polymer of Protomyces inundatus. A comparison of a stained gel (A) with its autoradiogram (B).

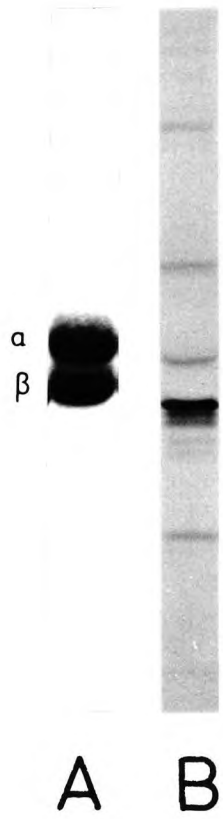

α
β
A
B

Fig. 109: Autoradiography of SDS-PAGE of HP_3 co-polymer preparations from two yeast species, on 6% gels.

A, Saccharomyces cerevisiae HP_3 co-polymer

B, Protomyces inundatus HP_3 co-polymer

A B

cerevisiae there appears to be no protein co-migrating with brain β-tubulin, however a diffuse band co-migrating with brain α-tubulin is present. The most strongly radioactive protein in the co-polymer migrates below the brain β-tubulin band.

The HP_3 co-polymers of brain microtubules with either Saccharomyces cerevisiae or Protomyces inundatus proteins are compared in Fig. 109. The 49,000 mol. wt. protein from Saccharomyces has a mobility on SDS gels slightly greater than the most strongly radioactive protein in the Protomyces co-polymer but appears to co-migrate with a less intense radioactive band below the major band from Protomyces. This strong band on autoradiograms of Protomyces co-polymer preparations may obscure any protein in this co-polymer which is equivalent to the Saccharomyces β-tubulin. Alternatively the strongly labelled protein band from Protomyces may be β-tubulin from this organism.

To identify proteins in a co-polymer of brain microtubules and Protomyces proteins as tubulins or MAPs it would be necessary to fractionate the preparation on phosphocellulose etc as performed with Saccharomyces co-polymer preparations. However certain comparisons and generalisations can be made considering the data available. Firstly there appears to be no co-polymerised protein co-migrating with brain β-tubulin in either yeast studied; secondly, the brain α-tubulin appears to be homologous with an α-tubulin-like protein from both yeast species.

DISCUSSION

It was evident from the growth studies that there was a difference in the sensitivity of Saccharomyces cerevisiae and Protomyces inundatus to the antibiotic griseofulvin. Protomyces was sensitive to micromolar concentrations of this drug in both defined and complex media, whereas the growth of Saccharomyces was not inhibited by concentrations of griseofulvin up to 100μM. Growth kinetics showed that there was at least a doubling of culture turbidity and dry weight before growth of Protomyces was arrested in drug-treated cultures. This suggests a drug-induced block at a specific stage in the cell cycle.

Light and electron microscopy suggest that cells were arrested in cell division, and light microscopy in particular illustrated the abnormal cell configurations produced by griseofulvin in Protomyces. Such configurations often appeared as Y- or V-shaped groups of cells.

A similar situation was seen in the case of Schizosaccharomyces pombe treated with colcemid (Lederberg & Stetton, 1970). This drug induced the formation of doublets of cells arranged in a characteristic V-shape. Such doublets were resistant to sonication, indicating that septum formation had not occurred.

Transmission electron microscopy confirmed that griseofulvin treated Protomyces cells were arrested in cell division and revealed some aspects of the nature of the inhibition. Nuclear division did not appear to have taken place within the characteristic drug-induced groups of cells although replication of DNA was suggested by the presence of multiple nucleoli which often result from multiple sets of ribosomal RNA genes in the nucleus. No septa were formed to divide the cells within a group. It is known that in Saccharomyces cerevisiae cytokinesis and eventual cell separation are

dependent upon the completion of nuclear division. If an analagous situation exists in Protomyces then it would appear that griseofulvin causes the cell cycle to be arrested at some stage of nuclear division. A major feature of drug-treated cells was that assembled microtubules were still present in the nuclei, even after prolonged exposure to the drug. However a normal spindle did not appear to be formed, and in many cases the microtubules formed abnormal aggregates in the nucleus. Grisham et. al. (1974) found that the inhibition of mitosis in HeLa cells by griseofulvin did not involve the disassembly of microtubules even after treatment of cells with 20-40μM griseofulvin for up to 24 hours. In their study, spindle microtubules appeared to be morphologically normal but disorganised, and in addition the number of microtubule organising centres (MTOCs) was increased, resulting in many multipolar mitotic figures. Cells were arrested in metaphase.

These findings, and those from Protomyces demonstrate that the effects of griseofulvin on nuclear microtubules in vivo are completely different from the action of colchicine. Colchicine also arrests cell division at metaphase, but in contrast no microtubules are seen in the nucleus. Very high concentrations of griseofulvin will cause the disappearance of pre-formed cytoplasmic microtubules from animal cells, as demonstrated by loss of microtubule immunofluorescence. A prolonged incubation is necessary however, e.g. at least 18 hours in 280μM griseofulvin (J. Havercroft, this laboratory, personal communication), and some studies have utilised drug concentration of up to 480μM (Spiegelman et. al., 1979).

It has been reported in earlier work (Malawista, Sato & Bensch, 1968) that treatment of Pectinaria gouldii (marine worm) oocytes with griseofulvin at approx. 10μM resulted in the dissolution of the meiotic spindle as evidenced by loss of birefringence in the polarising microscope. On

inspection using electron microscopy loss of birefringence was correlated with a decrease in microtubule number, and in addition no disorganised microtubules were seen. Similar findings were reported using isolated Pectinaria and Pisaster oocyte spindles (Malawista, Sato & Creasy, 1976). These results are not a complete refutation of the Grisham work, as the effects of the drug were studied only by application directly to pre-formed metaphase spindles and not by growth and development of the oocytes in the presence of the drug. In addition it was noted that a concentration of griseofulvin which caused a 50% inhibition of cell division (i.e. 1-2μM) produced a persistant, but reduced spindle, measured by birefringence, whereas higher concentrations of drug (approx. 10μM) caused a complete disappearance of the spindle. These results therefore have an analogy with reduction of cytoplasmic microtubules detectable by immunofluorescence, even though these appear to require a higher drug concentration for their disassembly. It is also apparent that the griseofulvin concentration used to some extent dictates the effects that one sees on microtubules i.e. concentrations which inhibit mitosis do not disassemble microtubules, but higher concentrations may cause disassembly. The absolute values for these concentrations may also depend upton the organism and 'type' of microtubule studied, i.e. spindle or cytoskeletal microtubules.

The similarity of griseofulvin effects in fungi and higher eukaryote cells suggests that the mechanism of griseofulvin inhibition of mitosis in sensitive cells may be functionally different from that of colchicine. Grisham et. al. (1973) have proposed that higher orders of microtubule arrangement or spatial control of microtubule assembly/arrangement may be the target for griseofulvin. Such a proposal is also made by Spiegelman et. al. (1979) as a result of experiments studying regrowth of microtubules in vivo by immunofluorescence after griseofulvin treatment of animal cells.

These authors have reported that, using techniques to improve resolution in areas of high microtubule density, different classes of MTOC could be distinguished by their order of initiation of regrowth after griseofulvin induced disassembly of the microtubules. Regrowth occurred firstly from a single site near the nucleus, thought to be the centriole or pericentriolar material. Regrowth from other sites followed subsequently. Regrowth after colcemid treatment followed a different pattern, hence the suggestion that griseofulvin may act on selected organising/initiation centres.

Recently griseofulvin has been shown to bind specifically to brain HMW-MAPs, and to inhibit microtubule assembly in vitro. MAPs are known to be necessary for initiation but not for elongation of microtubules, however a correlation between MAPs and functional MTOCs remains to be verified.

There is, at present, little information concerning MTOCs in the biochemical sense. Various cell components such as pericentriolar material, spindle pole bodies and kinetochores have been demonstrated to initiate microtubule assembly in vitro, however such apparently functional centres have not been analysed with respect to their protein components or kinetics of initiation. In the event of such an analysis, the effects of griseofulvin on such activities may be tested.

The effects of griseofulvin on growth and mitosis of Protomyces are superficially similar to the effects of MBC on Saccharomyces cerevisiae (R.A. Quinlan, this laboratory, unpublished data). This drug inhibits cell division of Saccharomyces at a point prior to bud emergence. Cells are arrested as doublets of equal-sized cells, and these are markedly enlarged compared with control cells. Ultrastructural studies showed similarities to griseofulvin inhibition of Protomyces in that septa dividing parent and daughter cells failed to form, however an inspection

of the nuclei of MBC treated cells no microtubules were found. MBC therefore exhibits a colchicine-like effect on cell division in Saccharomyces, and may be considered as a potential tool for the study of tubulin in yeast. Colchicine has long been used as an aid to the identification of tubulin in mammalian and other higher eukaryote tissues, however cell division in most fungi is resistant to colchicine hence the requirement for a colchicine-like molecule which is specific for fungi. MBC has already been used with success in the investigation of microtubule proteins in Aspergillus nidulans. Davidse (1977) used the MBC-binding property of Aspergillus tubulin to partially purify this protein from wild-type, MBC-resistant and super-sensitive mutants of this fungus. It was shown that tubulin from resistant strains bound the drug less efficiently whilst that of super-sensitive strains bound it more strongly than wild-type protein. MBC-resistant and repressor mutants have also been used by another group to study the protein changes involved in MBC resistance and its repression. Analysis of microtubule proteins by a sensitive two-dimensional PAGE technique has revealed that small changes in microtubule proteins were responsible for MBC resistance and led to the identification of the genes for α- and β-tubulin in Aspergillus (Sheir-Neiss et. al. 1978; Morris et. al., 1979). Saccharomyces cerevisiae is a well characterised organism from the point of view of its genetic manipulation and analysis. The use of MBC in similar studies to those mentioned above may reveal similar information on Saccharomyces microtubule proteins and their genes.

Protomyces is a convenient organism for the study of effects of microtubule inhibitors on yeast as it appears to be much easier to fix and prepare for electron microscopy than is Saccharomyces.

Uptake of griseofulvin

Griseofulvin was taken up into both Protomyces and Saccharomyces to similar levels and with similar kinetics. The small differences in extent of uptake (0.2 n.moles/mg c.f. 0.5 n.moles/mg for Saccharomyces and Protomyces respectively) are not sufficient to explain the complete insensitivity of Saccharomyces compared with Protomyces.

Similar uptake kinetics have been reported for griseofulvin accumulation into starfish (Pisaster) oocytes (Malawista et.al., 1976), i.e. a rapid initial phase over approx. 1 minute, followed by a slow subsequent accumulation thought to represent cellular binding of the drug. In contrast Protomyces shows a slow decline in cell radioactivity in some experiments after the initial rapid phase of uptake. The reason for this is not known. Values for griseofulvin accumulated by oocytes are similar to those taken up into Saccharomyces. Such concentrations are sufficient to completely inhibit mitosis in oocytes, however Saccharomyces is resistant to the drug. This suggests that some function other than permeability to the drug is responsible for the insensitivity of Saccharomyces.

The short-term uptake of griseofulvin into Protomyces and Saccharomyces showed different responses to a lowering of temperature. Reduction of temperature reduces the initial rapid phase of uptake of drug into Protomyces, but has no effect on uptake by Saccharomyces. It is possible that the initial binding at $4^{o}C$ is a measure of non-specific adsorption onto cell wall/membrane material, and thus the difference between values at $4^{o}C$ and $25^{o}C$ is a measure of 'true' uptake. If this is the case then it would suggest that there is no true uptake of drug by Saccharomyces, only non-specific binding. Alternatively the differences in temperature effects between the two yeasts could be a reflection of different mechanisms

of drug uptake in the two cases. Protomyces showed a further slow accumulation of radio-labelled drug after the initial rapid phase at 4°C - in the case of this organism it may be this phase which is temperature dependent, taking place much more rapidly at 25°C.

The time-course of colchicine uptake into human KB cells resembled that of griseofulvin into oocytes (Malawista et. al., 1976) and this was similarly found in the case of Protomyces and Saccharomyces which appeared to accumulate equivalent quantities of colchicine in a similar manner to the accumulation of griseofulvin. However, both yeasts are resistant to colchicine. Haber et. al. (1972) have also shown that the resistance of Saccharomyces cerevisiae to colchicine was not due to selective permeability, however their results did not take the form of a time-course, uptake being presented as a percentage permeability to the drug compared with tritiatied water, after 1.5 hours incubation. These results also showed that stationary phase cells of Saccharomyces were more permeable to both colcemid and colchicine. This is consistent with the increased permeability of Saccharomyces cells to griseofulvin.

Protomyces and Saccharomyces showed significantly different patterns of griseofulvin uptake during prolonged incubations with radio-labelled drug. Protomyces accumulated increasing quantities of labelled drug after approx. 4-6 hours, whereas Saccharomyces showed no such pattern. The rate of this long term phase of griseofulvin accumulation was extremely variable on comparing results from several identical experiments. In comparison, the short-term uptake results were very consistent with regard to the kinetics and extent of the drug accumulation. It is possible that the differences in griseofulvin uptake between Protomyces and Saccharomyces over long periods of time in non-growing conditions is a result, and not the basis of the differences in griseofulvin sensitivity between these two

yeasts, i.e. the effect of the drug on the sensitive yeast may cause the breakdown of cellular integrity in some way which in turn may result in an increased permeability to the drug. An indication that this may be occurring comes from the work of Trinci & Gull (1973) who report that cells of Protomyces inundatus do die as a result of incubation with griseofulvin as evidenced by a fall of culture viability beginning immediately after addition of the drug to cells. In contrast griseofulvin appears to have no such effects upon Saccharomyces and hence no long term increase in permeability to the drug.

Previously reported studies of griseofulvin uptake into sensitive fungi have been performed over long periods of time with mycelia under non-growing conditions (e.g. El Nakeeb & Lampen, 1965b). These authors have studied uptake by the sensitive dermatophyte fungus Microsporum gypseum and report that this fungus takes up large quantities of drug i.e. approx. 40 n.moles/mg over periods of up to 72 hours. They do report, however, that there appeared to be an immediate removal of small quantities of labelled drug from the medium which was independent of temperature and pH. This rapid removal was also demonstrated with Saccharomyces cerevisiae (El Nakeeb & Lampen, 1965c) which bound 0.05-0.1 n.moles/mg over 48 hours. Moreover, this 'immediate binding' was always seen when samples of Saccharomyces cells were repeatedly added to medium containing labelled griseofulvin. The discussion of these results by the authors states; 'From the present data, one cannot state whether or not this immediate binding is a prerequisite for the continued uptake of griseofulvin.' Results presented here suggest that in sensitive organisms the 'immediate binding' is the 'true' uptake and that subsequent permeability changes are a result of it. In the light of the present work it is possible to speculate that these previous authors were following the second phase

caused by breakdown of cell integrity. In previous work no lag is seen prior to this phase (c.f. results for Protomyces) however no determinations appear to have been made on Microsporum before 6 hours i.e. the time when Protomyces began the second phase of drug accumulation, therefore such a lag would remain undetected.

Many previously reported results may therefore be explained from the point of view of the present work on uptake. Data on griseofulvin uptake reported in the literature are shown in table 10.

Inhibition of microtubule assembly in vitro by Saccharomyces cerevisiae cell extracts

Attempts to isolate and purify microtubules from Saccharomyces cerevisiae by 'traditional' methods of assembly-disassembly were unsuccessful; however in the course of this investigation some of the factors impeding the assembly of microtubules in vitro from crude cell extracts were characterised and, to some extent, anulled. In particular, protease activity appeared to be an important factor, especially where the aim of the investigation is primarily to identify qualitatively the species of proteins involved in a system. The removal of HMW-MAPs from SDS gels of brain microtubule protein proved to be a very sensitive assay for protease activity in extracts of Saccharomyces. It allowed the effects of various inhibitors of protease activity to be examined rapidly and conveniently. This digestion of brain MAPs may partly explain the kinetics of inhibition of brain microtubule assembly in vitro in the presence of Saccharomyces extracts. MAPs have been found to be necessary for the initiation but not the elongation of microtubules in vitro (Murphy et. al., 1977), therefore the removal of MAPs would result in an increased lag (initiation) phase

Table 10 Summary of griseofulvin uptake data for sensitive and resistant organisms

* Harrison, R.G., Henson, A.F., Moore, R.H. unpublished data cited in Bent & Moore, 1966

Table 10

Organism	External [Griseofulvin] (μM)	Incubation time	Uptake n.moles/mg	Reference
Microsporum gypseum	56	6h	8	El-Nakeeb & Lampen 1965c
		24h	30	
		48h	44	
Microsporum gypseum	2.8	24h	0.006	Harrison, Henson & Moore 1966 unpublished *
	70.0	72h	6.0	
Saccharomyces cerevisiae	28	48h	0.06	El-Nakeeb & Lampen 1965c
Saccharomyces cerevisiae	2.8	24h	0.006	Harrison, Henson & Moore 1966 *
Saccharomyces cerevisiae	50	8h	0.2	
Saccharomyces cerevisiae	50	2min	0.2	
Protomyces inundatus	50	8h	0.8-4.0	
Protomyces inundatus	50	2min	0.2-0.5	
Pisaster ochraceous	20	2min	~0.15	Malawista, Sato & Creasy 1976

prior to microtubule formation, and a decrease in rate of assembly. These are observed features of microtubule assembly by yeast extracts.

Vallee & Borisy (1977) have studied the effects of trypsin on brain microtubule preparations. Exposure of intact microtubules to low concentrations of the protease results in the digestion of the HMW-MAPs and their concomitant disappearance from the top of SDS gels. In addition the lateral projections, seen in negatively stained brain microtubule preparations in the electron microscope, are removed. Digestion of the HMW-MAPs in this work resulted in a decrease in rate of microtubule assembly in vitro, however there was very little inhibition of the total extent of assembly as assayed by viscometry. Rings were also visible in trypsin-treated preparations, therefore an initiating activity in the MAP fraction was not totally destroyed and may possibly have been enhanced, as suggested by an 'overshoot' i.e. an increase in viscosity of protease-treated protein over and above the control. Their conclusion was that only part of the brain HMW-MAP molecule was essential for assembly, and that trypsin cleavage did not destroy the active fragment. Saccharomyces extract protease appeared to be much more effective than trypsin in removal of HMW-MAPs from SDS gels, and inhibition of brain microtubule assembly in vitro. It suggests that extract protease digestion of the brain MAP molecule was more extensive than that by trypsin, resulting in the loss of assembly initiating activity as evidenced by the decrease in assembly rate. In addition, Saccharomyces cerevisiae extracts reduced the total extent of microtubule formation, suggesting that microtubule elongation promoting activity was also affected.

Protease activity was not solely responsible for the inhibitory activity of cell extracts however, as evidenced by the fact that extracts of a protease-free strain of Saccharomyces still inhibited brain microtubule

assembly. It is not possible to discount the presence of specific and/or masked proteases in extracts of the 'protease-free' strain, which may attack tau proteins in brain preparations and thus inhibit assembly. Such a digestion of tau proteins would not be detected by the SDS-PAGE system used, in which the brain tau proteins are obscured by the α-tubulin band.

Other factors have been reported to inhibit microtubule assembly *in vitro*, in particular RNA (Bryan *et. al.*, 1975). Consistent with this finding, treatment of *Saccharomyces* extracts with RNase alieviated the inhibition of brain microtubule assembly.

The failure to take precautions to eradicate such inhibitory factors in cell extracts is obviously the reason for the failure of early attempts to isolate microtubule proteins from lower eukaryotes e.g. *Chlamydomonas* (Farrel & Burns, 1975). In this work it was found that *Chlamydomonas* tubulin could not be induced to co-polymerise with gerbil brain microtubules, and that the addition of *Chlamydomonas* extracts to brain microtubule protein caused the inhibition of assembly. This inhibitory activity could be fractionated by DEAE-cellulose chromatography and was found to be associated with putative *Chlamydomonas* tubulin fractions, hence the conclusion by these authors that the algal tubulin itself inhibited gerbil brain microtubule assembly. However, it seems more likely that RNA or an attendant protease may have been responsible.

Recently microtubule proteins have been purified from a variety of non-neural cell extracts by assembly, and the inhibitory activity of such cell extracts on microtubule assembly has been recognised. Few workers have attempted to characterise the inhibitory factors responsible, an exception being Nagle *et. al.* (1977). These authors describe a glycerol sensitive inhibitory activity in extracts of cultured cells which could be titrated by the addition of increasing concentrations of glycerol until

extracts no longer inhibited assembly. Extracts of neuroblastoma cells are inhibitory when no glycerol is present in the assembly mixture, but strongly stimulate assembly in the presence of 3.6M glycerol. Similarly we find that the residual inhibitory activity in Saccharomyces extracts (after RNase treatment and abolition of protease activity) may be greatly reduced by the addition of 4M glycerol in assembly mixtures. These findings are a vindication of the use of glycerol in the preparation of microtubules from lower eukaryote and non-neural sources. Microtubule protein preparation from brain by assembly in the presence of glycerol has been criticised as it is found that glycerol causes a reduction in MAP content of microtubule preparations i.e. protein prepared by the Borisy et. al. procedure (1973) contains 15-20% MAP whilst the Shelanski et. al. (1973) method, incorporating glycerol, produces protein containing only 5-10% MAPs. However, the effects of glycerol in reducing inhibitory activity in non-neural cell extracts and also the apparently protective effects of glycerol on tubulin sulphydryl groups (Mellon & Rebhun, 1976) provide an overriding argument for the inclusion of glycerol in lower eukaryote assembly mixtures, particularly in co-polymerisation mixtures.

All of the criteria for successful co-polymerisation of Saccharomyces and brain microtubule proteins apply also to any future attempts to isolate a homologous assembly system from Saccharomyces cerevisiae. The GTP hydrolytic activity of extracts remains, but it is possible that this problem could be overcome. This activity affects mainly the yield of microtubules and not necessarily the nature of the products, however in an homologous assembly system, where only small quantities of microtubule protein may be present any reduction in yield would become very undesirable.

Identification of microtubule proteins in Saccharomyces cerevisiae

Microtubule proteins from Saccharomyces cerevisiae have been identified by co-polymerisation with brain microtubules taking precautions to minimise inhibitory activities in the yeast extract, in particular the proteases. Co-polymerisation through three cycles of assembly-disassembly and subsequent phosphocellulose chromatography of the resultant co-polymer indicated that the Saccharomyces protein which co-migrated with brain α-tubulin, and the 52,000 mol. wt. protein behaved as, and are candidates for Saccharomyces tubulins. There was apparently no protein co-migrating with brain β-tubulin on SDS-PAGE.

One might expect yeast α- and β-tubulins to co-polymerise with brain microtubules in equal quantities as the building-block of microtubules is thought to consist of an $\alpha\beta$ dimer. However, it appeared from the intensity of bands shown on autoradiograms that the putative β-tubulin was present in smaller amounts than was the α-tubulin.

There are two alternative explanations of this phenomenon, assuming the protein in question to be Saccharomyces β-tubulin. Firstly that the two putative yeast tubulins were present in equal amounts but that the β-tubulin was not as strongly labelled, resulting in a reduced exposure of the photographic film during autoradiography compared with the yeast α-tubulin. It is reported that α- and β-tubulins from brain have similar amino acid compositions, however no comparison of the sulphur-containing amino acids has been reported. An analagous unequal exposure of autoradiograms by labelled α- and β-tubulins from cultured mammalian fibroblasts has been reported (Ben-Ze'ev et. al., 1979). In this case proteins were labelled with [^{35}S]-methionine, and cytoskeletal proteins examined by two-dimensional PAGE. The radioactivity of the α- and β-tubulins on such gels appeared to be quite different, the α-tubulin being labelled to a much

lower specific activity. In this respect the situation appears to be completely opposite to that in Saccharomyces. It does however indicate that differences in sulphur content of the two tubulin subunit proteins are plausible. A study of co-polymerised Saccharomyces proteins labelled with [^{14}C] or [^{3}H] amino acids would determine whether this is the case in yeast.

Alternatively the yeast β-tubulin, being apparently different from that of brain, may not assemble into the subunit lattice of a heteropolymer microtubule as efficiently as the yeast α-tubulin in a system where brain subunits are in enormous excess over those from Saccharomyces. This latter explanation assumes that tubulin reversibly dissociates into α- and β-protomers in solution. Consequently, during cycles of assembly and disassembly there is a selection against the yeast β-subunit. There is direct evidence for such a dissociation. PAGE of brain tubulin under non-denaturing conditions has shown the presence of the 55,000 dalton protomers as well as the 110,000 mol. wt. dimer (Lee, Frigon & Timasheff, 1973).

Other authors report the identification of Saccharomyces tubulins using a co-polymerisation technique, for example Water & Kleinsmith (1976) found yeast proteins which co-migrated with brain tubulins, however the quality of the autoradiograms presented in this work is such that their interpretation is equivocal, coupled with the fact that the electrophoretic analysis was performed using 5-15% acrylamide gradient gels, a system which tends to compress protein bands at the lower end of the molecular weight range thus possibly obscuring any slight differences in mobility between proteins.

In contrast Baum et. al. report the identification (again by co-polymerisation) of tubulins from Saccharomyces cerevisiae with estimated molecular weights of 46,000 and 45,000 daltons, i.e. much lower than brain

tubulins. Their estimate of brain tubulin molecular weight was however lower than usual reports i.e. 48,000 and 50,000 daltons. These differences may be a characteristic of the SDS-PAGE system used in the study. It was noted that these authors used a Laemmli (1970) gel system run at a pH of 9.2 compared with pH 8.3 as specified in the Laemmli recipe and as used in the present work. When protease deficient Saccharomyces co-polymer was subjected to electrophoresis under the conditions specified by Baum et. al. the 49,000 mol. wt. protein band appeared to split, producing a doublet of approx. equal intensity bands, these being the most strongly labelled bands on the co-polymer autoradiograms. It is possible that such a doublet was identified as tubulin by Baum et. al. in their experiments.

The interpretation of SDS-PAGE data especially with regard to molecular weight determinations will be discussed below.

Immunoprecipitation of yeast tubulins using antibody against Chlamydomonas flagellar tubulin as reported by Baum et. al. appeared to yield the putative yeast α-band preferentially - differential labelling of yeast α- and β-tubulin was proposed as an explanation of this apparent selectivity. However the antibody used in this study appeared to be by no means specific for tubulin judging by the multiplicity and range of other yeast proteins also precipitated.

A recent report by Shriver (1978) has identified a protein from Saccharomyces cerevisiae which co-polymerised with brain microtubules and co-migrated on SDS-PAGE with brain α-tubulin. This protein also behaved as a tubulin during phosphocellulose chromatography. No yeast protein was detected co-migrating with brain β-tubulin. These findings compare directly with the present work.

A similar situation was found in Aspergillus nidulans by Sheir-Neiss et. al. (1976). Co-polymerisation experiments with ^{35}S-labelled fungal

extracts yielded two Aspergillus proteins, one of which co-migrated with brain α-tubulin during SDS-PAGE, and one with a slightly greater mobility than brain β-tubulin. Isoelectric focusing of such co-polymer preparations gave similar results.

The evidence suggests that tubulins from Saccharomyces and Aspergillus differ from brain tubulin with respect to the β-subunit. It is possible that this is a feature of fungal microtubule proteins. In addition there is evidence that the tubulins from Physarum polycephalum, Tetrahymena pyriformis and Ascaridia galli all have β-tubulins with different electrophoretic mobility from that of brain (A. Roobol, C.M. Ireland, this laboratory, unpublished data), therefore this may be a feature of lower eukaryotes in general.

SDS-PAGE is universally used in investigations of microtubule proteins both as a means of identification, and to provide estimates of molecular weight. The behaviour of proteins in different gel systems may vary markedly, therefore comparison of data from different groups using different PAGE techniques should be made with caution. The presence of urea in SDS gels may alter the migrational properties such that the migration order of some proteins may be altered, in particular, there is evidence that the migration order of α and β tubulin is reversed on SDS-urea gels i.e. α-tubulin appears to be the faster migrating species on SDS-urea gels compared with SDS gels in which it is the slower-moving protomer (R.A. Quinlan, this laboratory, unpublished data).

SDS-PAGE has been shown to be a reliable method for the estimation of molecular weight (Weber & Osborn, 1969), however Bryan (1974) has shown that the migration, and thus the apparent molecular weight of α-tubulin in particular depends upon conditions used in the PAGE system. By varying the pH and ionic strength, the mol. wt. of α-tubulin may be estimated at

values between 52,000 to 59,000 daltons. The sensitivity of SDS-PAGE to small changes in protein composition is illustrated by Noel et. al. (1979) who report that a single amino acid substitution in a histidine transport protein alters its mobility on SDS-gels as if the molecular weight were greater by approx. 2,000 daltons. This finding would suggest that a molecular weight difference between similar proteins should not be inferred solely on the basis of SDS-PAGE.

The situation regarding MAPs in Saccharomyces cerevisiae is less definite. In order to identify and isolate such a protein it is necessary to define the behaviour and function by which a MAP may be recognised. As there is considerable uncertainty in the literature as to the function of MAPs this is not an easy definition.

The identification of MAPs in Saccharomyces was based on the ability of such proteins to remain associated with co-polymerised microtubules throughout three cycles of assembly, the behaviour of these proteins during phosphocellulose chromatography and their activity in stimulating assembly in vitro and their association with ring formation at $4^{o}C$. All of these activities are characteristic of brain MAPs.

Phosphocellulose chromatography confirmed that the charge characteristics of the yeast proteins in a HP_3 co-polymer were consistent with their designated identity i.e. the 52,000 and 55,000 mol. wt. proteins - the putative tubulins - remained unbound, whereas the remaining proteins of mol. wt. 49,000; 70,000; 110,000; 130,000 and approx. 200,000 bound to the ion-exchanger. The major MAP in Saccharomyces co-polymer preparations had an apparent mol. wt. of 49,000. This protein was eluted from phosphocellulose with 0.5M salt, and in this property resembles the behaviour of a protein

with a similar molecular weight reported to be a prominent MAP in homologous microtubule preparations from various cell culture lines (Nagle et. al., 1977). This protein appeared to be present in a variety of types of cells including neuroblastoma, glial and CHO cells.

Assembly kinetics of co-polymer preparations are more difficult to interpret especially in a heterologous system where brain tubulin is in enormous excess over yeast proteins. The brain tubulin used in the co-polymerisation experiments was depleted of at least the HMW-MAPs by assembly in DMSO, however evidence exists to suggest that there may be brain MAPs (possibly tau?) remaining associated with such protein. The critical concentration of MAP-depleted tubulin rises from 1 mg/ml to 2 mg/ml after two cycles of assembly in glycerol in parallel with the co-polymer. This would suggest a selective loss of assembly stimulating activity from the preparation during the cycles of assembly. Such a factor may be a MAP e.g. tau, which is not usually detected on SDS gels, or simply bound DMSO from the initial preparative assembly. Ideally tubulin purified by phosphocellulose chromatography is the material of choice as a basis for co-polymerisation experiments, however the DMSO method of tubulin preparation proved to be more convenient and economical from the point of view of technical manipulation and quantity of material required.

Kinetic experiments using such co-polymer preparations must be interpreted with caution therefore. It appears that co-polymerised proteins from Saccharomyces do tend to stimulate assembly of DMSO MAP-depleted tubulin, however the extent of the stimulation/lowering of Co must depend upon the amount of MAP-like material present.

The ability of brain tubulin to form ring-like structures in vitro in the cold depends on the presence and quantity of brain MAPs, therefore

depolymerised Saccharomyces co-polymer preparations were examined for the presence of such rings. These do appear to be formed in co-polymer preparations and are absent in the control. Many cationic proteins and other molecules demonstrate the stimulation of ring formation from tubulin in vitro, and the possibility that this non-specific activity is taking place in co-polymer samples cannot be discounted. It is not certain whether ring-shaped oligomers of tubulin are obligate intermediates in the pathway of microtubule assembly in vitro, however they do appear to participate in the initiation of assembly when they are present, and their presence in co-polymer preparations correlates with stimulation of assembly.

The co-polymerisation technique is therefore useful for identifying microtubule proteins from organisms or tissues from which it is not possible to prepare microtubules directly. This method has been successful in identifying both tubulin and MAPs from Saccharomyces cerevisiae. Preliminary experiments with Protomyces inundatus suggest that tubulins may be similar to those from Saccharomyces, whereas there appeared to be qualitative differences in the MAPs. Since griseofulvin has been shown to inhibit brain microtubule assembly in vitro by binding specifically to brain MAPs (Roobol et. al., 1977a) it may be possible to explain the difference in the sensitivity of these two yeasts to griseofulvin in terms of the differences in MAPs. A study of griseofulvin binding in Saccharomyces cerevisiae and Protomyces inundatus co-polymers may be useful in establishing this.

The preliminary identification of tubulins from the two yeasts studied appears to compare well with findings from other lower eukaryotes. It seems likely that differences in antimitotic drug sensitivity displayed by lower organisms compared with mammalian tissues, especially with respect

to colchicine, may be interpreted as a difference in microtubule proteins. It has been reported (Maekawa, 1978) that tubulin from Tetrahymena pyriformis, which is relatively resistant to the anti-mitotic effects of colchicine, has a markedly lower affinity for the drug than brain tubulin. In addition, it has recently been found that microtubules assembled in vitro from the colchicine-resistant slime mould Physarum polycephalum are also resistant to the drug, and that tubulin from this organism does not bind colchicine (A. Roobol, this laboratory, in preparation). In both these organisms tubulins appear to have altered mobility on SDS-PAGE compared with brain tubulin.

Analysis of tubulins by one-dimensional PAGE represents differences as a change in apparent molecular weight whereas in reality such changes may be of a more conservative nature. The application of more sensitive techniques such as two-dimensional PAGE may be useful in the resolution of minor differences between proteins and may reveal the presence of multi-tubulins as found in Aspergillus (Sheir-Neiss et. al., 1978). In particular, Saccharomyces cerevisiae possesses many advantages over other lower eukaryotes and fungi as an experimental organism for the investigation of microtubules. Firstly, it is a non-flagellated organism and the mitotic/meiotic spindle seems to be the only microtubule-containing structure present in the cell. Confusion is therefore unlikely to arise as a result of the possibility of different pools of tubulin subunits c.f. work with Naegleria gruberi which synthesises specific tubulins to form flagellae during differentiation. In addition Saccharomyces can be cultured easily and rapidly, and well characterised methods exist for genetic manipulation in this yeast.

Saccharomyces, in common with most fungi is insensitive to colchicine, however the anti-fungal agent MBC is emerging as a potential tool for the

study of tubulin in fungi, analagous to colchicine in mammalian cells. The use of this drug, in conjunction with mutant studies and two dimensional PAGE analysis of proteins may result in the characterisation of the genes for microtubule proteins in yeast.

Finally, the identification and characterisation of the inhibitory activities in cell extracts of Saccharomyces which prevent microtubule assembly in vitro not only enables the co-polymerisation experiments to yield meaningful results but also introduces the possibility that microtubules may be purified from Saccharomyces cerevisiae by assembly without the use of additional brain protein. If this is possible, then the sensitivit of the microtubules to various anti-mitotic agents may be tested directly. These techniques may also be extended for use with other lower eukaryotes.

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MICROTUBULE PROTEINS IN THE YEAST, *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Microtubule proteins, particularly tubulin, have been isolated and characterised from a range of higher eukaryote tissues [1,2] and cultured cells [3]. In virtually all the higher eukaryotes the main protein tubulin occurs in its native state as a heterodimer. The two constituent subunits, α and β tubulin, of ~55 000 mol. wt, can be separated on SDS–polyacrylamide gel electrophoresis. The microtubules and microtubule proteins of non-flagellated lower eukaryotes have received very little attention. However, organisms such as the yeast *Saccharomyces cerevisiae* have great potential as systems for the study of microtubule function. Studies with these organisms are hampered by the low tubulin concentration in cell extracts [4]. Tubulin from various cell types will copolymerise with brain microtubules. However, it is known that cell extracts inhibit the assembly of microtubules in vitro [5,6]. We have markedly reduced the inhibitory effects of yeast cell extracts by using RNase and DNase treatment, proteolysis inhibitors and a strain of yeast which posesses very reduced protease activity. Using the above precautions with brain microtubule protein depleted in microtubule-associated proteins we have been able to identify radiolabelled yeast proteins which, by their behaviour during repeated cycles of copolymerisation with brain microtubule and chromatography on phosphocellulose, are candidates for yeast tubulins and MAPs. These include a yeast protein comigrating with brain α-tubulin on SDS–polyacrylamide gels. The major possible yeast MAP has mol. wt 49 000. There is apparently no yeast protein which comigrates with brain β-tubulin.

Abbreviations: DMF, dimethyl formamide; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis (β-amino ethyl ether) tetra-acetic acid; MAPs, microtubule-associated proteins; PIPES, piperazine-*N*,*N*'-bis(2-ethane-sulphonic acid); PMSF, phenyl methyl sulphenyl fluoride; SDS, sodium dodecyl sulphate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl

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2. Materials and methods

Saccharomyces cerevisiae strain 20B-12, a mutant deficient in proteases A,B and C [7] was grown in a defined medium using $(NH_4)_2SO_4$ as the nitrogen source. Cultures were incubated for 16 h at 25°C on an orbital shaker, harvested by filtration onto a 1.2 μm Millipore membrane, washed with warm sulphate-free medium (a medium in which all the sulphates in the defined medium were replaced by chlorides) and finally resuspended in 50 ml sulphate-free medium containing 1 mCi Na_2 $^{35}SO_4$ (Radiochemical Centre, Amersham). Cells were incubated in this medium for 4 h at 30°C in a reciprocal shaking water bath.

Cells were collected by filtration, washed in sulphate-free medium and finally suspended in twice the pellet volume of PEMG buffer (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP, pH 6.9) containing 1 mM PMSF in 1% (v/v) DMF; 200 μM TLCK; 50 μg/ml leupeptin. The cells were broken by vortex mixing with an equal volume of cold 0.45 mm diam. glass beads. The extract was pipetted away from the beads and centrifuged at 130 000 × *g* for 30 min at 4°C.

Microtubule protein was prepared from fresh sheep brain as in [8]. Immediately before use

twice-polymerised microtubule protein was resuspended in cold PEMG buffer, depolymerised at 0°C for 30 min, and centrifuged at 130 000 × *g* at 4°C for 30 min. Tubulin was prepared from this supernatant using a modification of the method in [9] in which protein was polymerised in 0.4 M PIPES, 1 mM EGTA, 0.5 mM $MgSO_4$, 1 M glycerol, 10% DMSO, 0.5 mM GTP (pH 6.9) at 37°C for 20 min and collected by centrifugation at 130 000 × *g* for 20 min. Most of the MAPs remained in the supernatant and were discarded. The tubulin enriched pellet was resuspended in PEMG buffer, depolymerised and centrifuged as for the whole microtubule protein. The supernatant was added to ^{35}S-labelled yeast extract. At this stage ribonuclease A (EC 3.1.4.22) (2 μg/ml) and deoxyribonuclease 1 (EC 3.1.4.5) (1 μg/ml) (Sigma) were added.

Co-polymerised microtubules were formed by warming the mixture at 37°C for 30 min and centrifugation at 130 000 × *g* for 30 min at 26°C. This first 'hot pellet', HP_1, copolymer was resuspended in PEMG minus glycerol, depolymerised at 4°C for 30 min and centrifuged at 130 000 × *g* for 30 min at 4°C. The supernatant was adjusted with glycerol to final conc. 4 M, and the process repeated twice more to produce copolymerised pellets, HP_2 and HP_3. Protease inhibitors were present throughout at the above concentrations.

The three times polymerised copolymer pellet (HP_3) was resuspended in cold buffer (0.025 M PIPES, 0.5 mM EGTA, 0.25 mM $MgSO_4$, 0.1 mM GTP, pH 6.9) and applied to a Whatman P11 phosphocellulose column equilibrated in this resuspension buffer. Material bound to the column was eluted with a stepwise gradient of KCl in column buffer (further details in legend to fig.3). Fractions eluted from the column were collected, mixed with PCS scintillation cocktail (Hopkin and Williams Ltd, Chadwell Heath, Essex) and the radioactivity determined in a Packard 3003 Tricarb Liquid Scintillation Counter.

Phosphocellulose was prepared by initial swelling and de-fining in distilled water (15 ml/g powder) followed by successive 1 h equilibration in 0.5 M NaOH and 0.5 M HCl and washed with 1 litre of distilled water after each stage. The ion-exchanger was finally resuspended and stored in 0.025 M PIPES adjusted to pH 6.9 with NaOH.

SDS–polyacrylamide gel electrophoresis was performed by the Laemmli method [10] in 1 mm thick slabs of 6% acrylamide with a 2.5% acrylamide stacking gel. Samples were prepared by dilution of protein into Laemmli sample buffer, or by addition of sample buffer to acetone precipitates. These were then heated at 100°C for 90 s, cooled, loaded onto gels and subjected to electrophoresis at 12 mA for 2 h. Gels were stained with 0.1% Coomassie brilliant blue in glacial acetic acid : isopropanol : water (10:25:65), dried and exposed to X-ray film (Kodirex) for 5–25 days.

Protein was determined by the Lowry method [11] using bovine serum albumin as a standard.

3. Results and discussion

Brain microtubule protein depleted in high molecular weight MAPs by polymerisation in DMSO was used for copolymerisation experiments in order to expose the maximum possible MAP binding sites to the yeast extract.

Copolymerisation was performed in 4 M glycerol to facilitate the polymerisation of the MAP-depleted tubulin. Preliminary experiments have shown that yeast extracts inhibit the assembly of tubulin to a lesser extent in glycerol buffer. Also, the inhibitory activity of the extract is substantially reduced by the use of RNase and a strain of yeast deficient in proteases.

Figure 1 shows autoradiograms of SDS–polyacrylamide gels of the first and second copolymer pellets (HP_1 and HP_2, respectively). HP_1 contains a large number of yeast proteins, most of which disappear during the second cycle of assembly-disassembly. The major yeast proteins remaining associated with the microtubules throughout two cycles of assembly have mol. wt ~49 000 and ~55 000. Other less abundant proteins have mol. wt ~73 000, ~110 000, ~130 000, ~200 000 and ~230 000.

The 55 000 mol. wt yeast protein migrates as a diffuse band in the same position as brain α-tubulin. Direct comparison of the stained gel and its autoradiogram (fig.2) suggests that this yeast protein is homologous to the brain α-tubulin. Figure 2 also shows that one of the less abundant yeast proteins has a mobility just greater than the brain β-tubulin and mol. wt ~52 000. There is, however, apparently no

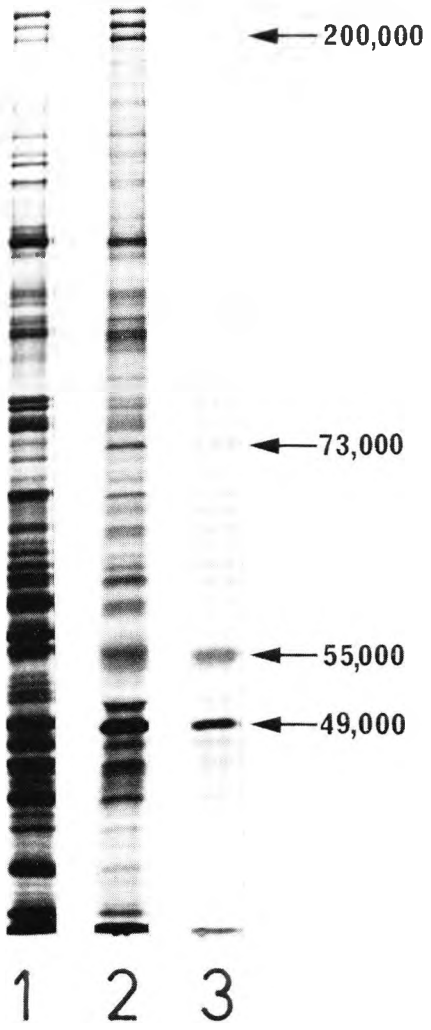

Fig.1. Autoradiograms of SDS–polyacrylamide gels of: (1) yeast soluble proteins; (2) HP_1 copolymer; (3) HP_2 copolymer. Figures denote molecular weights of major bands.

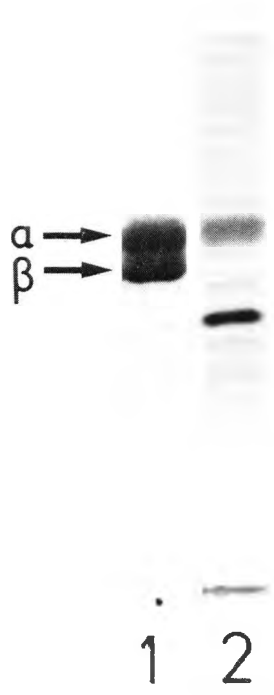

Fig.2. The tubulin area of an SDS–polyacrylamide gel of a HP_2 copolymer: (1) is the protein stained with Coomassie brilliant blue; (2) is the autoradiogram of the stained gel (1).

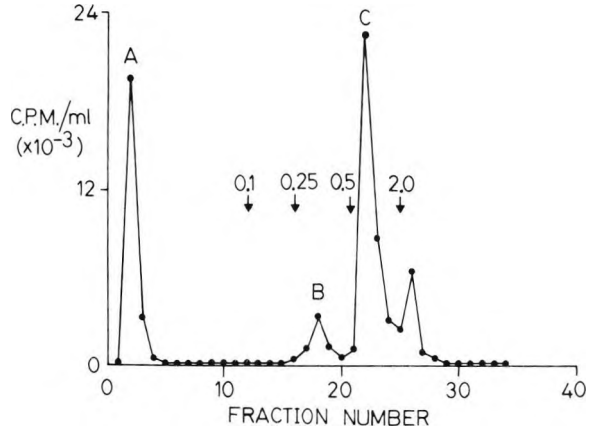

Fig.3. Separation of radioactive yeast proteins in a HP_3 copolymer. 2.4 mg protein in 1.6 ml were applied to a 3 × 1 cm phosphocellulose column at 4°C. Flow rate was ~ 20 ml/h; 2 ml fractions were collected. Arrows denote the addition of 10 ml each of 0.1 M, 0.25 M, 0.5 M and 2.0 M KCl.

yeast protein that comigrates with brain β-tubulin. The other major protein in this area of the gel has mol. wt ~49 000.

The elution of radioactivity from phosphocellulose after application of a copolymer HP_3 is shown in fig.3. The pattern of yeast proteins appearing in each peak, as compared with the starting material, is shown in fig.4. Peak A in fig.3 is the unbound fraction which contains the brain tubulin and two radioactive yeast proteins. These are the proteins which comigrate with the brain α-tubulin and the 52 000 mol. wt protein. The behaviour of these two proteins in copolymerisation and ion exchange chromatography makes them strong candidates to be yeast tubulins. Similar results in *Aspergillus nidulans* were shown

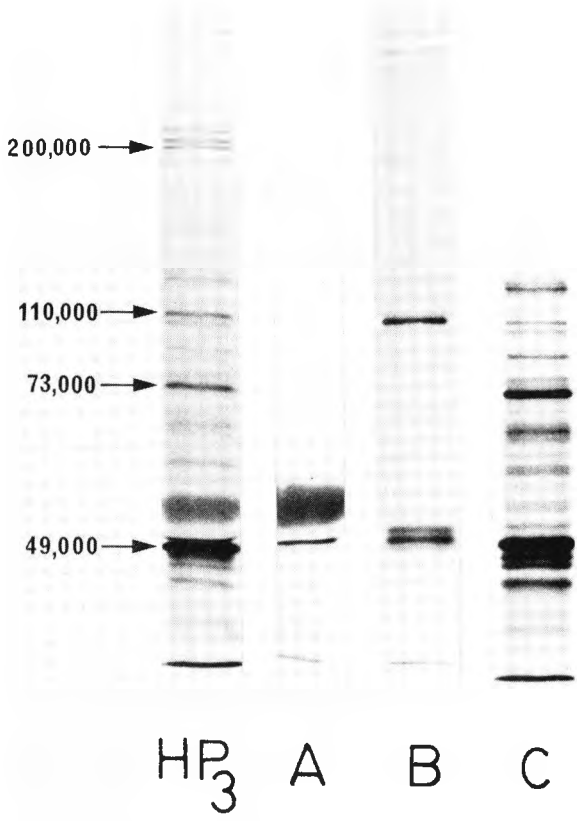

Fig.4. Autoradiograms of SDS–polyacrylamide gels of yeast proteins in the HP_3 copolymer and peaks A–C from the phosphocellulose column. Protein was precipitated from the column fractions by addition of an equal volume of cold acetone and a precipitate allowed to form overnight at −20°C. 50 μl Laemmli sample buffer was added to the resulting precipitate and treated as in section 2.

[12] where the α-tubulins show homology with brain α-tubulin. There is, however, less homology between the *Aspergillus* β-tubulin and brain β-tubulin.

Peaks B and C from the phosphocellulose column contain the majority of the associated proteins. The 49 000 mol. wt protein is eluted with 0.5 M KCl and thus behaves in a similar way to the 49 000 mol. wt protein identified as a MAP in neuroblastoma, C_6 glioma and CHO cells [3]. The behaviour of these proteins during repeated cycles of polymerisation and phosphocellulose chromatography suggests that the 49 000 and 110 000 mol. wt proteins may be yeast MAPs. Preliminary experiments indicate that the critical concentration for polymerisation of the copolymer is lower than that of a control. Also, we have evidence of ring-like structures in the cold depolymerisation copolymer whereas none are visible in our MAP-depleted brain protein preparations.

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