THE ROLE OF MICROTUBULES IN SECRETION

Alan Russell Prescott

Doctor of Philosophy Thesis

The University of Aston in Birmingham

October 1981

Summary.

The University of Aston in Birmingham

The Role of Microtubules in Secretion.

Microtubules have been implicated in the movement of secretory granules to the cell membrane in a variety of both exocrine and endocrine cells. A range of roles, from provision of the motive force to preventing pre-secretory movement, have been attributed to these organelles.

An *in vitro* model system to investigate the interaction between isolated exocrine pancreatic zymogen granules and brain microtubules has been devised. This system has been studied by electron microscopy, colchicine binding assay for the microtubule protein tubulin and radioimmunoassay.

These assays for microtubule proteins have been performed on mouse pancreas homogenates to test whether a correlation exists between the assembly of microtubules *in vivo* and secretion in response to a known secretagogue, Pilocarpine.

The *in vitro* model demonstrated an interaction between microtubules and the membrane of zymogen granules. Attachment was produced by the microtubule side arms and possibly a membrane component. This morphological observation was confirmed by the colchicine binding assay which showed that zymogen granules removed tubulin as assembled microtubules from solution upon sedimentation.

The investigation of the pancreas microtubule-free tubulin equilibrium during Pilocarpine-induced secretion showed that secretion was accompanied by a reduction in the assembled microtubules. A concomitant increase in the measurable free tubulin was initially produced followed by a rapid decrease.

These findings provide evidence for an important role for microtubules in exocrine pancreas secretion. Secretory zymogen granules appear to bind specifically to microtubules and Pilocarpine-induced secretion seems to induce microtubule disassembly allowing granules to migrate to the site of release under the influence of an unknown mechanism.

Microtubules Tubulin Exocrine pancreas Secretion

Alan Russell Prescott, Doctor of Philosophy Thesis, 1981.

List of Contents.

Tables, Photographs and Diagrams.

Diagrams :

Tables :

Photographs :

Acknowledgments

I would like to thank Dr. P. Sheterline of the University of Liverpool for supplying me with M.A.P. and tubulin antisera and Paul Wickstead for his cooperation on the *in vivo* pancreas granule number study. I would also like to thank the staff of A. Thompson(Aston)Ltd.,Pork Butcher for my ready supply of slaughter house material.

Technical assistance was provided by Messrs Kevin Hughes and Steve Southwick,typing was by Mrs. Weaver. Finally I would most like to thank Dr. Dave Starling for his assistance,advice and patience during his period as my supervisor.

Abbreviations.

Anovar	Analysis of variance
anti-T.	Tubulin antiserum
anti-M.A.P.	Microtubule associated protein antiserum
ADP	Adenosine 5'- diphosphate
ATP	Adenosine 5'-triphosphate
B.S.A.	Bovine serum albumin
c-AMP	Adenosine 3':5'-cyclic monophosphate
cpm	counts per minute
DMSO	Dimethyl sulphoxide
dpm	disintegrations per minute
EDTA	Ethylene diamine tetracetic acid
EGTA	Ethyleneglycol-bis (β-aminoethyl ether) N,N'- tetracetic acid
E.M.	Electron microscope
G	Zymogen granules
GARS	Goat anti-rabbit serum
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
^{3}H	Tritium
^{125}I	Iodine-125
K	1000
l.s.	longitudinal section
M.A.P.	Microtubule associated protein(s)
MES	2-(N-Morpholino) etane sulphonic acid
MS	Mean square (Deviance)
MT.	Microtubules
MTS-buffer	Microtubule stabilising buffer
NS	Not significant ($P>0.05$)
P	Probability
PBS	Phosphate buffered saline
^{226}Ra	Radium-226
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulphate
S.E.M.	Scanning electron microscope
SS	Sum of squares
T.	Tubulin
T.E.M.	Transmission electron microscope
TS-buffer	Microtubule disassembly buffer
t.s.	transverse section
VBL	Vinblastine
VCR	Vincristine

Protein secretion in the exocrine pancreas.

The functional unit of the mammalian exocrine pancreas is the pyramidal acinar cell which secretes a variety of proteins into a narrow lumen at the cellular apex. The acinar lumen, created by a small group of cells, then drains into a duct starting at the centro-acinar cells of the acinus and eventually, via intra- and inter-lobular ducts, ending in the main pancreatic duct. The pancreatic secretion produced by these cells is exclusively enzymatic, (Table 1.1) variation only in the relative proportions produced occurring between species. A typical acinar cell as observed in a conventional electron micrograph is predominated by the rough endoplasmic reticulum (RER) filling the whole of the basal half of the cell (Figure 1.1). The Golgi cisternae (GC) are less marked occupying the region between the RER and the granulated apex of the cell. Mature zymogen granules and condensing vacuoles are found throughout the apical region. The GC has two poles an outer convex face nearest the nucleus and an inner concave face. The outer cisternae are believed to form a continuous network of tubules. The boundary cisternae approaching the RER bear few or no ribosomes.

The route of protein secretion in the exocrine pancreas and most other cells is essentially the same although some protein secreting cells appear to by-pass certain areas of the cell. The secretory cycle has in the main been studied by cell fractionation (Siekevitz and Palade; 1958,a,b,c), radioautography (Caro and Palade; 1964) and pulse labelling techniques (Jamieson and Palade; 1967,a, b). The secretory process consists of several stages: uptake of amino acids, protein synthesis and segregation into the RER, transport from the RER via the GC to form condensing vacuoles (CV), formation and storage of zymogen

Table 1.1 : The protein constituents of pancreatic juice.

PROTEIN	Mol. Wt.	Mass proportion (%)
Proteolytic enzymes		
Chymotrypsin(ogen) 1 (EC 3.4.4.5)	25850	1.7
Chymotrypsin(ogen) 2 (EC 3.4.4.5)	25850	16.4
Trypsin(ogen) (EC 3.4.4.4)	24400	33.0
(Pro)elastase(pancreopeptidase) (EC 3.4.4.7)	28000	8.0
(Pro)carboxypeptidase A1 (EC 3.4.2.1)	45000	3.5
(Pro)carboxypeptidase A2 (EC 3.4.2.1)	45000	8.8
(Pro)carboxypeptidase B (EC 3.4.2.2)	47700	8.8
Amylolytic enzyme		
α-amylase (EC 3.2.1.1)	52000	3.6
Lipolytic enzymes		
Lipase(glycerol ester hydrolase) 1 (EC 3.1.1.3)	66000	8.5
Lipase(glycerol ester hydrolase) 2 (EC 3.1.1.3)	49700	3.4
Esterase(carboxyl ester hydrolase) (EC 3.1.1.1)		
(Pro)phospholipase A (EC 3.1.1.4)		
Cholesterol esterase(sterol ester hydrolase) (EC 3.1.1.13)		
Nucleolytic enzymes		
Ribonuclease(RNase) (EC 2.7.7.16)	13000	1.1
Deoxyribonuclease(DNase) (EC 3.1.4.5)		
Other proteins		
Colipase		
Trypsin inhibitor		
Sulphated polyanionic peptidoglycan(?)		

The bracketed prefix(pro) and suffix(ogen) indicate that these hydrolases are secreted as inactive precursors or zymogens which are activated in the gut lumen by peptide bond cleavage in the amino-terminal region of the precursors.(Case,1978).

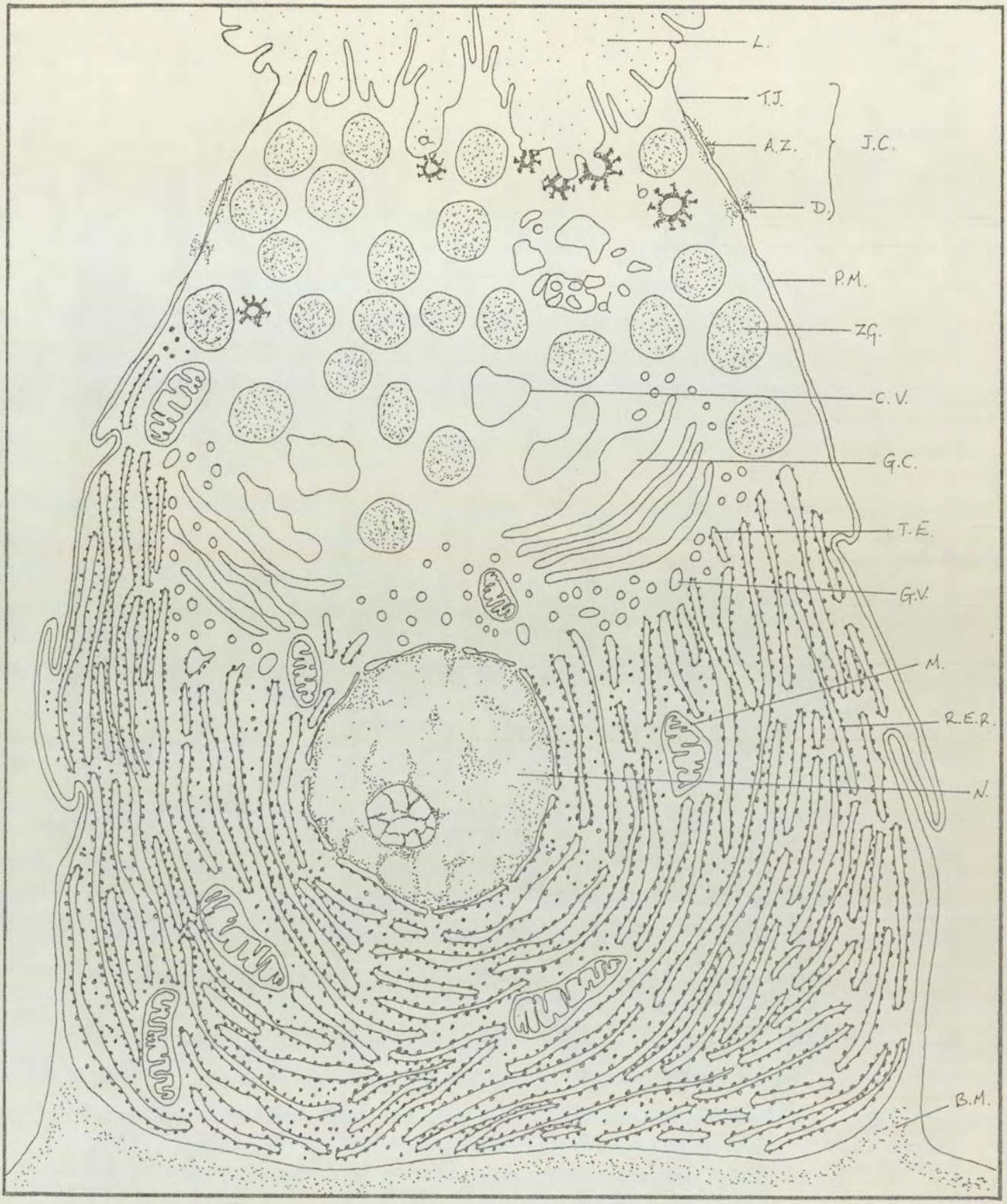


Figure 1.1 : Diagram of a pancreatic acinar cell.

L.-Lumen,T.J.-Tight junction,A.Z.-Adhering zonule,D.-Desmosome,J.C.-Junctional complex,P.M.-Plasma membrane,Z.G.-Zymogen granule,C.V.-Condensing vacuole, G.C.-Golgi complex,T.E.-Transitional element,G.V.-Golgi vesicle,M.-Mitochondrion, R.E.R.-Rough endoplasmic reticulum,N.-Nucleus,B.M.-Basement membrane.(Case,1978)

granules (ZG) and finally ZG content discharge and retrieval of ZG membranes. Each of these phases will now be considered briefly before coming to look in detail at the stages involving MT.

Amino acid uptake from the extracellular fluid is believed to be an active process requiring energy and the presence of a membrane bound carrier. The electrochemical gradient of Na^+ is also involved in this process but exactly how is uncertain. Schultz and Curran (1970) have suggested that Na^+ moving into the cell down a concentration gradient, created by a Na^+, K^+ - ATPase, drags with it other solutes (ie., amino acids). Amino acids entering the cell appear to be taken up directly from the membrane bound carrier by transfer RNA (t RNA), those amino acids not required for synthesis are then released into the intracellular pool where they can exchange fairly readily with the extracellular pool particularly with the unstirred layer near the cell membrane. In this way the cell rids itself of amino acids it does not require and increases uptake of those necessary for protein synthesis (van Venrocijet al, 1972).

Upon entering the cell amino acids are bound to t RNA by its terminal adenine nucleotide to form aminoacyl-t RNA. This in turn, according to its specific anticodon nucleotide sequence, binds to the corresponding codon triplet or messenger RNA (m RNA); these in turn bind to a ribosome. A single ribosome consists of two subunits with sedimentation coefficients of 40S and 60S. The 40S subunit binds to the m RNA-t RNA complex and this in turn binds to the 60S subunit to form a functional 80S ribosome. Numerous ribosomes can bind to a single m RNA strand to form a polysome with one ribosome every 90 m RNA nucleotides. Once synthesis is initiated GTP-dependent movement of the ribosome relative to the m RNA molecule and the attachment of further aminoacyl-t RNA molecules brings amino acids into position so that

ribosomal peptidyl transferase can enable peptide bond formation to produce a polypeptide.

The majority of this protein synthesis appears to occur on the RER, ribosomes being bound to the ER membrane. Blobel et al have suggested that, after the m RNA initiation codon of adenine-uridine-guanine, there is a signal sequence of codons whose translated peptide on emergence from the 60S ribosomal subunit, triggers the attachment of the ribosome to the ER. This signal probably causes the creation of a ribosome receptor on the ER membrane by membrane protein aggregation. This complex has a pore through which newly formed protein then passes into the RER cisternae. The signal protein is then cleaved before completion of the nascent polypeptide chain probably on the ER membrane. Once translation of the m RNA is completed a 'detachment factor' causes the ribosome to detach from the ER membrane the pore receptor closing as a consequence (Blobel and Dobberstein; 1975 a,b; Campbell and Blobel, 1976).

Proteins segregated into the RER are then transported to the peripheral GC and from here via the Golgi cisternae to the CV (Jamieson and Palade, 1967a). Transport from the RER to the GC is believed to occur by one of three mechanisms. Firstly, the smooth-surfaced protrusions from the transitional elements of the RER may be pinched off, travel across to, and fuse with the GC. This, however, seems unlikely since the membranes of the RER and the GC differ in lipid : protein ratio, lipid composition and enzymatic profile (Meldolesi, Jamieson and Palade, 1971). Secondly there may be fine tubular connections between the two organelles, these have been demonstrated in the liver (Claude, 1970), but differences in membrane chemistry mentioned above may preclude their presence in the pancreas. Finally it is possible that membrane bound vesicles may shuttle back and forth between the RER and

the GC, although as yet there is no direct evidence for this (Jamieson and Palade, 1967a).

On reaching the peripheral Golgi cisternae proteins then move from the outer face to the inner face of the GC. This may be brought about by direct movement of the Golgi cisternae or possibly via tubular interconnections between the cisternae (Slott et al, 1974). During their time in the GC proteins undergo a number of biochemical changes necessary for their final activity. These changes include glycosylation and sulphation of glycoprotein and mucopolysaccharide components of the ZG and granule membranes, together with sequestration of divalent cations important in enzyme action (eg. carboxypeptidase is a zinc metalloenzyme and α-amylase a calcium metalloenzyme). (Neutra and Leblond, 1966; Berg and Young, 1971; Pekas, 1971; Ceccarelli, Clemente and Meldolesi, 1975). Transformed protein finally leaves the GC in condensing vacuoles (CV) which arise from the inner Golgi cisternae. Since the membrane biochemistry of the GC and CV is different it appears that vesicle membranes are manufactured at the margins of the GC and on reaching a given size they break off, fusion of these CV possibly occurring (Völkl, Bieger and Kern, 1976). An alternative mechanism has been described by Novikoff (1976), he suggests that a distinct organelle the GERL (Golgi-associated smooth ER involved in lysosome function) situated close to the inner GC is not only involved in lysosomal formation but also in the packaging of secretion products, this has yet to be confirmed.

CV, leaving the GC eventually form native ZG, this is brought about by the passive aggregation of the proteins to form osmotically inert complexes which are sustained even if the ZG membrane is experimentally removed. Aggregation by the formation of inter-protein bridges may be brought about by soluble acidic lipoproteins, divalent cations or more

likely in the case of ZG, mutual interactions between the great variety of proteins present (Koenig,1974; Tinoco,1957; Howell,1974).

The penultimate step in the secretory pathway involves the movement of the ZG from their site of formation adjacent to the inner Golgi cisternae to the apical cell membrane where the ZG contents are discharged. The actual mechanism of movement will be considered later since it involves the microtubule-microfilament system. On reaching the apex of the cell apposition of the vesicle membrane to the plasma membrane produces a five-layered membrane structure, elimination of two layers produces a trilaminar membrane (single unit membrane). This becomes attenuated to a single layered diaphragm which ruptures to release the ZG contents into the lumen (Palade and Bruns,1968). Membrane fusion may be controlled by the intrinsic membrane proteins since freeze-fracture studies indicate the localised clearing of membrane particles at the sites of exocytosis. Microfilaments inserted into the plasma membrane may regulate the mobility of these particles (Poste and Allison,1973). The fate of ZG membranes is uncertain although three processes appear to remove the excess membrane from the apex of the cell (Kramer and Geuze,1974). Most important in the pancreas appears to be the vacuolization of the deep caveolae or folds of the apex. Secondary removal appears to be by lateral movement of the membrane and pinocytotic removal of the baso-lateral membranes. Fine regulation of membrane removal then occurs by the formation of coated vesicles by micropinocytosis and retrieval by coating and subsequent coat removal. Retrieved membranes first undergo degradation by lysosomal proteases and phospholipases in multi-vesicular bodies. Whether the membrane constituents are only degraded to macromolecular subunits or further is unknown, either way the membrane proteins are re-utilized eventually since membrane protein turnover is less than for

secretory protein (Geuze and Kramer,1974).

The pathway which has just been described is one which has been built up from the study of numerous tissues between which there is a great deal of variation. Other pathways have been suggested to explain discrepancies in this 'segregation' model. The most useful challenge has come from the 'equilibrium' hypothesis which suggests that proteins are not segregated into distinct organelles but move freely across cellular membranes maintaining an equilibrium, (Rothman,1975).

No detailed consideration has been made of the way in which the pancreatic secretagogues cholecystokinin-pancreozymin (CCK-PZ), released from the duodenal mucosa in response to a meal, and acetylcholine (ACh) from the vagal nerve, produce acinar cell secretion. A detailed description of stimulus-secretion coupling is given by Bonfils, Fromageot and Rosselin,(1977) and second messenger homeostasis is reviewed by Berridge (1975). Briefly CCK-PZ but not ACh. activates membrane bound adenylate cyclase but cyclic AMP does not appear to regulate enzyme discharge in the acinar cell. Ca^{2+} appears to be the major intracellular second messenger, CCK-PZ and ACh. raising the intracellular Ca^{2+} concentration by causing its release from intracellular stores possibly in the mitochondria or ER. The role of Ca^{2+} in the discharge process is as yet unknown although it may involve the microtubule-microfilament complex discussed in the next section. The intracellular calcium regulator, calmodulin may play a part here in controlling MT. assembly/disassembly in association with cellular calcium (Means and Dedman,1980).

The actions of CCK-PZ and ACh. on the exocrine pancreas can be mimicked by a variety of parasympathomimetic agents. These include the synthetic analogues of acetylcholine: Methacholine, Carbachol and Bethanechol. In addition a variety of natural alkaloids have similar

effects, these are Pilocarpine, Muscarine and Arecoline. These compounds primarily have their effect at muscarinic receptors of autonomic effector cells although some ganglionic effects are also observed, (Koelle,1975). Pilocarpine is the chief alkaloid obtained from the leaflets of South American shrubs of the genus Pilocarpus. It is well known to cause smooth muscle contraction throughout the body as well as effecting secretion by the sweat, salivary, lacrimal, gastric, pancreatic and intestinal glands. In addition its mimicry of ACh. produces effects on the cardiovascular system and the central nervous system. The effect on the exocrine pancreas has been widely used to study the changes occurring during active secretion. Alm,(1971) used Pilocarpine to study the turnover of L-DOPA in the rat exocrine pancreas, while Kramer and Poort (1968) used it to study pancreatic protein synthesis during secretion. The drug induces almost complete depletion of pancreatic zymogen granules within an hour of administration (Atkins et al.,1975). Nevalainen (1970) noted not only complete granule depletion but a number of other effects. Cellular volume reduction was greater than could be attributed to the effect of granule extrusion possibly via an effect on water loss. Ultrastructurally the acinar cells showed a reduction in mature zymogen granule size and number, later development of prozymogen granules and small smooth-walled vesicles in the enlarged Golgi region, followed by filling of the apical cytoplasm with mature zymogen granules. Watari and Baba (1968) have shown the presence of complex dense bodies showing acid phosphatase activity in the exocrine pancreas of dogs treated with Pilocarpine.

Migration of zymogen granules.

Palade (1975) has ascribed granule movement in the acinar cell to diffusion since it has been shown that new and old ZG are discharged at random (Jamieson and Palade,1971). However, it seems unlikely that

granules should move in one direction only towards an area of high granule concentration. Early suggestions (Matthews,1970) that granule movement occurred by Brownian motion have been refuted by micro-cinematographic recordings showing that granules remain stationary over long periods, only when cells are dying is Brownian motion observable, (Douglas,1974). Granule interconnections have also been suggested as being responsible for directional movement in other tissues as well as pancreas (Makita et al.,1974). However, despite numerous models for the mechanism of granule movement within a variety of cell types the most popular is still the microtubule-microfilament association. This model will be considered now together with details of the structure of microtubules, their relationships to other cell organelles and their role in protein secretion.

Introduction to microtubules.

Microtubules (MT.) have recently been described by Dustin (1978) as "proteinaceous organelles, present in nearly all eukaryotic cells, made of subunits assembled into elongated tubular structures, with an average exterior diameter of 24nm and an indefinite length, capable of rapid changes of length by assembly or disassembly of their subunit protein molecules or tubulins, sensitive to cold, high hydrostatic pressures and some specific chemicals such as colchicine and vinblastine, building with other proteins complex assemblies like mitotic spindle, centrioles, cilia and flagella, axonemes, neurotubules, and intervening in cell shape and cell mobility." This definition implies the large amount of work that has been involved in delineating the nature of MT; the majority of this work has been done in the last ten years but its historical background goes back to the end of the 19th century when the drug colchicine, later shown to be an MT. inhibitor, was shown to poison cell mitosis (Pernice,1889).

Research into the MT, despite its relatively recent impetus, has been enormous, here only points which are stictly relevant to the secretory process will be considered in detail. More extensive reviews include those edited by Soifer (1975), Borgers and De Brabander (1975), Goldman, Pollard and Rosenbaum (1976), the excellent books by Dustin (1978) and Roberts and Hyams (1979).

Microtubule structure and biochemistry.

As was stated in the definition above MT. are made up of protein subunits which assemble to form competant MT. under certain conditions. These subunits are found in conjunction with a number of other proteins and other molecules but the basic 'backbone' of the MT. consists of the protein tubulin (T). The properties of T. are shown in Table 1.2; this shows a representative selection of properties, however, these vary according to the source and method of T. extraction. Two main types of T. have been characterised, α and β tubulin; these are present in roughly equal amounts in MT. forming the heterodimer, with a dis-association constant of 8×10^{-7} M at $4.6^{\circ}C$ (Detrich and Williams,1978), responsible for the characteristic geometry of MT.

The α and β tubulin subunits have also been resolved into a number of subspecies.Marotta , Strocchi and Gilbert (1978) have resolved two forms of α and β tubulins by isoelectric focusing, each of the α subspecies separating into at least three forms when subjected to a second dimension SDS-polyacrylamide gel electrophoresis. Kobayashi and Mohri (1977) have separated the α and β subunits each into four subspecies. This heterogeneity of the T. subunits may be due to post-translational modification of these proteins since Bryan, Cutler and Hayashi (1978) have shown that only three types of messenger-RNA can be isolated from chick brain polysomes coding for two types of α and one β. However, Portier, Milet and Hayes (1979), using one and two

Table 1.2 : Properties of the tubulin molecule

Molecular weight of dimer	
sedimentation equilibrium	120,000
gel filtration	115,000 ± 5,000
Molecular weight of monomer	55,000 ± 2,000
Sedimentation coefficient	5.8-6.0 S

Amino acid composition, from chick embryo brain and sea urchin sperm outer doublets :

	Chick	Sea urchin	Chick	Sea urchin
Lys	16	24	16	21
His	11	14	9	11
Arg	20	26	20	23
CMCys	10	10	8	7
Asp	43	48	46	55
Thr	28	29	28	32
Ser	26	21	31	24
Glu	56	69	53	69
Pro	20	25	20	28
Gly	37	41	38	42
Ala	32	41	29	34
Val	33	33	31	33
Met	7	10	10	17
Ile	22	25	18	19
Leu	30	36	31	36
Try	15	ND	15	ND
Phe	19	21	20	24

ND-not determined

Amino acid sequences of NH_2-terminal regions of α and β tubulins from chick brain and outer doublet of sea -urchin sperm

Chick brain α tubulin Met-Arg-Glx-Ser?-Ile-Ser?-Ile-His-Val-Thr-
Sea urchin α tubulin Met-Arg-Glu-Ser?-Ile-Ser?-Ile-His-Val-Thr-
Chick brain β tubulin Met-Arg-Glu-Ile- Val-His -Ile-Gln-Ala-Thr-
Sea urchin β tubulin Met-Arg-Glu-Ile- Val-His -Met-Glx-Ala-Thr-

Chick brain α tubulin Gln-Ala-Thr-Val-Gln-Ile-Thr-Asx-Ala-Ser?-
Sea urchin α tubulin Glx-Ala-Thr-Val-Glx-Ile-Thr-Asx-Ala-Ser?-
Chick brain β tubulin Gln-Ser-Thr-Asx-Gln-Ile-Thr-Ala- ? -Phe -
Sea urchin β tubulin Glx-Ser-Thr-Asx-Glx-Ile-Thr-Ala- ? -Phe -

Chick brain α tubulin ? -Glx-Leu-Try-Ser?
Sea urchin α tubulin ? -Glx-Leu-Tyr-Ala?
Chick brain β tubulin Trp-Glx-Val-Ile-Ser?
Sea urchin β tubulin ? - ? -Val-Ile-Ser?

Conformation(pH 6.5, 4°C)-22% α helix, 30% β structure, 40% random.
Conformation(37°C) -50% β helix, 50% random coil.

Molecules that bind to tubulin	Stoichiometry (molecules bound per tubulin dimer)	Competition	Affinity (liter/M)
Drugs			
colchicine	1.07±0.05	podophyllotoxin assembly site	2.0×10^6
vinblastine	1.0	GTP-binding site, other vinca alkaloids	$2\text{-}3 \times 10^5$
podophyllotoxin	1.0	colchicine assembly site	3.0×10^6
Phosphate	0.08-1.0		
GTP	2.0		
Magnesium	1.15(maximum)		

Molecules that copolymerise with tubulin	Molecular wt. (daltons)	% of total microtubule proteins
High molecular weight components	350,000	6
	300,000	20
tau	70,000	1
Protein kinase	280,000	-

(Snyder and MacIntosh, 1976 and Dustin, 1978)

dimensional electrophoresis and isoelectric focusing, have resolved several α and β T. bands in proteins produced by two separate cell-free systems (wheat germ extract and a rabbit reticulocyte lysate) under the direction of Tetrahymena pyriformis poly (A)- containing RNA. They also confirmed that α and β T. were coded for by separate m-RNA's. Forgue and Dahl (1979) isolated 14 distinct T. bands localised near pH 5.4 by isoelectric focusing in a gradient established with a very narrow range of ampholyte mix. Comparative peptide mapping of trypsin digested α- and β- T. showed 16 peptides unique to each subunit and 23 common ones (Nellis and Bamburg,1979). When T. was isolated from rat brain at different ages after birth and compared by isoelectric focusing, changes that occurred in electrophoretic character postnatally were most pronounced 8 - 12 days postnatal with little change after 22 days. These differences may reflect age dependent changes in assembly properties or changes in the relative contributions of the spindles, axons, dendrites and glia to the total pool of T. in the brain.(Dahl and Weibel,1979).

The polymerisation of T. subunits into MT. has been investigated in detail with conflicting results since the initial discovery by Weisenberg (1972) that T. extracted from rat brain could be purified by ultra-centrifugation and made to form normal MT. in vitro. Since this discovery MT. have been polymerised in vitro from a variety of species, tissues and using various purification techniques. The main source of MT. proteins has been mammalian brain although a variety of other sources have been used including dogfish brain (Langford,1978), cultured cell lines (Nagle, Doenges and Bryan,1977; Weatherbee, Lufty and Weihing,1978), marine eggs (Kuriyama,1977) and sea urchin sperm tail (Farrell and Wilson,1978). MT. preparations from isolated mammalian brain cells differ in character, for instance C_6 glial cell MT.preparations

exhibit few rings at 0°C, have low polymerisation yield and low M.A.P. content. These alterations are probably the result of M.A.P.differences, since the C_6 cell T. is indistinguishable from brain T. with respect to molecular weight, amino acid composition and colchicine binding activity. Wiche, Honig and Cole (1979) have suggested that C_6 M.A.P's are more labile than their brain counterparts, brain M.A.P's being capable of restoring the polymerisation abilities of the C_6 MT.proteins after decay. When T. from different sources are compared the differences are less apparent, Anderson (1979) isolated 3 carboxymethylcysteine-containing peptides from calf brain T. by digestion with elastase. Peptides homologous to these could be isolated from mouse brain and 3T3 cells indicating that the T's from these three sources are homologous in the regions of amino acid sequence accounting for the peptides examined.

In general, in vitro polymerisation of T. requires a number of conditions to be met. The protein concentration must be high, ie., greater than 1mg/ml according to the other conditions. An optimal pH of 6.9 is necessary, together with the presence of GTP, although MT. will form in the pH range from 6.2 to 7.0 (Matsumura and Hayashi,1976). The Ca^{2+} concentration should be lowered using a strong chelator such as E.G.T.A. This chelator should not remove Mg^{2+} ions (as does E.D.T.A) since they appear to be necessary for MT. assembly although concentrations greater than 20mM are inhibitory. Finally the MT. protein solution should be warmed to 37°C since initiation of assembly appears to be endothermic even though MT. elongation may be otherwise (Sutherland and Sturtevant,1976). Hinz et al.(1979) have shown that the heat capacity change, ΔCp, of the MT. growth reaction was - 1600± 500 cal./(deg.mol) per 110,000 molecular weight T. dimer incorporated into the MT. The assembly reaction is characterised by a complex heat uptake pattern

comprising both endo- and exo- thermic processes. Johnson and Borisy (1979) monitoring MT. self-assembly by turbidity changes have shown that the kinetic behaviour above and below 20°C is different. Above 20°C MT. assembly is a steady state balance of irreversible reactions coupled to hydrolysis of GTP (see later). Below 20°C there is a marked decrease in the equilibrium constant as a result of an additional reaction involving the association of M.A.P's at specific sites on the MT. lattice. MT. assembly is also influenced by high molecular weight microtubule-associated proteins (HMW's or MAP's) and the so-called 'tau' protein which can be isolated from MT. preparations (see below). Certain MT. extractions also require nucleation centres for MT. formation, these can simply be small aggregates of T. or fragments of MT, or the MT. organising centres (MTOC) commonly found in the cell such as the centrioles, basal bodies, kinetochores, axonemes, etc. (Pickett-Heapes,1975). Intact centrosomes, kinetochores and chromosomes from mitotic PtK_2 cells pretreated with colcemid to remove MT. and lysed with Triton X-100 were capable of nucleating MT. growth from purified T. at 37°C with 1mM GTP. This growth could be blocked with T.-antibody but not control antibody or buffer alone (Pepper and Brinkley,1979). Summers and Kirschner (1979), using dark field light microscopy, have shown that polymerisation can occur at both ends of the MT. One end growing at three times the rate of the other. These opposite ends showed differential sensitivity to low temperature, Ca^{2+} and colchicine. The distal ends of ciliary axoneme MT. were the fast growing ones as were the ends facing away from metaphase chromosomes. Kirschner (1980) has suggested that this <u>in vitro</u> "treadmilling" of MT. is a manifestation of the <u>in vivo</u> mechanism designed to use GTP or ATP hydrolysis to control the spatial organisation of MT. in the cell. MT. "treadmilling" requires higher T. concentrations than for polarized polymerisation of

anchored MT, hence anchored polymers are stabilised while spontaneous polymerisation is suppressed. Bergen and Borisy (1980) have identified similar polarity in MT. from the A subfibre of flagellar outer doublets by measuring the rate of elongation using flagellar axoneme seeds and porcine brain subunits. Such polarity has been demonstrated *in vivo* in six neurones of the nematode *Caenorhabditis elegans*; short MT. present in these cells have morphologically distinquishable ends (Chalfie and Thomson, 1979). Farrel, Kassis and Wilson (1979) have shown that net T. addition and loss occurs at opposite ends of MT. (see later section). Also certain non-physiological factors can influence MT. assembly, these include polycations, dimethylsulphoxide and glycerol, all of which aid polymerisation *in vitro*. In contrast with the small biochemical constrasts between different species T's, Murphy and Hiebsch (1979) have shown that the difference in optimum pH necessary for MT. assembly from porcine (pH 6.94) and bovine (pH 6.62) brain T's is attributable to the T. and not the M.A.P's.

MT. assembly *in vivo* could be controlled by any of the factors necessary for *in vitro* polymerisation. Among the candidates suggested are MTOC, Ca^{2+}, Calmodulin, M.A.P's, tau, natural MT. acting drugs (eg., colchicine, *Vinca* alkaloids, benzimidazoles), GTP and C-AMP. Certainly the mechanisms of control are very complex possibly varying from species to species and also even within the same cell. Marcum et al.(1978) have shown the presence of a calcium dependent regulator protein associated with control of MT. in the mitotic spindle.

T. subunits assemble into MT. in conjunction with their associated proteins (MAP's, tau) and other molecules (eg.,GTP). MT. assembled *in vitro* are generally morphologically similar to those found *in vivo*, their structure having been studied in both states. The macrostructure of the MT. cytoskeleton has been investigated using indirect

immunofluorescence (Weber,1975; Brinkley, Fuller and Highfield,1975; Osborn and Weber,1977) and immunohistochemical staining (Karsenti et al, 1978; De Brabander et al,1977) to make the MT. thick enough to see in the light microscope; in both cases the structures seen were confirmed in the electron microscope (Eckert and Snyder,1978). Cytoskeletons of PtK2 cells grown on gold grids and visualised first for light microscopy by the indirect immunofluorescence procedure and then for E.M. by uranyl acetate staining showed direct correspondence of the fluorescent fibres in the light microscope with uninterrupted fibres of diameter $\sim$550 Å in the E.M. This diameter corresponds to that of a single MT. decorated around its circumference with two layers of antibody molecules (Osborn, Webster and Weber,1978). These methods show that the whole cytoplasm is supported by a complex cell web of MT, often in association with other fibrous cellular elements such as microfilament bundles, tonofilament-like bundles and thinner fibres (30 - 40 Å), (Webster et al, 1978; Fujiwara and Pollard, 1978). This structure has been confirmed directly in microinjected fibroblasts using MT. proteins labelled with dichlorotriazinyl fluorescein which does not affect its polymerisability or affinity for unlabelled proteins (Keith, Feramisco and Shelanski,1980). Conventional electron microscopy has shown MT. to have a diameter of about 25nm; their maximal length has not been measured, however, MT. in the nervous system (called 'neurotubules') may be as long as neuronal axons (greater than 10m in the whale). The subunits comprising the MT. are in the size range 4 - 5 nm corresponding to a globular protein of 55,000 daltons molecular weight. This molecule may be split symmetrically into two lobes and slightly elongated (Erickson,1974).

The MT. are made up of T. heterodimers comprising 13 protofilaments producing three, five and eight start left handed helices which are arranged to form a tubular structure (Figure 1.2). Although 13 proto-

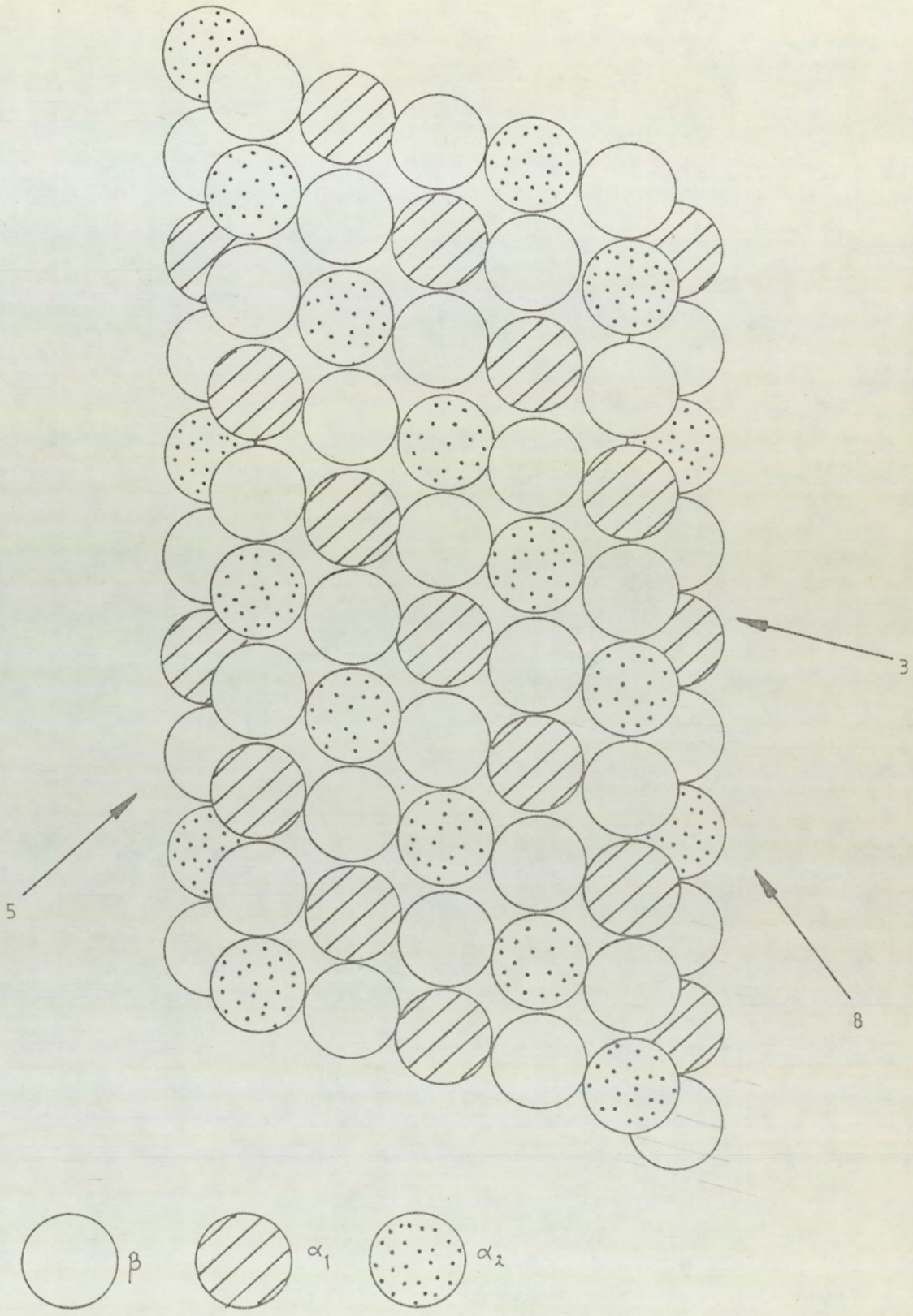


Figure 1.2 : Possible location of α1 and α2 tubulins in microtubules. Three-start and five-start helices of alternating α1-β and α2-β tubulin dimers. Homogeneous eight-start helices of α1-β and α2-β dimers. (Bibring et al, 1976).

filaments are normally found both in vivo and in vitro (Tilney et al. 1973), 12, 14 and 15 have been reported (Burton et al. 1975; Pierson, Burton and Himes,1978); 14 protofilament MT. reassembly in vitro. The increasing in number with consecutive cold dissociations/ MT. protofilament numbers of crayfish nerve cord seems to be inherent in the T. or associated proteins (Pierson, Burton and Himes,1979); axonal MT. have 12 protofilaments while the surrounding glial cells have 13, T. isolated from nerve cord forms both types of MT. in vitro. Temperature of assembly also appears to affect the angle of binding of adjacent protofilaments altering the number required to complete the circumference of the MT; at 20°C 13 are required while at 40°C only 10 protofilaments. The intra-protofilament bonds are stronger than the inter-protofilament bonds since single protofilaments can be isolated which are cold resistant (Delacourte et al.1977), also protofilaments are often seen splayed out from the ends of MT. The T. molecule does appear to be sufficiently flexible to accommodate slight distortions of its lateral bonding sites while the bonding of α and β-T. monomers seems similar enough to allow $\alpha-\alpha$ and $\beta-\beta$ bonds, (Langford,1980). This can be concluded from the fact that 14 protofilament MT. formed from dogfish brain T. have a slightly altered helical pitch angle and a discontinuous dimer lattice to accommodate the 14th protofilament.

Not only will T. subunits form MT. under the right conditions but they are also capable of forming other oligomers. T. solutions isolated from brain contain at least two fractions with different sedimentation coefficients. Always isolated is the 6S T. dimer together with the 30S oligomer (sometimes found to be 36S) which is believed to consist of a ring or spiral of dimers. These two oligomers are believed to be in equilibrium in solutions kept in the cold (Marcum and Borisy,1978). Kirschner et al.(1974) have shown the presence of two fractions of T.

isolated from mammalian brain : X consisting mainly of 6S dimers and Y consisting of 36S and 6S oligomers forming rings and spirals. It is possible that these two types of oligomer are involved in MT. formation, however Rodinov, Gel'fand and Rozenblat (1976) have shown that the 32S (equivalent to 30S) oligomer is resistant to factors suppressing MT. polymerisation (colchicine, Ca^{2+}, cooling and the absence of GTP), indicating that two types of interaction occur between T. molecules : MT. bonds and more stable oligomer bonds. Oligomers isolated from a cold T. solution consist of, in addition to the 6S dimer, an 18S aggregation of 4 - 6 dimers and one or more HMW molecules, a 20S ring of dimers and tau and a 30S double ring of 29 dimers and 9 HMW's (Burns,1978). However, the presence of these various oligomers may depend on solution variables; in such a solution at pH 5.8 - 6.5 mainly 6S dimers are present, from pH 6.5 - 7.4 mainly 30S oligomers and from pH 7.4 - 8.2 mainly 18S dimers. Similarly increasing Na^{+} concentration causes a shift from 30S to 18S and 6S oligomers (Marcum and Borisy,1978).

In addition to these oligomers found in cold T. solutions other aggregates occur. Isolated protofilaments are rarely seen; sheets of T. subunits can be induced by Zn^{2+} ions (Baker and Amos,1978); double walled MT. result from sheets spirally wrapping normal MT; macrotubules are formed in the presence of MT.poisons. Zinc-induced sheets have been particularly useful in 3-D image reconstruction and X-ray diffraction studies of the structure of the protofilaments and subunits in assembled MT. Internal contact between subunits within the T. heterodimer and less extensive contact between dimer units have been demonstrated together with inter-protofilament joints (Tamm, Crepeau and Edelstein, 1979; Amos and Baker,1979). The two classes of T. subunit can also be distinguished by one having a prominent cleft. The occurrence of T. polymorphisms under various Ca^{2+} concentrations and at various pH's

is summarised in Figure 1.3. The role of these polymorphs in MT. assembly is uncertain, Figure 1.4 summarises the possible in vitro situation. In vivo only one step in the MT. assembly pathway has been demonstrated, this is the C-MT, a curved sheet of protofilaments.

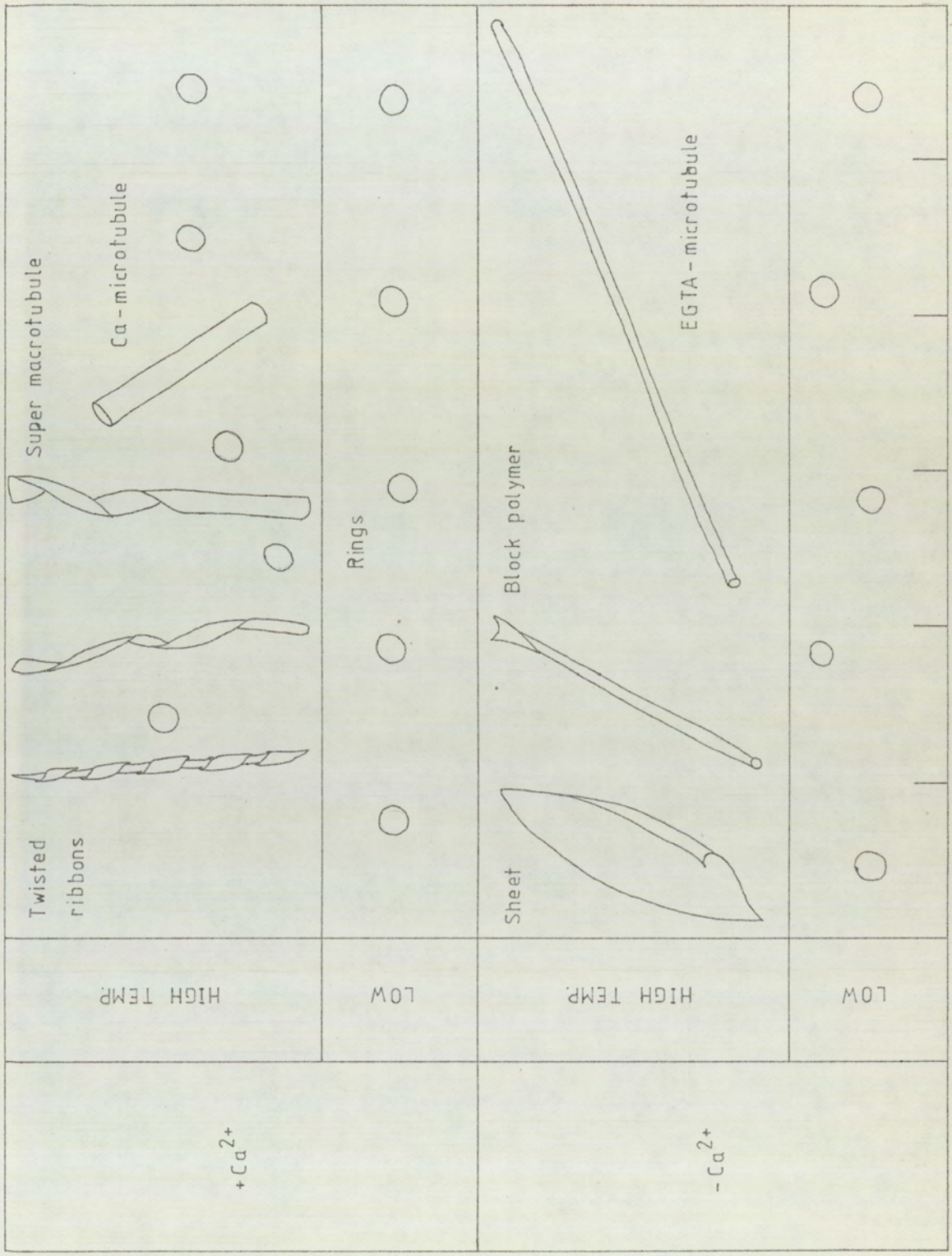
Microtubule associated molecules.

In addition to α and β tubulin MT. also contain a number of other molecules which are necessary for their characteristic structure and function. The already mentioned M.A.P's and tau proteins are the most important together with the guanosine nucleotides (GDP and GTP). In addition to these commonly isolated molecules T. has been shown to bind polycations, basic proteins, glycerol, lipids (probably phospholipids) and carbohydrates; however many of these do not appear to be of physiological importance.

When MT. are polymerised and depolymerised in the presence of glycerol, which promotes assembly in the absence of added nucleotides (Shelanski et al.1973), some glycerol remains bound to the T. This glycerol is not removable by gel filtration or prolonged dialysis, five moles binding per mole of T. dimer, three moles of which are exchangeable upon polymerisation-depolymerisation. Aggregates bind eleven moles of exchangeable and eleven moles of non-exchangeable glycerol per mole of T.

Various polycations have been shown to aid MT. assembly, substituting for M.A.P's and tau proteins (see below). In addition polyanions inhibit MT. assembly possibly removing heat-stable cationic proteins necessary for polymerisation (Bryan,1976). Polycations shown to promote MT. assembly are poly (L-glutamic acid), poly (L-lysine), poly (A-agarose) and diethylamino-ethyl dextran (DEAE dextran). The latter compound under non-assembly conditions promotes the formation of clusters of curved sheets or filaments which under assembly conditions form

Figure 1.3 : Formation of different forms of polymerised tubulin under varying conditions of temperature, pH and calcium concentration. (Matsumara and Hayashi, 1976)



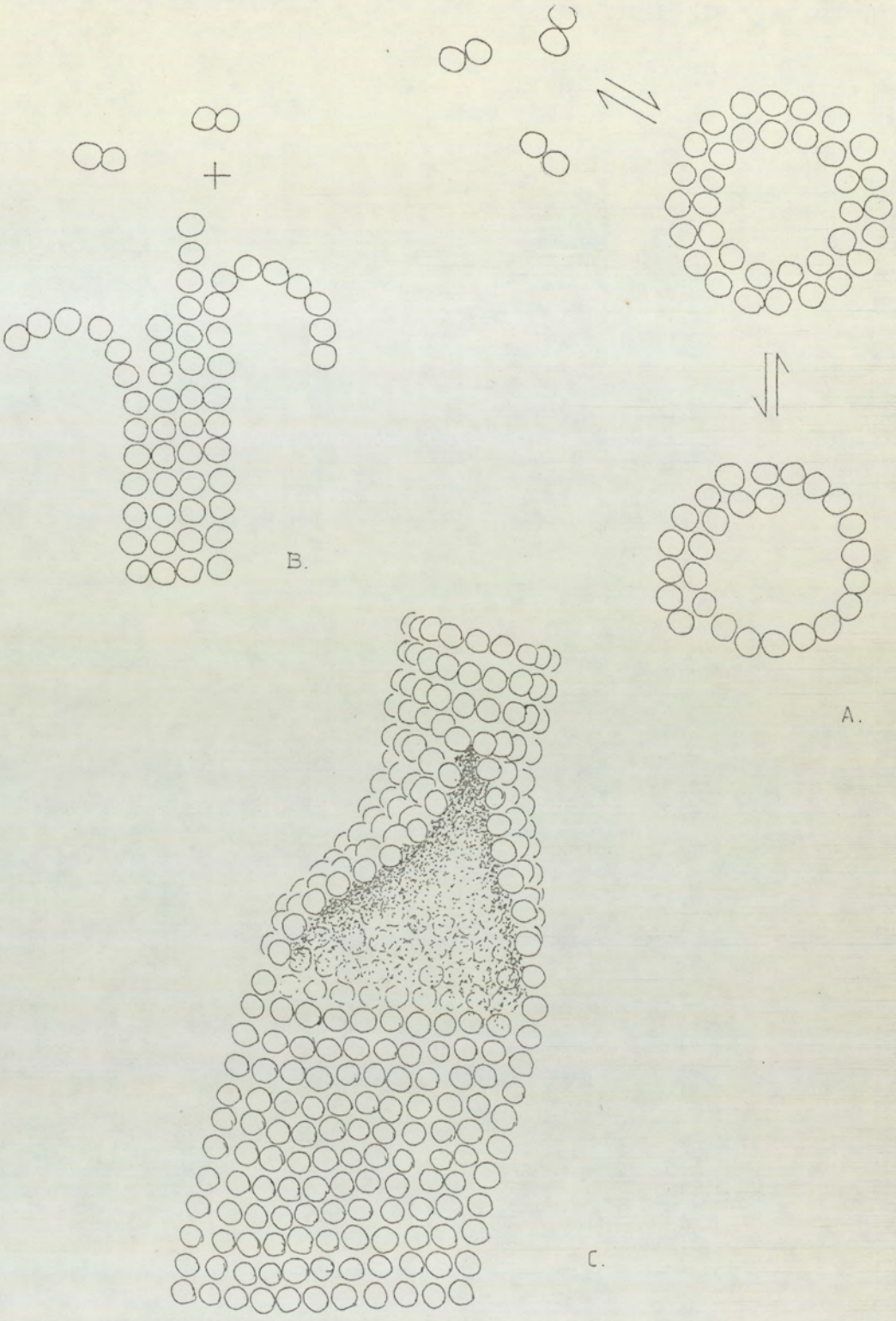


Figure 1.4 : Possible steps in microtubule assembly.
A. Equilibrium between tubulin dimers, rings and short helices.
B. Assembly of dimers at the end of short protofibrils.
C. Formation of a microtubule from a more or less flat sheet of protofilaments.
(Penningroth et al, 1976)

double-walled MT. otherwise identical to normal MT. (Erickson and Voter,1976). Where polycations mimic M.A.P's and/or ta Lee, Tweedy and Timasheff (1978) have suggested that they are involved in MT. stabilisation. Basic proteins such as R Nase A also increase MT. polymerisation in a similar way to polycations (Erickson,1976).

Phospholipids have indirectly been shown to be associated with MT; Daleo, Piras and Piras (1977) have suggested, in the light of their experiments with detergents and phospholipases, that phospholipids may have a role in determining the association of T. and/or the final dimensions of assembled MT. Early reports of MT-associated carbohydrates proved to be due to incomplete purification of MT. proteins. However, indirect evidence for the presence of mucopolysaccharides on the surface of the MT. comes from the fact that lanthanum hydroxide, ruthenium red, and dialysed iron all stain the outer coat of MT. (Lane and Treherne,1970; Stebbings and Bennett,1975). All these stains have an affinity for mucopolysaccharides and other anionic surfaces. Prus and Mattison (1979) analysed phospho-cellulose-purified T. for neutral and amino sugar content and found 8.3 ± 0.11 and 0.8 ± 0.02 mol/mol of dimer respectively. They also confirmed the presence of complex carbohydrates by showing that MT. diameter increased upon treatment with ruthenium red, Alcian blue and lanthanum hydroxide. Concanavalin A reactive sites were also detected on both the surface and in the lumen of the MT.

The T. molecule itself seems to have intrinsic associated enzymic activity; Larssen, Wallin and Edström (1979) have demonstrated MT. associated acid and alkaline phosphatases in bovine brain together with M.A.P. phosphatase activity associated with the larger M.A.P. The rapid turnover of carboxyl-terminal tyrosine of the α-T subunit seems to be dependent on the presence of assembled MT, Thompson,Deanin and Gordon,(1979)

estimated the half-life of terminal tyrosine for MT. as less than 37 minutes while the value for T. itself was greater than 48 hours. Indeed post-translational modification of the T. molecule may be important in phenylketonuria effects on the brain. Rodriguez and Borisy (1979) have shown that the substitution of α-T carboxyl-terminus tyrosine with phenylalanine in hyperphenylalaninaemia of newborn rats alters the polymerisation capacity of the T. Some T-associated enzymic activity appears to result from hypotonic shock of mitochondria during isolation. The anionic character of T. leads to ionic association of mitochondrial glutamate dehydrogenase; using a new extraction protocal in sucrose medium. Karr, White and Purich (1979) have isolated MT. proteins with lower associated mitochondrial enzymes. The resulting M.A.P's had altered electrophoretic behaviour and higher yields. In addition lower critical MT. protein concentrations were necessary for MT. assembly although the MT. still behaved normally under the influence of Ca^{2+}, drugs and cold depolymerisation.

An important protein known to bind to T. is the calcium dependent regulator protein or calmodulin. This binds to T. in a calcium dependent manner preventing MT. assembly (Kumagai and Nishida,1979; Nishida et al.1979). This protein has been proposed as the regulator of a wide variety of cellular processes involving calcium (Means and Dedman,1980), of particular interest here are its roles in mitosis and cyclic nucleotide metabolism.

Each T. dimer has bound to it two molecules of guanosine nucleotides (GDP or GTP). Two binding sites have been identified, one which binds GDP firmly and is not readily exchangeable and another freely exchangeable GTP binding site. Jacobs (1975) has proposed a model for the role of these 'E' (exchangeable) and 'N' (non-exchangeable) sites in MT.assembly:-

$$n\ GTP + n_{E}T_{N}GDP \longrightarrow n\ GTP_{E}T_{N}GDP \longrightarrow (GDP_{E}T_{N}GDP)\ n + nP.$$

Weisenberg, Deery and Dickinson (1976) have suggested that the apparent existence of an N site may be due to the fact that upon the formation of T. ring aggregates the E site becomes blocked; reported trans-phosphorylation and hydrolysis of N site nucleotide could then be explained under conditions of polymerisation and depolymerisation when the E sites freely exchange with the medium. The E site of the T. dimer has been localised by polymerising T. in the presence of the photoaffinity analogue of GTP, 8-azidoguanosine triphosphate (8-N_3 GTP). Photolysis of MT. formed in the presence of (β, γ - ^{32}P) 8-N_3 GTP resulted in the covalent incorporation of radioactivity onto the β tubulin monomer (Geahlen and Haley,1977). GDP and GTP bind to the same E site, although GTP binds with nearly three times the affinity of GDP (Zeeberg and Caplow,1979). The N site nucleotide does not appear to play any role in MT. polymerisation and may function as a stable structural co-factor of the T. molecule (Spiegelman, Penningroth and Kirschner, (1977). Neither is it the site of transphosphorylase activity as had previously been reported (Zeeberg and Caplow,1978). T. polymerisation has been shown to be possible with guanosine 5'-methylenediphosphonate a GDP analogue resistant to hydrolysis. Using this analogue Sandoval et al (1978) have shown that polymerisation can occur in the absence of γ-phosphate and phosphate bond hydrolysis at the E site. The B, γ-imido and B,γ-methylene GTP analogues have been used by Karr, Podrasky and Purich (1979) to show that GDP supports MT. elongation but poisons nucleation; GTP is involved in early nucleation while GDP or GTP act to stabilise the MT. The affinity of the MT. ends for T. appears to be governed by the nucleotide ligand at the E site. The GTP α-β bond could play a role in MT. polymerisation. Removal of GTP from the E site also enables the binding of other non-hydrolysable nucleotide analogues (adenylyl imidophosphate, adenylyl B,γ - methylene-

diphophosphonate) which enable MT. assembly (Penningroth and Kirschner, 1978). However Giesel, Fasold and Haase (1978) have shown that MT. assembly prevention by N-ethylmaleimide is not due to blocking of the GTP binding site but alkylation of two SH groups near the binding site on the monomer which in turn prevents hydrolysis to GDP. GTPase activity has been shown to occur at the ends of MT. at equilibrium (David-Pfenty, Laporte and Pantaloni, 1978; David-Pfenty, Erickson and Pantaloni, 1977).

Terry and Purich (1979) have shown that MT. assembly in vitro can be supported by ATP and UTP rather than GTP by the action of brain nucleoside -5'-diphosphate kinase. The rates of GDP and GTP release (0.68 and 0.32 min^{-1}) are sufficiently fast to allow GDP release, phosphorylation and GTP binding as the sole mechanism of nucleoside -5'- diphosphate kinase action in MT. assembly. However Selkoe (1979) has shown that the ATP-transphosphorylase system seems to only function for one assembly cycle phosphorylating GDP at the N site leaving the E site vacant. Assembly-disassembly leads to a randomisation of the N and E sites and a subsequent cycle leads to a T. fraction unable to accept additional phosphate. These effects are conformational since they are not reversed by addition of more GTP although glycerol appears to reduce denaturation of the T. The fact that GTP is exchangeably bound to flagellar outer doublet T. and then becomes non-exchangeable with exogenous GTP upon incorporation of T. into MT. has been used to study MT. at steady state (Farrell, Kassis and Wilson,1979). This has shown that net addition and loss of T. occurs at oppo site ends of the MT. resulting in a unidirectional flux of T. from MT. assembly ends to disassembly ends.

Not only does the T. molecule undergo phosphorylation but Coughlin, White and Purich (1980) have demonstrated that M.A.P's are flash

phosphorylated through the sequential action of protein kinase and phosphop rotein phosphatase present in MT. protein; M.A.P-2 (see below) is the major site of phosphorylation. Obviously the role of guanosine nucleotides in the regulation of MT. assembly is very complex, under certain conditions GTP hydrolysis is required but in others it is not; the exact role of N site nucleotides also has to be defined.

Two main groups of protein have been identified which co-purify with tubulin from MT. through several cycles of assembly and disassembly. These are the high molecular weight MT-associated proteins (M.A.P's) and the tau factor. These proteins which are often found in stoichiometrical relations with T. should not be confused with other proteins which can be extracted with T. from complex structures such as cilia, nor with aggregates of T. molecules such as rings and helices mentioned above. Murphy, Vallee and Borisy (1977) identified 35 proteins associated with brain T. after two cycles of polymerisation-depolymerisation, however, after six such cycles only a few remain (including two M.A.P's). 60% of MT. assembly stimulation was due to M.A.P's, 40% to other proteins.

Tau protein co-purifies with T. through several cycles of assembly-disassembly and migrates during polyacrylamide gel electrophoresis as four closely spaced bands with molecular weights between 55,000 and 62,000. The four peptides have been shown to be closely related by peptide mapping and amino acid analysis, and have no relationship to the HMW-MAP's. Tau has a sedimentation coefficient of 2.6S and is highly asymetric with ellipsoid axial ratio of 20 : 1. The peptides are neutral or slightly basic and are phosphrylated by a protein kinase co-purifying with MT. Tau factor is required for both initiation and growth of MT. (Witman et al,1976) and promoting ring formation (Weingarten et al,1975), under polymerisation and depolymerisation conditions. It is believed to bind to several T. molecules increasing the local concentration of T.

and inducing the formation of longitudinal filaments (Cleveland, Hwo and Kirschner, 1977b). The critical concentration of T. for MT. assembly in vitro is a function of the tau concentration and can be lowered to as little as 30µg/ml (Cleveland, Hwo and Kirschner, 1977a). Lockwood (1978) has purified one of the tau factors known as tubulin assembly protein (TAP) and, using immunofluorescent antibody to the protein, has demonstrated its occurrence throughout the cytoplasmic MT. network and the mitotic spindle. TAP appears to form an MT. assembly competent complex stoichiometrically with T. required throughout MT. elongation and occurring along the entire length of assembled MT. Solomon, Magendantz and Salzman (1979) have isolated a tau-like protein from the detergent-extracted cytoskeletons of NIL8 hamster cells. Several polypeptides were isolated when T. was removed by Ca^{2+} or colchicine treatment but only the tau-like protein consistently associated with calf brain T. through several cycles of MT. assembly and disassembly.

The high molecular weight M.A.P's also copolymerise with T. in the proportion of one M.A.P. molecule per $\overset{15}{\wedge}$T. dimers. There are at least two M.A.P's with molecular weights between 300,000 and 350,000 daltons composing between 20% and 6% respectively of total MT. protein by weight (Sloboda et al, 1976). These proteins have been shown to be important in both MT. initiation and elongation (Sloboda, Dentler and Rosenbaum, 1976) and particularly in the formation of ring structures (Murphy and Borisy, 1975). Indirect immunofluorescence using antibody to M.A.P's has shown that they are associated with the MT. cytoplasmic network in interphase cells (Connolly et al, 1978). They can be extracted from fibroblasts, in the same way as from brain, as an HMW phosphoprotein (Klein, Willingham and Pastan, 1978). Murphy, Johnson and Borisy (1977) have suggested that HMW-MAP's are required for MT. initiation

but not elongation, rather they affect the extent and net rate of polymerisation by binding to the polymer, thereby stabilsing the formed MT. and consequently shifting the equilibrium to favour assembly. M.A.P's are also believed to form structural components projecting from the surface of the MT. wall (Murphy and Borisy,1975) which are absent when MT. are formed in non-physiological conditions in the absence of M.A.P's. Of the two forms of M.A.P, M.A.P.2 (Molecular weight 30,000) has a c-AMP dependent endogenous protein kinase which may also play a role in MT. assembly (Francon et al,1978). Bulinski and Borisy (1980a,b) have isolated M.A.P's from HeLa cells and used them to prepare antisera to the two main types of M.A.P. the 210,000 and 125,000 dalton molecular weight proteins. The presence of these M.A.P's has been demonstrated on HeLa MT. assembled in vitro and they are present in vivo. Antibody to the 210,000 dalton M.A.P. was used to examine its presence in primate cells. It was associated with cellular MT. in ten human and four monkey cell lines and tissues but did not appear in the two rodent, ungulate, marsupial or chicken cells examined. This M.A.P. thus appears to be confined to MT. of primate origin. M.A.P's are identifiable in T. oligomers, 30S double rings require the presence of HMW-MAP's and 18S aggregates tau protein. There is twice the amount of M.A.P's to T. in the 30S oligomer compared to MT. although the organisation in the rings is conserved in the MT. (Vallee and Borisy, 1978). Sandoval and Cuatrecasas (1976) have shown that one M.A.P. is released from T. complexes when colchicine binds irreversibly to T.

As was mentioned above, M.A.P's appear to be responsible for the formation of structural components on the surface of the MT. Depolymerised MT. proteins can be separated after several cycles of assembly-disassembly into a T. and a M.A.P. fraction by phosphocellulose chromatography (Herzog and Weber,1977; Himes et al,1976). The T.fraction

can then be induced to form MT. in the absence of M.A.P's using either high Mg^{2+} concentration (10mM) or 10% dimethylsulphoxide. When such MT. are compared to MT. formed in the presence of M.A.P's, the so-called 'side-arms', 'links' or 'bridges' are absent (Sloboda et al,1976), also the MT. pack more tightly on centrifugation (Dentler, Granett and Rosenbaum,1975). The presence of these side-arms has been confirmed by freeze-fracture studies of axostyles of the flagellates Saccinobaculus and Pyrsonympha. Here 6 bridge-binding sites were present with as many as 4 interrow bridges. The diameter of the MT. and their spacing was greater than for sectioned material possibly due to the removal of water and intertubule material during dehydration. MT. subunits had a periodicity of 16nm as did the intrarow; interrow bridges lacked periodicity being orientated at varying angles to the MT. axis (Bloodgood and Miller,1974). In conventional sectioned material Amos (1977) has shown that the side-arms are spaced 96nm or 12 T. dimers along the MT. protofilaments, side-arms on neighbouring protofilaments are staggered to produce a helical superlattice superimposed on the underlying T. lattice. Depolymerised MT. retain their side-arm attachment to protofilaments in spiral and ring formations. Trypsin treatment of MT. removes the side-arms but does not cause MT. disassembly, the remaining proteins still being capable of cyles of assembly-disassembly. However, the removed portion of HMW-MAP (molecular weight 255,000) cannot bind to assembled MT. This indicates that one part of the M.A.P. protein promotes MT. assembly and the other the formation of side-arms (Vallee and Borisy,1977). Whole M.A.P's can attach to pre-assembled MT. without M.A.P. side-arms to produce normal MT; this attachment occurs even in the presence of the MT-inhibitor colchicine (Sloboda and Rosenbaum, 1977).

M.A.P's attach to MT. after T. polymerisation shifting the equilibrium

towards MT. formation by stabilising the assembled T. (Sloboda and Rosenbaum,1979). M.A.P-free MT. formed with high T. concentrations became decorated with added M.A.P's, increasing M.A.P. concentration increased the concentration of M.A.P. sedimenting with MT. linearly to the ratio found in MT. formed in the presence of M.A.P's. The M.A.P-free MT. did not depolymerise and form new M.A.P. decorated MT. since decoration occurred in the presence of 10^{-4} M colchicine. Also MT. became more cold stable the more M.A.P's were added. The two M.A.P's, M.A.P-1 (molecular weight - 350,000 daltons) and M.A.P-2 (molecular weight - 300,000 daltons), seem to play different roles in MT. assembly. During MT. assembly, initially only M.A.P-2 is associated with the protofilaments, subsequently the other non-T. proteins are associated with sedimented MT. In this study Stearns and Brown (1979) showed that T. rings are not necessary for MT. initiation although they may rapidly dissociate releasing M.A.P's to interact with T. Also they believed that M.A.P-2 may specify the binding site for other M.A.P's since MT. assembled with only M.A.P-2 lacked surface filaments which rapidly appeared on addition of the total M.A.P. fraction. However in contrast to this, Kim, Binder and Rosenbaum (1979) produced decorated MT. with heat-stable M.A.P-2 devoid of M.A.P-1. This M.A.P-2 stoichiometrically provided MT. assembly lowering the critical T. concentration to 0.05mg/ml, MT. saturated with M.A.P-2 containing $\sim$1 mole of M.A.P-2 to 9 moles of T. dimer. When stained with tannic acid they showed a decoration with axial periodicity of 32 $\pm$ 8 nm. Zingsheim, Herzog and Weber (1979) demonstrated differences in the texture of the MT. walls when assembled in the presence of M.A.P-2, tau and T. alone. M.A.P-2 - MT had a rougher surface than tau - MT. which in turn were rougher than T.-alone MT. M.A.P-2 produced globular protusions up to 30nm from the MT. wall producing an overall diameter of

100nm. They suggested that the halo effect seen *in vivo* around MT. could be the result of a similar arrangement to that seen *in vitro* with M.A.P-2-MT. The role of M.A.P's in controlling the microenvironment of the T. molecule is shown by the fact that in the presence of M.A.P's MT. formed stable spiral structures upon addition of vincristine while without M.A.P's, MT. were completely disassembled. Also at $0^{o}C$ MAP-free T. formed amorphous aggregates while with M.A.P's spirals were formed upon vincristine addition (Donoso, Haskins and Himes,1979).

Microtubule-microtubule interactions and their interaction with other cellular organelles.

The most well known MT-organelle interactions are found in the mitotic spindle and in cilia and flagella. Details of these structures can be found in the book by Dustin (1978) and those edited by Sleigh (1974) and Goldman, Pollard and Rosenbaum (1976). However here only the more simple associations likely to be involved in cellular secretion will be dealt with; the point worth noting from cilia, flagella and the mitotic spindle is that MT. can be associated with a number of structural protein molecules notably dynein, nexin and axin (Mohri,1976).

MT. not only associate with other cellular organelles but also form complex geometric structures when associated with one another. These structures, when viewed in transverse section, reveal a variety of linkage patterns varying from side by side linkage to form a large sheet to an almost crystalline six link pattern. Sheets of MT. are found in the Km fibres of the ciliate *Stentor coeruleus*, these are responsbile for cellular extension brought about by MT. sliding (Huang and Pitelka,1973). Inter-MT. three and four links are found in the axonemes of *Gym nosphaera albida* (Jones,1975) and the axopodia of *Acanthocystis*, *Raphidiophrys* and *Heterophrys* (Bardele,1977). Finally six links are found between MT. in the pharyngeal baskets of ciliates,

where they perform a mechanical function, and in the axonemes of heliozoa. In the latter structure MT. links are both long (31nm) and short (2nm) producing a curious double spiral (Roth, Pihalaja and Shigenaka,1970). It is important to note that the linkage patterns mentioned above are those seen in transverse section and may lie in different planes since MT. are three-dimensional helices.

In the ovaries of the insect Notonecta glauca ('Backswimmer') trophic tubes carry ribosomes from the syncytial trophic region to the oocytes. These tubes contain a dense mass of MT. which are separated from one another and the ribosomes by an electron non-opaque region. This region contains no inter-MT.connections and may represent a mucopolysaccharide coat or a region of electrostatic repulsion (MacGregor and Stebbings,1970). This 'exclusion zone', as it has been called, appears to be penetrated by stain due to its break up on cooling which produces the appearance of cross-bridges in HeLa cell cultures (Bhisey and Freed,1971). Electrophoretic analysis of the proteins constituting the insect ovary nutritive tubes failed to demonstrate the presence of actin, myosin or dynein,also the MT. in these tubes were not depolymerised by cold, Ca^{2+} or colchicine. Hyams and Stebbings (1979) suggested that the stability of these trophic tubes was maintained by accessory proteins in the 'exclusion zone'. A peculiar MT-MT association is found in the cyst cell of the testis of Gerris remigis ('Water strider'), here the MT. have two curved projections which join near the apex of the cell. (It is interesting to note that colchicine treatment results in only the loss of the curved side-arms and not the MT.(Turner, 1972) .

A study of the three-dimensional occurrence of inter-MT. connections in the axostyle of Saccinobaculus and chick testis (sperm tail and spermatid perinuclear helix) has indicated the presence of periodic and non-periodic bridges. Non-periodic bridges arise because not all

periodic binding sites are filled, these sites are distributed according to the substructure of the MT. wall (McIntosh,1974). Godula (1979) has identified a specific MT. organising centre in 'Firebug' (Pyrrhoceris apterus L.) spermatid maturation. This organises the MT. involved in formation of the perinuclear manchette and circummitochondrial ring mediated by an MT. self-linkage mechanism. The formation of inter-MT. links appears dependent on MT. assembly and disassembly since long links formed between manchette MT. in late spermiogenesis are formed too early under the action of the MT. poison colcemid (MacKinnon, Abraham and Svatek,1973). The axostyle of the termite flagellate Pyrsonympha vertens consists of about 2,000 MT. arrayed in semi-crystalline manner (Langford and Inoué, 1979). These MT. generate saw-tooth waves by the action of interrow bridges active in the straight regions of the waves. MT. linkages are found in a variety of organisms and orgenalles and have a variety of functions, from the mobile axopods and axonemes to the initiation of action potentials in cultured chick embryo dorsal root ganglia cells (Bird and Lieberman,1976). Mandelkow and Mandelkow (1979) have suggested that MT. protofilaments are capable of combining in two distinct ways, one leading to MT. walls and the other forming junctions between walls. Image analysis of negatively stained MT. sheets has led them to attribute the two types of bonding to an inherent property of T. and not of M.A.P's. Although they are not directly related to secretory systems the abundance of interconnections provides indirect evidence for the role of MT. in organelle movement within the cell.

Another analagous system provides evidence for MT. mediated intracellular movement, this is the infection of cells by virus particles. Here MT. appear to be involved both in the initial cell infection and later with virus replication (very much analagous to cell protein

secretion) (Dales,1975). Dales has shown that labelled viral RNA is found in close association with MT. soon after infection. The mitotic apparatus of L strain culture cells becomes coated with dense granular or fibrous viral material and aggregates of progeny virus particles following reovirus type 3 infection (Dales,1963), interphase MT. are also similarly coated. Adenovirus type 5 are rapidly transferred from HeLa cell surface to the uncoating site (nuclear pore complex). Vinblastine (an MT. poison inducing the formation of T. paracrystals) delays the infection cycle of adenovirus 5 and also produces paracrystals which have virus particles associated with them. Dales and Chardonnet (1973) have suggested that this indicates that MT. are involved in virus particle movement, however the interaction must be complex since colchcine, another MT. poison, has no effect on the infection cycle. No arms appear to attach the virus particle to the MT. but arms are visible on the virus when attached to vinblastine induced paracrystals. Reovirus 3 particles associate with neurotubules (MT. in the nervous system) in perikarya and dendrites of the CNS of mice with encephalitis (Gonatas, Margolis and Kilham,1971) and such associations can also be mimicked <u>in vitro</u> (Luftig and Weihing,1975). Finally bean pod mottle virus and cowpea mosaic virus particles infecting leaves are bound in tubules, associated with MT. These tubules may themselves be altered MT. with virus particles in their central core; MT. may have a role in inter- and intra-cellular movement and assembly of virus particles as well as the plants' reaction to infection (Kim and Fulton,1975).

Marchant (1978) has shown that MT. are attached to the cytoplasmic face of the plasma membrane of the green alga <u>Mougeotia</u>,dense bodies being present at the MT. ends. This confirms previous indications of a colchicine binding protein (tubulin) attached to the cell membrane (Zenner and Pfeuffer,1976; Westrum and Gray,1976). MT. also seem to

have a role in the distribution of intramembranous particles such as receptors within the membrane (Oliver, Ukena and Berlin,1974), however results in this field are contradictory and require far more work before any clear pattern emerges. MT. may be involved in maintaining the position of the nucleus, certainly MT. connect the nucleus to the cell membrane (Puck,1977). In certain organisms MT. are also associated with nuclear movement; during basidiosporogenesis of Shiitake(Lentinus edodes) MT. are found in close relationship to the nuclei migrating from the basidium via the sterigmata to the basidiospores (Nakai and Ushiyama,1978). When baby hamster kidney (BHK 21-F) cells are infected with parainfluenza virus SV5 they produce syncytia in which the nuclei migrate to the centre at rates of 1 - 2μm/min. This movement is prevented by colchicine, leaving the nuclei randomly scattered throughout the syncytia; rows of nuclei can normally be isolated intimately associated with MT. (Holmes and Choppin,1968).

In addition to their associations with nuclei MT. are also found associated with almost every other cellular organelle, this is hardly surprising since, as Osborn and Weber (1977) and De Brabander, De Mey, Joniau and Geuens (1977) have shown by immunofluorescent studies, MT. form a complex cytoskeleton coursing throughout the cytoplasm. MT. are aligned parallel to mitochondria during meiosis in Nephrotoma spermatocytes and may be responsible for the equal distribution of mitochondria into the four spermatids (La Fountain,1972). Organelle, particularly mitochondrial, movements in cowpea rust fungus (Uromyces phaseoli var. vignae) are correlated with non-random associations with MT. (Heath and Heath,1978). Hegyenex, Simon and Singer (1978) have identified MT-mitochondrial associations, which can be disrupted by colcemid, in a variety of cell lines by indirect immunofluorescence. Mitochondria in the cell cortex of Tetrahymena thermophila are associated with cortical

MT. bands. A characteristic electron-dense area in the mitochondrial membrane not seen elsewhere is found in the region of MT. association (Aufderheide,1979). The Golgi apparatus also has associated MT, in Euglena gracilis MT. are present in the intercisternal space, within the cisternae and vacuoles and associated with the endoplasmic reticulum (Mollenhauer,1974). Membrane-bounded vesicles have also been identified associated with MT. (Allen,1975), an association which withstands MT. bending due to staining techniques; the association between MT. and cell vesicles will be discussed in detail below.

Besides these sepcific MT-organelle associations MT. are involved in other cellular processes such as cytoplasmic streaming (Rebhun, 1972; Tucker,1978), muscle conductance (Makita and Kiwaki,1978) and orientation of cellulose microfibrils (Robinson and Herzog,1977; Robinson,1977). Finally MT. are associated with the ingestion of food particles in the suctorian ciliates (Tokophyra sp., Rhycheta sp.,Choanophyra sp.and Discophyra sp.). The tentacles of the organisms bear MT. with projections contacting with the inner food canal or funnel which move the food particles into the suctorian body. (Hitchen and Butler,1973 and 1974; Tucker,1974; Tucker and Mackie,1975; Curry and Butler,1976).

As was indicated earlier the favourite source of MT. proteins, particularly tubulin, is brain tissue; this is not surprising since MT, or 'neurotubules' as they are called, are found in abundance in all nerve cells. Here MT. are associated with other fibrous organelles, the neurofilaments, within the axons and dendrites of neurons. Within axons there are two main types of transport, fast and slow, neuronal MT. probably being responsible for fast transport. This is shown by the fact that MT. poisons inhibit axoplasmic transport, discrepancies in the literature concerning the action of anti-MT. drugs results from the long period of exposure necessary before maximum inhibition is seen

(Paulson and McClure,1975; Banks and Till,1975). Brimijoin, Olsen and Rosenson (1979) compared the velocity of transport of dopamine-β-hydrox ylase and MT. numbers in bullfrog and rabbit unmyelinated adrenergic nerves incubated at various temperatures. Transport velocity was exponentially related to temperature over a wide range although it fell off abruptly below 13°C for rabbit and 10°C for frog nerves. However, MT. numbers fell off rapidly with temperature before transport was inhibited indicating that MT. in these nerves are present in excess even though they determine transport capacity. Black and Raymond (1980) have identified two slow moving groups of axonally transported proteins in guinea pig retinal ganglion cell axons. Slow component a (SCa) moves at 0.25mm/day and consists of T. and neurofilament protein while slow component b (SCb), moving at 2-3mm/day, consists of actin and associated polypeptides. This slow transport represents movement of structural complexes of proteins; SCa the MT-neurofilament network and SCb the microfilament network and complexed proteins. MT. are also responsible for membrane excitability in squid giant axons since depolymerisation by 0.2mM Ca^{2+} perfusion destroys this; however excitability can be restored by a variety of perfusates including T-tyrosine ligase, T, ATP, Mg^{2+}, K^{+}, c-AMP and axonal 300K proteins. By looking at the effects of a variety of MT. inhibitorson squid giant axon resting potential, height of propagated action potentials and threshold to evoke action potentials and comparing them with the in vitro effects on MT., Matsumoto and Sakai (1979) have demonstrated a good correlation between conditions supporting maintenance of membrane excitability and MT. assembly. Associated with the plasma membrane of squid giant axons are MT. which appear to regulate in part both resting and action potentials. Within the nervous system MT. associate with cellular organelles in the same way as they do in other cell types. Associations, involving side-arms in

some cases, have been noted between MT. and mitochondria in the CNS and the peripheral nervous system of a number of species (Raine, Ghetti and Shelanski,1971). Cross-bridges (80-200Å) connecting MT. and mitochondria are visible in lamprey nerve cord, the variation in length of the bridges being due to the uneven mitochondrial surface (Smith, Järlfors and Cameron,1975). Smith, Järlfors and Cayer (1977) have shown the presence of particle arrays, probably responsible for side-arms, in the mitochondrial membrane in the central axons of Periplaneta americana. MT-mitochondria associations are not only found in the axons but also in synaptosomes, Chan and Bunt (1978) have identified a horse-shoe-shaped mitochondria flanked by an arc of 3-10 MT. in the synaptic terminals of the rat cerebral cortex. Synaptosomal membranes isolated from rat cerebral cortex contain significant amounts of T, α-T.being much more tightly associated with the membrane. Gozes and Littauer (1979) have suggested that this membrane-bound T. may function by ways other than polymerisation into MT. and that agents which prevent MT. assembly may not necessarily interfere with this role in synaptic transmission. Within the cell body of neurons MT. are associated with nuclear pores and the coated pits of the dendrites (Gray and Westrum,1976) as well as forming lamellae with one another at the origin of the stem process (possibly initiating action po tentials) (Bird and Liebermann,1976). MT-MT interactions in the axons are characterised by filamentous inter-connections (Burton and Fernandez,1973) which are particularly obvious after lanthanum staining techniques. Lockwood (1979) has separated two molecules from mammalian brain which interact with the colchicine binding sites on T. Of these species, a protein and heat-stable peptide, the smaller one inhibits MT. assembly; both may play a significant role in the regulation of cellular MT. function and assembly in the brain.

A 68,000 dalton molecular weight protein found in early cycles of

purification of brain T. has been identified as being derived from 10nm filaments similar in morphology and protein composition to neurofilaments (Runge, Detrich and Williams,1979). When boiled these 10nm filaments produce a number of proteins which stimulate phosphocellulose-purified T. polymerisation and may correspond to T. assembly protein. Thorpe et al,(1979) has separated 10nm filaments from a variety of mammal brain sources, identifying a major 50,000 dalton protein and three other polypeptides (210,000, 155,000 and 70,000 daltons), identical to those polypeptides responsible for the 10nm filaments co-isolated with T. The 10nm filament fraction from brain appears to have cyclic nucleotide phosphodiesterase activity which is activated by calcium dependent regulator (CDR) protein (Runge,et al, 1979). This activity was present in both the 10nm filament fraction co-isolated with T. and isolated alone. Boiling this fraction also released an endogenous filament-associated CDR. The role of the 10nm filament-MT. interaction is unclear, however Wang, Cross and Choppin (1979) have suggested that they could be active in positioning intracellular nuclei. This follows the observation that MT. and 10nm filaments form long bundles between rows of nuclei in syncytia induced in baby hamster kidney (BHK 21-F) cells by parainfluenza virus SV5. These MT. are disrupted by colchicine preventing nuclear movement. MT. are also associated with a variety of vesicles in nerve cells, electron dense (Järlfors and Smith,1969), electron luscent (Smith, Järlfors and Beránek, 1970) and lipoproteinaceous (Spoerri,1978). Retinal receptor cells also appear to have MT-vesicle and MT-synaptic ribbon associations (Glees and Spoerri,1977 a and b).

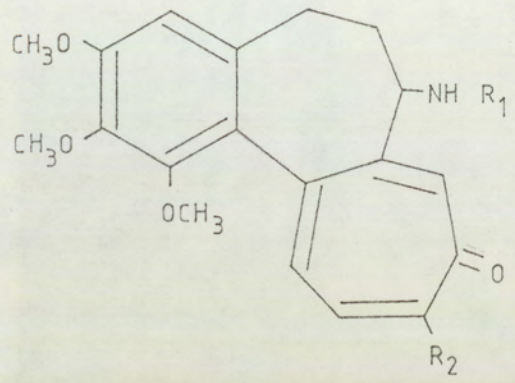
The importance of MT. and particularly T. synthesis to brain development has been demonstrated in visually deprived hooded rats by Cronly-Dillon and Perry (1979). The levels of T. synthesis in the visual cortex

of normal light reared rats increased during the period from eye opening (13 days) to 35 days after birth. However animals reared in the dark showed no such increase in T. synthesis in the visual cortex although allowing them 24 hours light during the critical period did produce significant increases. The number of synapses and cytoplasmic processes maintained by the visual cortex cells was limited by the T. pool available. Overall the nervous system provides an excellent model for the study of MT. influenced cellular movement particularly in simple systems such as the squid giant axon (Schmitt,1968). Consideration will now be given to other cell types with particular reference to cellular secretion.

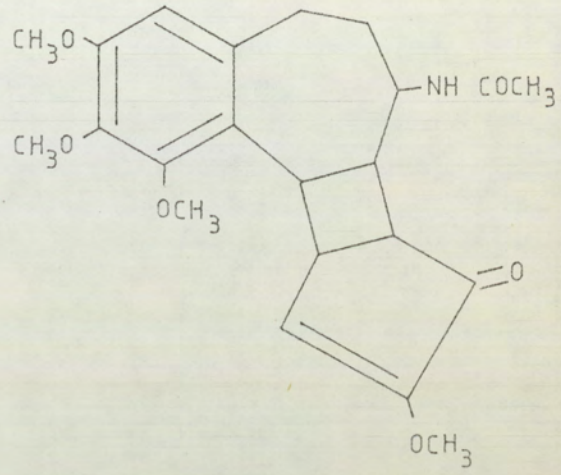
The role of microtubules in various cell types.

By far the most useful tools in the study of MT. both in vivo and in vitro have been the MT. poisons or 'antimitotic drugs' as they are sometimes known. Perhaps the most well known of these, as has already been mentioned, are colchicine and the Vinca alkaloids (vinblastine and vincristine). Other drugs are also used in the study of MT. action notably podophyllotoxin and griseofulvin and their derivatives. Together with these better known MT. poisons a wide variety of chemicals have been shown to have effects on MT, the majority acting to prevent MT. formation but some having an antagonistic action on other antimitotic drugs. Figure 1.5 shows the structure of the more useful MT. poisons and their derivatives, Table 1.3 lists other chemicals known to have an effect on MT. The important points worth noting about MT. poisons are that both colchicine and podophyllotoxin appear to bind to tubulin at the same site, the Vinca alkaloids bind at another distinct site, griseofulvin appears to bind to M.A.P's and finally an abundance of methoxy groups appear to be necessary for their action. Colchicine appears to form copolymers with T. which are incorporated at random into the MT.

Figure 1.5 : The common microtubule poisons and their derivatives



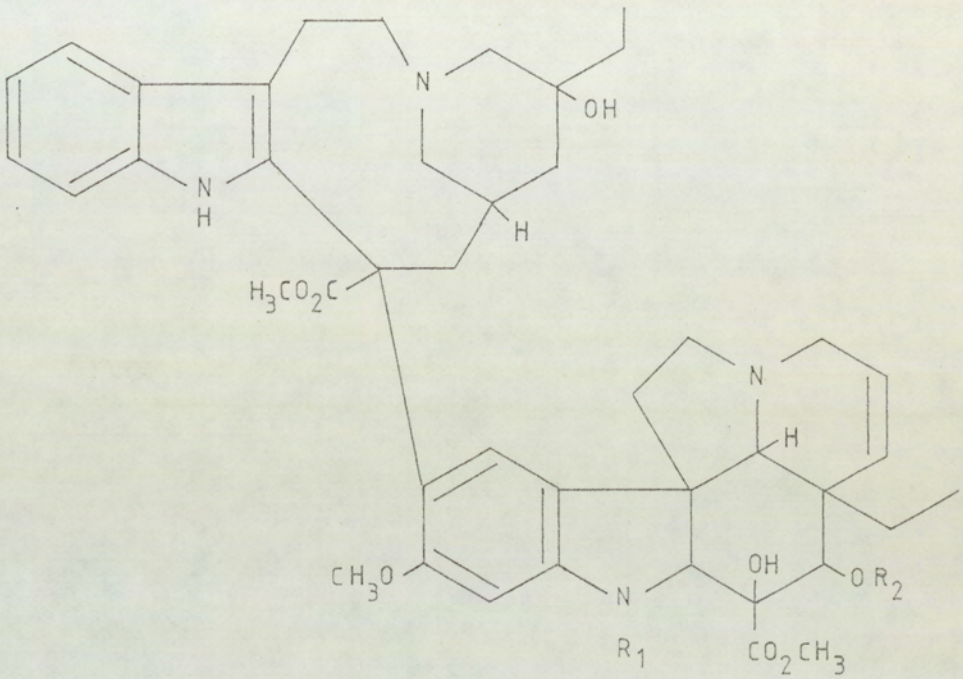
COLCHICINE



LUMICOLCHICINE

The principle derivatives of colchicine :

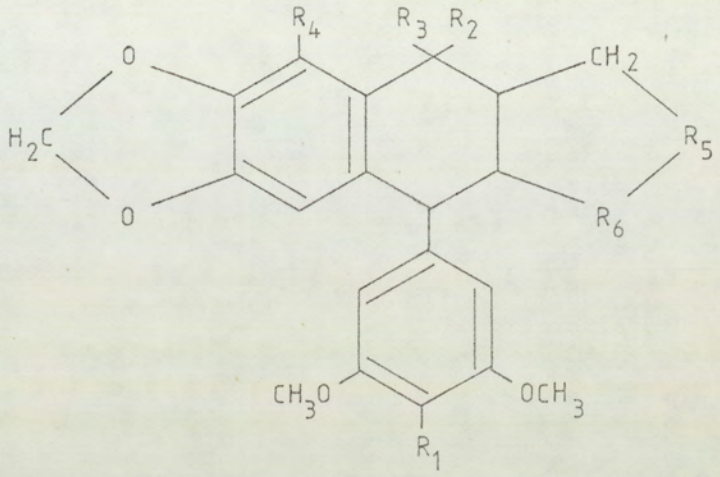
R1	R2	Name of drug
$CO-CH_3$	$O-CH_3$	colchicine
CH_3	$O-CH_3$	desacetyl-N-methyl-colchicine (demecolchicine, colcemide)
H_3	$O-CH_3$	desacetyl-amino-colchicine
$CO-CH_3$	CH_2-CH_2-N	colchicamides
$CO-CH_3$	$S-CH_3$	thiocolchicine
$CO-CH_2F$	$S-CH_3$	fluorthiocolchicine
$CO-CH_2Cl$	$S-CH_3$	chlorthiocolchicine
$CO-CH_2F$	$O-CH_3$	N-fluoro-desacetylcolchicine
$CO-CH_3$	OH	colchiceine
$CH_2-CHCl-CN$	$O-CH_3$	chlorocyanoethyl derivative of N-desacetyl-colchicine
H	$O-CH_3$	trimethylcolchicinic acid
$CO-CH_2-Br$	$O-CH_3$	bromocolchicine



VINBLASTINE

VBL: R_1 = $-CH_3$ R_2 = $-CO-CH_3$

VCR: R_1 = $-COH$ R_2 = $-CO-CH_3$

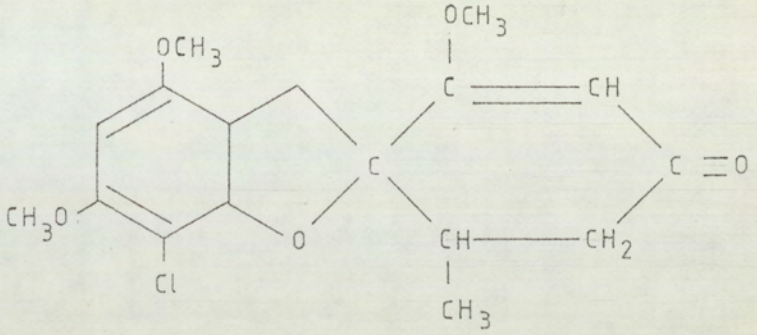


PODOPHYLLOTOXIN

The principle derivatives of podpphyllotoxin :

Name of drug	R1	R2	R3	R4	R5	R6
podophyllotoxin	OCH_3	OH	H	H	O	C=O
epipodophyllotoxin	OCH_3	H	OH	H	O	C=O
deoxypodophyllotoxin	OCH_3	H	H	H	O	C=O
β-peltatin	OCH_3	H	H	OH	O	C=O
4'-demethylpodo.	OH	OH	H	H	O	C=O
4'-demethylepipodo.	OH	H	OH	H	O	C=O
4'-demethyldeoxypodo.	OH	H	H	H	O	C=O
α-peltatin	OH	H	H	OH	O	C=O
VM-26	OH	H	*	H	O	C=O
VP-16-213	OH	H	£	H	O	C=O
podo.-cyclic ether	OCH_3	OH	H	H	O	H_2
deoxypodo.-cyclic ether	OCH_3	H	H	H	O	H_2
deoxypodo.-cyclopentane	OCH_3	H	H	H	H_2	H_2
deoxypodo.-cyclopentanone	OCH_3	H	H	H	C=O	H_2
podo.-cyclic sulphide	OCH_3	OH	H	H	S	H_2
deoxypodo.-cyclic sulphide	OCH_3	H	H	H	S=O	H_2
podo.-cyclic sulphone	OCH_3	OH	H	H	S=O	H_2

* thenylidene glucoside £ ethylidene glucoside



GRISEOFULVIN

References : Dustin,1978;Loike et al,1978

Table 1.3 : The wide variety of microtubule poisons

Name of drug	Reference
Acenaphtene	Bajer, 1968
Aldehydes	
Formaldehyde	Sentein, 1971
Gluteraldehyde	Sentein, 1975
Amiprophos methyl	Collis and Weeks, 1978
Anaesthetics	
Chloral hydrate	Sentein and Ates, 1974
Halothane	Allison and Nunn, 1968
Procaine	
Etidocaine	Haschke, Byers and Fink, 1974
Nitrous oxide	Brinkley and Rao, 1973
Lidocaine	Byers et al, 1973
Arsenical derivatives	
Arsenious oxide	
Sodium cacodylate	Dustin, 1947
Barbituates	Edstrom et al, 1975
Benzimidazoles	Davidse, 1973, 1975; Davidse and
Carbendazim	Flack, 1977; Ireland et al, 1979
Oncodazole	DeBrabander et al, 1975
Isopropyl(N-(3-chlorophenyl)carbamate	Oliver, Krawiec and Berlin, 1978
Mebendazole	Borgers and DeNollin, 1975
Fenbendazole	Friedman and Platzer, 1978
Noncodazole	Samson et al, 1979; Mareel and
	DeBrabander, 1978a, b
Chlorpromazine	Rao, Hare and Cann, 1978;
	Thyberg, Axelson and Hinek, 1977;
	McGuire, Quinn and Knutton, 1974
Colchicine and derivatives	Margulis, 1973; Banerjee and
	Bhallacharyza, 1979
Cordycepin	Deitch and Sawicki, 1979
Cytochalasin A	Himes and Houston, 1976
Digitonin	Olah and Hanzely, 1973
DL-2-oxo-3-(2-mercaptoethyl)-	
5-phenylimidazolidine	DeBrabander et al, 1978
DNA	Vater, Muller and Unger, 1978
5-fluoropyrimidine-2-one	Oftebro et al, 1972
Griseofulvin and derivatives	Mir et al, 1978; Wehland, Herzog
	and Weber, 1977
Hydantoin drugs	MacKinney, Vyas and Walker, 1978;
5,5-diphenylhydantoin	MacKinney, vyas and Powers, 1978;
5-(4-hydroxyphenyl)-5-phenylhydantoin	
Isoprenaline	Radley, 1974
Isopropyl-N-phenylcarbamate	Brown and Bouck, 1974
Maytansine	Kupchan et al, 1972
Melatonin	Jackson, 1969
Metal ions	Roth and Shigenaka, 1970;
Cu	Shigenaka, 1976
Ni	
Hg	
Cd	
Zn	
Nerve growth factor	Monaco, Calissano and Mercanti, 1977
Organomercurials	
Methylmercury	
Phenylmercury	Morris and Oakley, 1979

Name of drug	Reference
p-fluorophenylalanine	Morris and Oakley, 1979
Podophyllotoxin and derivatives	Loike et al, 1978
Quinoline	Sentein, 1970
Rotenone	Bibring and Baxandall, 1971
Steganacin	Wang, Rebhun and Kupchan, 1976; Schiff, Kende and Horwitz, 1978
Sulphhydryl binding drugs	
Dimercaptopropanol	
Dithioiodoethylcarbamate	Dustin, 1949
Diamide	Mellon and Rebhun, 1976
Taxol	Schiff, Fant and Horwitz, 1979
Trialkyltins(e.g.triethyltin chloride)	Tan, Ng and Kumar Das, 1978
Trifluralin	Bartels and Hilton, 1967
Urea	Shigenaka, Roth and Pihlaja, 1971
Vinblastine and analogues	Zavala, Guenard and Portier, 1978;
Vincristine	Starling, 1976
Withaferin	Shohat, 1973

lattice. However, as the copolymer to unlinked T. ratio increases in the MT. the critical T. concentration for MT. assembly increases. (Sternlicht and Ringel,1979).

MT. poisons have been particularly useful in studying the role of MT. in cellular activities, especially secretion. MT. action has been implicated in secretory and other cellular movements of a wide variety of cell types. From the secretory point of view the best studied system is the pancreas, outside secretory mechanisms excluding cilia, flagella and the mitotic spindle, the movement of pigment granules in the melanophores of fish and amphibians are the best understood.

The study of the mechanisms of pigment granule movement in melanophores illustrates the two main conflicting theories on the role of MT. in cellular movements. Melanophores darken and lighten in response to a number of factors such as hormones and changes in ionic concentrations. Darkening is brought about by the spreading out of the pigment granules into stellate expansions of the cytoplasm, lightening by their aggregation in the cell centre. Darkening agents include melanin-stimulating hormone (MSH) and Na^+, lightening results from MSH removal or an increase in K^+ concentration; c-AMP is believed to have a role as the second messenger of MSH. It is important to realise that pigment cells in insects, crustacea, teleosts and amphibia are all of different embryonic origin and cannot necessarily be compared when considering their mechanisms of action. However, looking at the overall role of MT. in pigment cells two main theories have been proposed, which may apply to other types of cell movement.

The first mechanism proposed by Murphy and Tilney (1974) is the "sliding filament" theory which suggests that pigment granules move along the MT. under the influence of an active associated protein such as dynein (found in cilia and flagella). The granules are believed to

be joined to the MT. by labile links although these have not as yet been demonstrated ultrastructurally. One problem with this theory is that granule movement is in two directions therefore one must propose the presence of two populations of MT. each responsible for either pigment aggregation or dispersion.

The second mechanism of granule movement proposed by Porter (1973) and Schliwa and Bereiter-Hahn (1974) is the "MT. assembly-disassembly" theory. Here it is suggested that pigment aggregation is brought about by the rapid disassembly of MT. into T. dimers and that pigment dispersion is brought about by MT. assembly pushing the granules out towards the cell periphery. The latter half of this scheme is quite easy to imagine but how MT. disassembly provides a centripetal force is much harder to explain. Porter (1973) has identified a flocculent gel matrix which coats both the MT. and the pigment granules and may undergo spontaneous syneresis or contraction, once its supporting MT. are removed, taking the granules with it to the cytocentre. This would certainly explain the very rapid (100µm/min) pigment aggregation as opposed to the much slower (30µm/min) and less uniform dispersion. Schliwa (1978) has characterised the MT. system of the melanophores of the angelfish (Pterophyllum scalare), the cell processes appear to contain a cortical array of MT. constituting an MT. "palisade" originating in dense aggregates at the cytocentre. As will be seen later these two models of cellular movement can be used to explain such mechanisms in a number of cell types and will be referred to frequently.

Consideration will now be given to a number of other cell systems, mainly secretory, but also involving other cell types. The presence of colchicine binding activity (tubulin) has been demonstrated in the adrenal cortex of several species, and was found to be similar to brain T. when compared by polyacrylamide gel electrophoresis (Strott and Ray, 1977).

Hadjian, Guidicelli and Chambaz (1977) have demonstrated that 3% of adrenal cortex cytosol protein is T. Comparison of bovine adrenocortical and brain MT. proteins indicated that T. from both sources was identical when compared by colchicine binding and tyrosylation studies (Ray and Strott,1978). However, the M.A.P's differed considerably, adrenocortical M.A.P's having molecular weights of about 36,000, 60,000 and 68,000 daltons while brain M.A.P's were all above 200,000 daltons, these larger proteins being absent in adrenal preparations. In Y-1 adrenal tumour cells colchicine, vinblastine and podophyllotoxin all stimulated steroid secretion to an extent that equalled a maximal ACTH response (Temple et al,1972). Study of the adrenal medulla is complicated by the fact that colchicine and vinblastine both appear to have an anticholinergic action so that their inhibition of acetylcholine-induced catecholamine production cannot be explained simply by an action on MT. (Trifaro et al, 1972).

Colchicine and other MT. poisons have long been used as cures for gout, this is explained by their actions on the polymorphonuclear leucocytes which are responsible for this inflammatory reaction caused by precipitation of sodium urate microcrystals in the vicinity of the joints. These microcrystals are phagocytized by the leucocytes where they puncture the lysosomal membranes liberating hydrolases into the cell resulting in its death. This in turn attracts further polymorphonuclear leucocytes producing the inflammation (Shiraham and Cohen,1974). MT. poisonsact by destroying leucocyte MT. and preventing phagocytosis, fusion of the lysosomes with the phagosomes and movement of the polymorphonuclears to the microcrystal deposits (Malawista,1968; Malawista, 1975). Lysosomal enzyme release in response to particles or an immune reaction are MT. mediated since they are inhibited by colchicine, however, release in response to the ionophore A23187 and calcium is under the

influence of the microfilaments (Hoffstein and Weissmann,1978). MT. assembly appears to occur in polymorphonuclears in response to phagocytosis, Oliver et al,(1977, 1978) have indicated that glutathione or one of its derivatives is necessary for concanvalin A-induced MT. assembly. Burchill et al,(1978) have shown that MT. assembly in human polymorphonuclear leucocytes is induced by particle (opsonized zymosan) contact and is maximal after 3 minutes of phagocytosis. After 5 - 9 minutes of phagocytosis disassembly is induced by a rise in glutathione disulphide and the formation of protein-disulphide-glutathione complexes. Chemotactic activity of polymorphonuclear leucocytes is modified by the colchicine effects on cellular MT. Ascorbic acid which increases chemotaxis of PMN's also promotes the assembly of intracellular MT, an effect which is blocked by colchicine. In addition ascorbic acid at physiological concentrations promotes in vitro MT. assembly. (Boxer et al,1979). The reduction of chemotactic factor (CF = Gly - His - Glyc)-induced chemotactic activity by pre-incubation with CF is also inhibited by colchicine. This was due to the arrest of the CF-induced increase in centriolar MT. not an alteration in the specific binding of CF. Spilberg, Mandell and Hoffstein (1979) have suggested that chemotaxis is prevented by the inappropriate organisation of MT. proteins. The chemotactic factor N-formylmethionyl peptide (FMLP) promotes MT. assembly in PMN at concentrations which are chemotactic for the cells. Boxer et al,(1979) have suggested that this could be important physiologically since oligopeptides similar to FMLP are formed in bacteria. Human leucocyte cytoplasmic MT. "appear to impose constraints on the plasma membrane, limiting lectin-receptor complex lateral mobility and expression of hormone sensitive adenyl cyclase". This was shown by Malaw ista, Oliver and Rudolph (1978) since the β-adrenergic agonist isoproterenol and the phosphodiesterase inhibitor isobutylmethylxanthine caused greater

increase in leucocyte c-AMP levels in colchicine-pretreated cells than controls. Also colchicine-induced concanavalin A capping was time and dose dependant in a similar way to c-AMP generation.

In another type of connective tissue cell, the mast cell, histamine release in response to antigen, IgE or IgG is reduced by colchicine, colcemid and vinblastine. D_2O on the other hand stimulates release and antagonises the inhibitory effects of c-AMP (Gillespie and Lichtenstein, 1972). Uncouplers of oxidative phosphorylation, sodium azide, dinitrophenol and oligomycin, also alter the location of MT. in murine mast cells. MT. parallel to the plasma membrane in cell surface folds and intranuclear MT. are both seen (Bergstrom-Porter and Shelton, 1979). Macrophage response to migration inhibitory factor (MIF) appears to be determined by the state of the MT. system (McCarthy, Shaw and Remold, 1979); colchicine and vinblastine both inhibit MIF's effect, while D_2O enhances it if preincubated with the macrophages before MIF addition. The presence of a colchicine-binding protein, characterised as T. has been shown in blood platelets (Castle and Crawford, 1978 a,b). Colchicine seems to inhibit β-glucuronidase release from blood platelets in response to thrombin, trypsin and sodium arachidonate although ADP, adrenaline and collagen-induced 5-hydroxytryptamine release is unaffected (Kenney and Chao, 1978). In the mixed lymphocyte response (where lymphocytes from two genetically different members of the same species are mixed; one group, the stimulator cells, treated to prevent DNA synthsis, induce increased DNA synthesis in the other group, the responder cells) colchicine and vincristine prevent the action of the stimulator cells by interfering with the MT. controlled antigens on the cell surface (Ranney and Pincus, 1976). Lymphocyte structure and function is also affected by MT. poisons; Center, Wasserman and Austen (1978) have demonstrated a chemokinetic response (in the absence of a chemical gradient)

to colchicine and vinblastine indicating that rat splenic lymphocytes require MT. disassembly for such a response. Stimulation of human and mouse lymphocytes with concanavalin A induced an increase in cellular MT. while colchicine reduced MT. numbers by 25%. However caution must be exercised in attributing the effects of colchicine wholly to its action on MT. since it was also shown to inhibit DNA and RNA synthesis in these cells although colchicine can act well after DNA synthesis has begun (Rudd et al,1979). In fact the presence of colchicine binding protein comigrating electrophoretically with T. has been demonstrated in bovine lymphocyte chromatin (Farr, Lucknow and Sundharadas,1979). Finally in consideration of fibroblasts Rudolph and Woodward (1978) have demonstrated the presence of many MT. running parallel to the plasma membrane in myofibroblasts from pig skin wound and around human silicone implants. The orientation of these MT. suggests a bracing function. Cytoplasmic MT. have been extracted from mouse embryo fibroblasts using detergent treatment (Triton X-100), they were found to be in close association with microfilaments (Bershadsky et al, 1978). The role of fibroblast MT. may be important in a number of inherited disorders; Shay and Fuseler (1979) have demonstrated a dramatic reduction in intact interphase MT. in fibroblasts from dystrophic muscle explants of chickens by immunofluorescence. However the suggestion that abnormally large ly sosomes and enhanced concavavalin A capping, in fibroblasts and macrophages from mice with a Chediak-Higashi-like syndrome, being due to MT. abnormality has not been confirmed by immunofluorescence studies (Frankel et al,1978). Pharbol myristate acetate (PMA) stimulated pinocytosis in mouse macrophages is abolished by cochicine and cytochalasin B. In Thioglycolate-elicited macrophages PMA causes the extension and radial organisation of MT. and 10nm filaments and promotes movement of secondary lysosomes from perinuclear location to the peripheral cytoplasm. Lysosomes radiating from the

centrospheric region are arranged linearly along MT. and 10nm filaments (Phaire-Washington, Silverstein and Wang,1980).

Collagen release from chick embryo frontal bone and 3T3 fibroblasts is inhibited by colchicine and vinblastine even though amino acid incorporation into collagen is unaffected, hence MT. are involved in secretion (Diegelmann and Peterk ofsky,1972). Immunofluorescence study of cells derived from tumours, normal cells and hybrids between the two showed no differences in their MT. network morphology (Wolin and Kucherlapati,1979). Comparison of the MT. and depolymerised T. levels in normal and transformed 3T3 cell lines showed that four out of five transformed cell lines had significantly higher total T. content than non-transformed cells and all had higher MT. content although this T. pool was only significant in three cases. This was in comparison with the fact that no changes in the T. or MT. pools were produced by alterations in cell density, growth, transformation or dibutyryl -c-AMP treatment of transformed cells (Eickhorn and Peterkofsky,1979). MT. are active in the regulation of events coupling receptor occupancy to the activation of adenylate cyclase in intact S49 lymphoma cells since colchicine and vinblastine both enhance β-adrenergic- and protaglandin-E stimulated c-AMP accumulation in these cells (Kennedy and Insel,1979). MT. inhibitors have potent actions on tissue culture cells, colchicine, vinblastine and vincristine all reduce lysosomal proteolysis in rat embryo fibroblast cultures by affecting the vacuolar system (Amenta, Sargus and Baccino,1977). Colchicine, vinblastine and podophyllin inhibit long saltatory movements of lysosomes, pinosomes, ingested carbon particles, lipoidal granules and other granules in HeLa cells; MT. can be demonstrated radiating from the centriolar cytocentre (Freed and Lebo w itz,1970). MT. protein has been extracted from HeLa cells by cycles of polymerisation and depolymerisation in the absence of glycerol

(Bulinski and Borisy,1979). After two cycles 95% of the protein was shown to be T. making up 4-5% of total protein; M.A.P's of 210,000 and 120,000 dalton molecular weight which stimulated MT. formation were also present. The uphill transport of α-aminoisobutyric acid into Ehrlich ascite tumour cells is inhibited by vincristine and vinblastine (Goldman et al,1977). Low density lipoprotein uptake and degradtion by cultured human skin fibroblasts and aortic medial cells was also reduced by colchicine (Ostlund, Pfleger and Schonfeld,1979).

In rat lacrimal glands the importance of MT. in protein secretion has been shown by the fact that colchicine inhibits only intracellular transport and has no other biochemical effect on discharge, protein biosynthesis nucleotide levels and phosphate incorporation into nucleotides, proteins and phosphlipids (Chambaut-Guérin, Muller and Rossignol, 1978). Also in the eye, the elongation of cultured lens epithelial cells occurred in the absence of MT. after nocodazole treatment. Colchicine was also shown to induce cell elongation by increasing cell volume at a concentration too low to effect MT; this indicates that colchicine may have another non-MT. effect on cell volume regulation by altering plasma membrane fluid transport.

In the gut MT. are involved in lipid absorption since colchicine induces a build up of lipid in the apical region of enterocytes from the small intestine. An unusual point is that the occurrence of enterocyte MT. in fasting rats is greater than in fed rats, somehow MT. are 'used up' during chylomicron transport and shifted towards a depolymerised state (Reaven and Reaven,1977). Acid and pepsinogen secretion from the bull frog gastric mucosa is inhibited by vinblastine but colchicine transiently stimulates acid secretion and then causes inhibition, by contrast cytochalasin B (microfilament inhibitor) has no effect indicating that only MT. are actively involved in secretion (Kaskekar,1978). In contrast,

cytochalasin B inhibits vitamin D-induced transepithelial phosphate transport in the chick jejunum while MT. inhibitors have no effect. The active metabolite of vitamin D_3 - 1,25 dihydroxy D_3 increase the amount of microfilament protein (actin) associated with the brush border.

MT. have been implicated in the action of vasopressin on the canine renal medualla and the toad urinary bladder. Colchicine inhibits the uptake of water, stimulated by vasopressin or dibutyryl c-AMP into renal medullary slices; colchicine, vinblastine and podophyllotoxin inhibit similar uptake in the toad urinary bladder. Lithium also inhibits the antiduiretic effect of vasopressin but lumi-colchicine (the MT-inactive isomer of colchicine) has no effect. Colchicine has no effect on basal or vasopressin-induced c-AMP levels. Thus it appears that vasopressin has no real effect on MT. but requires their presence for its action (Iyengar,Lepper and Mailman,1976; Dousa, Hui and Barnes, 1978; Taylor et al,1978). However, Kachadorian, Ellis and Muller (1979) have found that both MT. and microfilaments are necessary for initiation of vasopressin-induced osmotic water flow in the toad urinary bladder, but only microfilaments are necessary for its maintenance. Vinblastine sulphate has a structural effect in the kidney, after two hours exposure to this MT. poison MT. were almost completely lost from podocyte cell bodies of the renal glomerular epithelium and crystalline inclusions became present. Loss of MT. from the podocyte cell processes caused their collapse as they lost much of their cytoplasm to the nucleated cell body. MT. thus seem to be responsible for the maintenance of cell shape and the podocyte major processes (Andrews,1977). T. has been purified from bovine renal medulla by Barnes and Roberson (1979), they have suggested that its differences and similarities to brain T. support physiological studies which indicate that MT. are required for vasopressin

action in the kidneys.

In the liver two colchicine-binding sites have been isolated, a high affinity, low capacity one representing T. and a low affinity, high capacity non-specific site. 1% of soluble protein is T. representing 80% of the total colchicine binding activity of hepatocyte homogenates. 9.0×10^{-14} dimers are present per microgram of protein with an average of 3.1×10^{7} dimers per hepatocyte. This would produce an MT. 1.9cm long, however only 0.28cm are demonstrable. This gives a figure of 15% for the amount of T. in the form of MT, Patzelt et al,(1975) have reported a higher value of 40% (Reaven,1977; Patzelt et al,1975). The MT-poisons vincristine and colchicine are effective in inhibiting the secretion of very low density lipoproteins and proteins (globulin, albumin and small polypeptides) without having any action on other liver functions such as ureogenesis, glucose production, ATP levels, oxygen consumption, uptake and oxidation of fatty acids, esterification of oleate, lipogenesis or protein synthesis. Treated hepatocytes remain ultrastructurally identical to control cells except for an accumulation of vesicles and very low density lipoprotein particles, the disappearance of MT. and formation of paracrystals (in the case of vincristine). In addition lumicolchicine has no effect on secretion even though it binds to plasma membranes in the same way as colchicine (Le Marchand et al, 1973,1975). Very low density lipoprotein triglyceride (VLDL-TG) secretion has been measured by hepatic perfusion at various colchicine and vinblastine concentrations (Reaven and Reaven,1980). The reduction in MT. numbers was correlated with reduced VLDL-TG secretion the nadir of secretion (10-30% of controls) being reached when MT. comprised <0.005% of the cytoplasm (or <10% of control values). Under these reduced secretory conditions the number of Golgi complex dictysomal membranes were reduced to 15% of controls the few remaining being associated

with remaining MT. fragments. MT. have also been suggested as the site of regulation of hepatic protein secretion (Le Marchand et al, (1974). The secretion of coagulation factors V and VII and triglycerides into blood plasma is also inhibited by the action of colchicine and vinblastine on hepatocytes; by contrast the secretion of bilirubin-glucuronides and fluid into bile is not affected (Gratzl and Schwab 1976) eventhough biliary lipid secretion is (Gregory et al,1978). MT. are certainly important in hepatic secretory processes, however the number of MT. or amount of tubulin is not increased upon stimulation. For instance, increasing secretion of very low density lipoproteins in acute experiments (2 hours increased fatty acids) or chronic experiments (1 week on hyperlipidemic diet) produced no concurrent increase in MT. numbers or colchicine binding protein (Reaven and Reaven,1978).

In the lung colchicine and vinblastine inhibit the release of lung surfactants (Delahunty and Jonston,1976) and colchicine together with cooling to $4^{\circ}C$ suppresses the secretion of histamine and slow reacting substance, mediators of analphylaxis, induced by antigen-IgE interactions on human lung tissue mast cells (Kaliner,1977). In the latter case the action of colchicine is prevented by c-GMP; c-AMP appears to initiate MT. phosphorylation and c-GMP dephosphorylation, this controls MT. action on histamine and slow reacting substance secretion.

MT. have been demonstrated in heart muscle cells being particularly abundant in the perinuclear region, running parallel to the myofibrils and also within the myofibrils (Rybicka,1978). MT. are more numerous in adult cardiac muscle than has previously been recognised and may perform functions other than the cytoskeletal one. MT. encircle the nucleus, are associated with myofibrils in a helical arrangement and network that runs transversely at the level of the I band and axially between the myofibrils (Goldstein and Entman,1979). Treatment of heart muscle cells

in culture with dibutyryl c-AMP prevents their contraction, colchicine restores their ability to contract though lumicolchicine and dibutyryl c-AMP addition to colchicine pretreated cells each have no effect. Dibutyryl c-AMP treated cells have MT. arranged in parallel arrays as opposed to the random arrangement found in control cells. (Nath, Shay and Bollon,1978). MT. run longitudinally throughout frog skeletal muscle but within the bare regions of the nodes of Ranvier annular or helical MT. bundles run in a marginal band at right angles to the more central longitudinal MT. Gray and Westrum (1979) have suggested that these annular bundles support the pronounced convexity of the muscle in this region during muscle contraction. In addition the outermost MT. appear to be anchored to the muscle membrane by fine threads. Arterial wall smooth muscle cells secreting extracellular matrix components are affected by colchicine in two ways, one group accumulate secretory granules while the other undergoes rough endoplasmic reticulum cisternae dilation. Chaldakov,Nikolov and Vancov (1977) have suggested that two types of secretion are represented here, one involving the Golgi apparatus and the other direct exocytosis from the R.E.R., the latter not involving the MT. system. Cells probably differentiate which system is used on the basis of the cellular status of the MT, controlled in turn by cyclic nucleotides.

The secretion of progesterone by the ovine corpus luteum is inhibited by colchicine with a concomitant loss of luteal MT. and accumulation of secretory granules (Gemmell and Stacy,1977). The action of gonadotropins and prostaglandin E_2 on cultured graafian follicles is also effected by MT. and microfilament poisons. Release of c-AMP induced by LH and choleragen is mediated by microfilaments, prostaglandin E_2 by MT and FSH by microfilaments and MT. (Zor,Strulovici and Lindner,1978).

Prostaglandin $F_{2\alpha}$ and prostaglandin E levels in pseudopregnant rats *in vivo* and their release from uterine slices from such rats *in vitro* are both increased by colchicine and vinblastine. Hall and Robinson, (1978) have postulated that MT. removal enables enhanced accessibility of membrane phospholipids to phospholipase A_2 and thus increases the availability of prostaglandin precursors.

Parathyroid hormone secretion in response to vitamin A is inhibited by vinblastine but not in response to low Ca^{2+} concentrates *in vitro* (Chertow et al,1975 a,b). Colchicine treatment of the parathyroid induced an increase in secretory granules and a decrease in MT.numbers in the chief cells. The secretagogue, phosphate, however induced an increase in MT. numbers but had no effect on granule numbers (Reaven and Reaven,1975). The role of MT. in the parathyroid chief cells could be in transport from the endoplasmic reticulum to the Golgi apparatus since colchicine and vinblastine both prevent the conversion of pro-parathyroid hormone to parathyroid hormone (Kemper et al,1975); this is also indicated by the temperature dependence and energy requirements of the conversion (Chu, MacGregor and Cohn,1977).

MT. are certainly present in anterior pituitary cells since they can be isolated from this source (Sheterline and Schofiled,1975) and are also visible throughout prdactin cells of lactating rats particularly associated with secretory granules (Warchol et al,1975). Indeed MT. are seen in the early stages of the development of the thyroidectomy cells of the anterior pituitary associated with secretory granules and may have a role in the hypersecretory state of these cells (Shino and Rennels,1975). However, it appears that MT. are not involved in basal secretion of anterior pituicytes although they may have a role in stimulated secretion. Sheterline et al,(1977) have shown that growth hormone secretion from bovine somatotrophs in response to non-physiological

secretagogues (3-isobutyl-1-methylxanthine and barium) is accompanied by MT. depolymerisation and both secretion and MT. disassembly are prevented by the MT. stabiliser 2-methylpentan-2,4-diol. In the light of this information Sheterline et al propose that secretion is controlled by a sol-gel mechanism whereby exocytosis is prevented by a complex web of MT. and secretion involves depolymerisation of MT. to enable granules to reach the plasma membrane. A further explanation of the less typical effect of MT-poisons on anterior pituitary is given by Howell and Tyhurst (1978); if colchicine or vinblastine is added to anterior pituitary fragments after pulse labelling immediately, or after a further hour, growth hormone secretion is inhibited for 5 hours. However, addition of these compounds 2 hours after pulse labelling has no effect on growth hormone secretion; hence MT. are only involved in transport of secretory granules from the Golgi apparatus to the cytoplasmic pool and not in final secretion by exocytosis. Khar, Kunert-Radek and Jutisz (1979) have shown that MT. and microfilaments are involved in the Gn RH-induced release of gonadotropins in cultured rat anterior pituitary cells although not in the effect of high K^+ on such cells. The levels of T. in the medial basal hypothalamus (MBH) are greater in male rats than female rats. Castration brought about a decrease in MBH. T. while testosterone and oestradiol both augmented the T. levels (Valenti, Vacas and Cardinali,1979).

Secretion by the posterior pituitary is related to axonal transport mentioned previously since secretion is via the tractus hypophyseus from the hypothalamus. Secretory granules are produced in the cell bodies in the hypothalamus and carried along modified axons to the posterior pituitary lobe in association with many MT. (or neurotubules). MT-poisons are effective in inhibiting anti-diuretic hormone transport and secretion even though MT. remain visible. However, MT. are certainly

involved in neurosecretion since rats treated with hypertonic saline or with congenital diabetes insipidus both have increased numbers of MT. in the axons of tractus hypophyseus cells as a result of increased protein secretion (Grainger and Sloper,1974). In the case of rats with congenital diabetes insipidus the increase in MT. numbers does not arise until 4 days after birth, ruling out a possible congenital MT. abnormality (Grainger and Sloper,1976).

The thyroid gland has a peculiar form of secretory process involving a two-way movement through the cell. A protein thyroglobulin is synthesized in the endoplasmic reticulum and then passes to the edge of the cell where it is iodinated and stored as colloid in extracellular vesicles. Before thyroid hormones (Triodotyronine and thyroxine) can be secreted thyroglobulin has to be recaptured by the cell by phagocytosis and transported to lysosomes where hydrolysis releases the hormones so that they can be released into the blood. MT. and microfilaments appear to be necessary both for phagocytosis of thyroglobulin and its transport within the cell. Certainly MT-poisons prevent the liberation of thyroid hormones (Williams and Wolff,1970), however they seem to have effects on other cellular processes besides MT. formation (Grenier et al,1975; Chiraseveenuprapund and Rosenberg,1975). Microfilaments seem more important in phagocytosis than MT, MT. may only play the role of structural scaffolding (Neve and Wollman,1972); this will be discussed further in consideration of the action of MT. in pancreatic secretion.

Microtubules and the secretory process in the endocrine pancreas.

The role of MT. in secretion has been best studied in the endocrine pancreas, particularly in the pancreatic β-cell. Here the release of insulin in response to secretagogues such as glucose, Ca^{2+} and gliclazide produces a biphasic pattern consisting of an early, rapid secretory phase and a later, more prolonged phase. Most studies on the pancreatic β-cell have made use of the isolated perfused pancreas or purified isolated islets. Early work by Lacy et al,(1968) resulted in the proposal of a scheme for insulin secretion involving MT. and microfilaments (Lacy,1970). The biphasic pattern of secretion was explained by the fact that initial rapid release was of β granules bound to the MT. and the slower, prolonged release occurred because stored and newly synthesised granules had to become associated with the MT. system (Lacy, Walker and Fink,1972). The evidence for this was that vinblastine and deuterium oxide (D_2O) prevented both phases of insulin release but colchicine only inhibited the second phase. Later work by the groups of Malaisse and Orci (Malaisse et al,1971, 1975 a,b; Malaisse,1973; Malaisse-Lagae et al,1975) cast doubt on some of the MT-poison data of Lacy. Small doses of colchicine (10^{-5}M) were found to increase both phases of glucose-induced insulin secretion (van Obberghen et al,1974), this is in direct contradiction of Lacy's finding (Lacy et al,1968) that 10^{-6}M colchicine inhibits glucose-stimulated insulin secretion by isolated islets. Vincristine also stimulates glucose-induced insulin release (Devis et al,1974), although only in the first 30 minutes. The explanation of this apparent discrepancy is that the action of MT-poisons on the β-cell MT. takes a long time to occur, MT. not disappearing from the cytoplasm for at least 60 minutes (Devis et al, 1974; Malaisse-Lagae et al,1971). D_2O also effects β-cell MT. resulting in their stabilisation, preventing their correct function, this in turn

inhibits glucose-induced insulin secretion (van Obberghen et al,1974); however, Holze and Beckman (1977) have suggested that this inhibition can be explained by D_2O's role in inhibiting glucose oxidation by the β-cell. Vinblastine also inhibits glucose-,glibenclamide- or L-isopropyl-noradrenaline-induced insulin secretion producing cellular paracrystals, however the action of vinblastine on other aspects of secretion is acknowledged (Ericson and Lundquist,1975). MT-poisons have been shown to have effects on c-AMP metabolism unrelated to their MT. effects (Grill and Cerasi,1977). A study using isolated rat islets (Malaisse-Lagae et al,1979) has shown, by using increasing preincubation periods with colchicine in vitro, that the release of preformed insulin was progressively inhibited and that of newly synthesised hormone delayed. Subsequent ultrastructural autoradiographic study of islets preincubated with colchicine showed that the export of newly synthesised proinsulin out of the rough E.R., its transit through the Golgi complex packaging in secretory granules and conversion to insulin were all retarded. These effects were shown to be on the MT. system since under the same conditions colchicine failed to effect glucose oxidation and adenylate cyclase in the islets and were not reproduced by lumicolchichine.

Microfilaments are also involved in insulin secretion, their presence as a cell web below the plasma membrane has been demonstrated by actin immunofluorescence (Gabbiani et al,1974). The fungal metabolite cyto-chalasin B disrupts this cell web resulting in an increase in insulin secretion (van Obberghen et al,1975), it has been suggested that microfilaments control access by insulin granules to the cell membrane (Orci, Gabbay and Malaisse,1972) even though its effects are only on stimulated secretion and not the basal level (van Obberghen et al,1973). Orci, Malaisse-Lagae and Perrelet (1977) have observed changes in the membrane prior to granule exocytosis which are probably due to the effects

of MT. or microfilaments, MT. and microfilaments are also involved here in the mobility of insulin and growth hormone receptors (van Obberghen, de Meyts and Roth,1976).

The exact role of MT. and microfilaments in insulin secretion still remains confused due to the uncertain action of MT-poisons. However, MT. certainly play a role in the pancreatic β-cell; for instance, variations in insulin release in fed and fasted rats are accompanied by concomitant changes in T. polymerisation (Pipeleers, Pipeleers-Mariahal and Kipnis,1976). MT. connections with secretion can be shown by the fact that glucose and c-AMP induce increased T. synthesis (Pipeleers, Pipeleers-Marichal and Kipnis,1976), in addition the same agents increase the amount of T. found as MT, as shown by a decrease in the subunit pool (Montague, Howell and Green,1976). MT. are found throughout the pancreatic β-cell (Kern,1975), extending from the outer nuclear membrane to the plasma membrane. They run between the endoplasmic reticulum, the citernae of the Golgi complex and in close proximity to mitochondria and secretory granules. They are also connected to microfilaments and desmosomes at the plasma membrane. Removal of these MT. by colchicine incubation of isolated islets, causes a reversible disruption of the Golgi complex and the appearance of numerous dictyosomes due to rough endoplasmic reticulum disruption. This certainly indicates a role for MT. in maintaining the structural integrity of the cell (Moskalewski, Thyberg and Friberg,1976).

No one conclusive mechanism can yet be proposed for the role of MT. in the secretion of insulin, Ostlund (1977) has produced a model for β-cell secretion drawing on other secretory systems for evidence. In his model MT. act as guides to orient saltatory granule movement towards the plasma membrane. Granules are bound to MT. by M.A.P's acting as MT. surface arms. The MT. carry the granules to the cell web consisting of

microfilaments coating the inner plasma membrane. Secretory granules and the plasma membrane bind to the microfilaments by lipophilic interaction. Secretory stimulation causes an accumulation of cytoplasmic Ca^{2+} which induces the cell wall to contract and extrude the granules, their membranes fusing with the plasma membrane. Continued secretion results from granule movements towards the cell web along the MT. Movement could result from contraction of the microfilaments, electrostatic interactions, MT. polymerisation or granule-MT. interaction. The first phase of insulin secretion results from expulsion of granules already close to the cell web and the second phase from delivery of granules along the MT.

As was mentioned above colchicine disrupts the cellular morphology of the pancreatic β-cell, this is also true of the α-cell which secretes glucagon. Colchicine induces MT. loss, dispersal of dictyosomes and reduction in the number and size of Golgi cisternae in the α-cell (Moskalewski, Thyberg and Friberg,1976). The MT-poisons (colchicine and vinblastine) as well as the microfilament disruptor (cytochalasin B) increase arginine-induced glucagon release from pancreatic A_2 cells but have no effect on unstimulated release (Leclercq-Meyer, Marchand and Malaisse,1974); as with the β-cell the role of the MT-microfilament system in these cells is as yet unclear.

Microtubules and pancreatic exocrine secretion.

MT. are certainly present in the exocrine pancreas acinar cell having a similar pattern to that found in the pancreatic B-cell (Kern,1975). They originate in the outer nuclear membrane and course between the rough endoplasmic reticulum and between or around the Golgi complex finally inserting into the lateral and apical plasma membranes (Seybold, Bieger and Kern,1975). Initially MT. inhibitors, particularly colchicine, were thought to have no effect on acinar cell secretion (Benz et al,1972).

However, work on α-amylase release from the parotid gland threw suspicion on these results; since incubation of parotid slices with colchicine inhibited adrenalin-induced amylase secretions (Butcher and Goldman, 1972).

Vinblastine inhibits pilocarpine-induced acinar cell secretion in vivo if given 3 hours before stimulation. It also causes inhibition if given 2 hours after stimulation (Nevalainen,1975a). Vinblastine and vincristine both induce autophagocytosis of the exocrine pancreas suggesting that their effects on enzyme secretion could be due to their cytotoxic action which becomes evident after only 3 hours (Nevalainen, 1975b). In vitro also, the effects of Vinca alkaloids cannot be attributed directly to their effects on MT. (Williams and Lee,1976). However, Seybold, Bieger and Kern (1975) have shown that vinblastine induces a dose responsive inhibition of protein discharge in isolated pancreatic lobules. Protein release is inhibited by between 40% (10^{-6}M) and 90% (10^{-4}M) but protein synthesis is only inhibited by about 20% (10^{-6} - 10^{-5}M) and 55% (10^{-4}M). By contrast it has been shown that dibutyryl c-AMP has a secretagogue action on the exocrine pancreas in vitro which is potentiated by vinblastine, however spontaneous enzyme release is unaffected (Stock, Lannay and Grenier,1977).

Colchicine only inhibits protein synthesis at 10^{-2}M concentration yet concentrations between 10^{-3} and 10^{-5}M induce a 50% reduction over controls in the discharge of newly synthesised protein (Seybold, Bieger and Kern,1975). Colchicine and vinblastine at all doses inhibit amylase release by 30% and prevent movement of protein radioactivity from microsomal cell fractions to the zymogen granules. The MT-poisons also delay peak radioactivity in the smooth microsomal fraction (Golgi). Reduction in secretory activity induced by colchicine and vinblastine occurs concomitantly with ultrastructural changes in the acinar cell.

Secretory material is deposited in the cisternal space of the rough endoplasmic reticulum as small crystals, paracrystals of tubulin are also induced by vinblastine. Colchicine produces an enlargement of condensing vacuoles in the Golgi complex (Seybold, Bieger and Kern,1975).

Cytochalasin B disrupts the terminal web, resulting in the loss of microvilli from the acinar lumen (Seybold, Bieger and Kern,1975). This produces an inhibition of acinar cell secretion (Williams,1977), although at low concentrations resting secretion may be increased (Bauduin et al,1975). Yet again cytochalasin B is an example of an inhibitor which may have effects other than those on the microfilaments. Although it does not effect protein synthesis in the acinar cell (Seybold, Bieger and Kern,1975), it does inhibit monosaccharide transport (Bauduin et al,1975).

As was seen in the endocrine pancreas the role of the MT-microfilament system in the acinar cell is a complex one which is unlikely to be fully delineated using unspecific poisons. However, MT. certainly appear to have a role in the secretory process, either in transferring protein from the rough endoplasmic reticulum to the Golgi complex or in transporting mature zymogen granules from the Golgi complex to the plasma membrane. The role could be simply a structural one providing the scaffolding to support contractile events or they could provide the actual contractility themselves. Microfilaments may be involved as the contractile component of the system or may simply act as a barrier to exocytosis in the unstimulated cell. The model system proposed for the endocrine pancreas could also explain acinar cell secretory transport, in both cases more work is necessary to discover how the secretory products interact with the MT-microfilamentous system.

In vitro studies of microtubule associations.

In vivo biochemical and morphological studies have shown MT. associated with a wide range of cellular organelles, in vitro studies enable confirmation to be made of these observations and isolated study to be carried out on them. Early studies on the mechanism of insulin release across pancreatic β-cell membranes led to in vitro mixing of β-granules and membrane fragments. This interaction caused the release of free insulin into the incubation medium (Davis and Lazarus,1976). A later study by Formby and Capito (1977), using plasma membranes and β-granules from non-inbred ob/ob mice and measuring insulin sedimenting with the plasma membranes, showed that glucose, ATP or Ca^{2+} alone or in combination failed to release insulin from the membranes but c-AMP or phosphoenolpyruvate did affect release. A means of studying membrane and MT. subunit interactions was proposed in 1975 (Becker, Oliver and Berlin) but has not been much used, it involved the labelling of membrane and subunits with different fluorescent moieties such as fluorescein and rhodamine.

As was mentioned previously MT. are often associated in vivo with virus particles. Luftig and Weihing (1975) have shown that in vitro adenovirus particles will specifically bind to MT. to within ± 4nm of their edge; other particles such as reovirus, latex and coliphage F2 bind only at random and directly at the MT. edge. Adenovirus adhesion appears to involve the hexon capsomere rather than the pentons or fibres. Babis et al,(1979) have shown that MT. from chick or rabbit brain or HeLa cells bind to reovirus type 1 in preference to type 3. This binding in vitro is inhibited by reovirus type-1-specific antiserum and is correlated with the presence of a minor outer capsid polypeptide. The greater MT. binding of type 1 reovirus particles appears to reflect the increased accessibility of certain of its outer capsomeres, and thereby polypeptides,

to the MT. since type 1 reovirus types appear to have more distinct capsomeres than type 3 which appear diffuse in the E.M. These *in vitro* observations are confirmed *in vivo* when reovirus infected cells are examined; type 1-infected cells contained about eight times as many viral factories as type 3-infected cells, these factories were characterised by extensive viral particle-MT. associations. MT. *in vitro* also show a high affinity for DNA mediated by the M.A.P's (Corces et al,1978), M.A.P's binding better to eukaryotic - rather than phage - DNA. This M.A.P. affinity is more effective for satellite DNA (short base sequences in long tandem repeats close to the centromere) than bulk DNA.

Thus *in vitro* studies can throw light on simple interactions which are complicated by other outside associations *in vivo*. As far as the MT-microfilament system goes a number of studies have been done on MT-microfilament associations and microfilament-secretory granule associations but little consideration has been given to *in vitro* MT-granule associations. Izant and McIntosh (1977) showed that T. could be co-extracted with actin (the microfilament protein) from CHO cell culture using a copolymerisation procedure with brain T. Griffin and Pollard (1978 a,b) have subsequently studied the interaction of actin and MT. proteins *in vitro*. Using low shear viscometry methods they have shown that the viscosity of the combined actin and MT. is ten to twenty times greater than the sum of their viscosities alone. MT-actin mixtures are thixotropic, their viscosities increasing with increasing protein concentration and time of incubation at 37°C. (optimal polymerisation temperature). In addition MT. sediment with actin conjugated to agarose beads. Association of actin with T. lacking M.A.P's (assembled in DMSO) produce mixtures with viscosities approaching the sum of their viscosities. M.A.P's alone also cause an increase in actin viscosity. Thus it seems

likely that the MT-actin microfilament associations, demonstable ultrastructurally, are mediated by the M.A.P's; they are also inhibited by ATP and related compounds but enhanced by c-AMP. Muscle actin and M.A.P's, particularly M.A.P-2 form discrete bundles in vitro (Sattilaro, Dentler and LeCluyse,1981). These bundles, 26nm in diameter are composed of 20% M.A.P-2 with 80% actin (by weight) and have 28nm-spaced striations and 12nm length projections at 28nm intervals. Their formation in inhibited by ATP, β, γ -methylene-ATP and pyrophosphate but not by AMP, ADP or GTP. ATP dissociation produces actin filaments with attached M.A.P-2 particles along their length. MT. proteins also bind to myosin; Fujii and Tanaka,(1979) have shown that both T. and gross MT. proteins bind to rabbit skeletal muscle myosin and that they will inhibit the superprecipitation of actomyosin as well as preventing actin activation of myosin Mg^{2+}ATPase, these effects being enhanced by preincubation of T. with the myosin. Rods of skeletal muscle myosin are dispersed by brain MT. (Hayashi, 1979), decorating the MT. and eventually depolymerising them by its Mg^{2+}- GTPase activity.

Actin-granule interactions in vitro have been shown using muscle actin and pancreatic β-cell granules (Howell and Tyhurst,1978). Dilute (0.2 - 0.7mg/ml) solutions of actin prevent the sedimentation by centrifugation of isolated β-granules, Ca^{2+} reduced this effect but ATP enhanced it. The authors suggested that such an association may reflect a role for actin in the cell web or cytoplasmic transport of granules via the MT-microfilamentous system.

Whatever model mechanism is considered to function in moving secretory granules within the cell be it the sliding filament model, the assembly-disassembly model, the sol-gel system or the MT-microfilamentous system, in each case MT-granule interaction is necessary at some stage. However, as yet very little work has been done on the interaction of secretory

granules with MT. in vitro, observations have been based on in vivo ultrastructural studies. One study has been done in vitro by Sherline, Lee and Jacobs,(1977). This involved the incubation of brain MT. with porcine pituitary secretory granules in an MT. buffer. MT. were then pelleted by centrifugation and the amount of MT. protein left in the supernatant measured by colchicine binding assay. The amount of MT. protein pelleting increased as a function of the number of granules added. This interaction was inhibited by nucleoside triphosphates but enhanced by adenosine 5'-monophosphate and adenosine. Depolymerised tubulin (kept unpolymerised at 37°C by addition of 6mM Ca^{2+}) did not bind to the granules but the M.A.P's did do so. Sherline et al also showed that isolated secretory granule membranes sedimented lower in a discontinuous sucrose density gradient when MT. were added and concluded that MT. have a role in the intracellular movement of secretory granules possibly by way of M.A.P. cross-bridges linking the T. backbone and granule surface.

An analogous association of MT. has been demonstrated by Caron and Berlin (1979) with manufactured phospholipid vesicles. Proteins from high speed supernatants of brain homogenates were selectively adsorbed onto unilamellar dimyristoyl phosphatidylcholine liposomes. The composition of adsorbed proteins was similar to that of MT; the adsorption inducing stacking of the liposomes into multilamellar structures. This stacking was, however, demonstrated to be due to the metal staining (Melchior, Hollingshead and Cahoon,1980). Phosphotungstic acid or sodium tungstate staining both induced the formation of a network of multilayered lamellae while uranyl oxalate maintained the unilamellar dimyristoyl phosphatidylcholine or egg lecithin vesicles; this was regardless of the sample concentration, incubation time, temperature or presence of T. Nonetheless T. was still adsorbed onto the bilayer vesicles.

Caron and Berlin,(1980) have since characterised this T. binding by showing that MT. proteins bind to unilamellar phospholipid vesicles made from both acidic (phosphatidyl serine) and neutral (Phosphatidyl choline) phospholipids. Binding to acidic phospholipid vesicles is irreversible causing inhibition of MT. assembly whereas binding to neutral phospholipid vesicles is reversible, 'desorption' being driven by MT. assembly.

Phospholipid MT. protein binding has also been demonstrated using membranes from rat liver fractions (Azhar and Reaven,1980; Reaven and Azhar,1981). Membrane fragments from the liver cell golgi complex, plasma membrane, rough and smooth endoplasmic reticulum, nucleus and mitochondria all inhibited MT. assembly by binding MT. protein. Binding to golgi, plasma membrane and mitochondrial membranes was particularly strong; this was shown to be due to the presence in the membranes of negatively charged phospholipids (diphosphatidyl-glycerol, phosphatidyl serine and cardolipin). MT. binding to clathrin-coated vesicles has also been demonstrated in vitro. This association seems to be due to M.A.P's, particularly M.A.P-2. Coated vesicle MT. binding was stimulated between two and four times by c-AMP, since it stimulates in vitro M.A.P-2 phosphorylation, while ATP reversed binding. Solubilsed coated vesicles bound to activated Sepharose beads bound M.A.P's which were released by ATP whereas Separose-bound B.S.A. failed to bind M.A.P's although it non-specifically bound T. (Sattilaro, LeCluyse and Dentler,1980).

The separation and estimation of depolymerised tubulin and assembled microtubule tubulin in tissues.

Sherline, Bodwin and Kipnis (1974) developed a sensitive and simple procedure for assaying T. Prior to this the sepearation of T-bound-and free-colchicine had to be done using gel filtration or DEAE cellulose filter paper adsorption (Weisenberg, Borisy and Taylor, 1968; Borisy,1972), both methods being inapplicable to multiple determinations. The modification necessary to enable large numbers of rapid T. assays was to remove the free colchicine by adsorption onto activated charcoal. To enable estimation of the levels of T. in the cellular MT. pool and as free unpolymerised T. Pipeleers et al, (1977) modified this colchcine-binding T. assay by altering the conditions under which tissues were homogenised. MT. were stabilised using a homogenisation buffer containing glycerol and dimethylsulphoxide, while the unpolymerised T. was collected in a sucrose-phosphate buffer. Using this procedure on rat liver cells showed that in the fed state about 31% of cellular T. was in the polymerised form.

Consequently Pipeleers, Pipeleers-Marichal and Kipnis (1977) went on to examine the levels of polymerised and depolymerised T. in rat and mouse liver, rat islets, human lymphocytes and platelets. The percentage of total T. present as MT. varied from about 30% in the liver of fed rats to 89% in human platelets; these levels were modified by fasting and feeding in the case of the liver, by lectin-stimulation in the case of the lymphocytes and by storage at $4^{o}C$ in the case of the platelets. Thus the total cellular T. content and degree of polymerisation seems to be modulated by a vaiety of physiological factors. Ostlund, Leung and Hajek (1979) have measured the two T. pools in a variety of tissue culture cells and shown that MT. contained between 34 and 41% of total cell T; this was reduced to 6% of the expected level upon colchicine

or cold treatment. More recently the same authors (1980) have demonstrated that the levels of MT-derived T. in cultured, diploid human skin fibroblasts rose as the cultures neared confluence while the levels of total cell T. fell. Cold, concanavalin A and cytochalasin A all reduced the MT-derived T. component but cyclic nucleotides, ascorbate, glucose, insulin, Ca^{2+} or A23187 all failed to effect MT. assembly. Ben-Ze'er, Farmer and Penman (1979) have suggested that T. synthesis is controlled in cultured fibroblasts by the action of unpolymerised T. on the levels of translatable T-messenger RNA.

Montague, Howell and Green (1976) have correlated the level of insulin release from the endocrine pancreas with the size of the T. subunit pool. Vinblastine and cold treatment increased the T. pool while D_2O reduced it. Stimulated insulin release, by glucose or raising intracellular c-AMP levels, produced a reduction in the isolated islet T. subunit pool whereas reducing insulin release, by removal of Ca^{2+} from the incubation medium, increased the pool. These observations have been confirmed by McDaniel et al,(1980) by studying the levels of polymerised T. during glucose-induced biphasic insulin release in isolated islets. They found that at 2.5 minutes during the rapid onset of insulin release the level of polymerised T. altered significantly from 24% to 33%. This was followed by a reduction correlated with the disappearance of first phase release. Second phase insulin release was then correlated with a second increase in polymerised T. over the prolonged period of release. The enhancing effect of cytochalasin B on insulin release at submaximal glucose levels was also correlated with increased islet polymerised T. Thus it seems that the biphasic pattern of insulin release is well correlated temporally with the MT, free T. equilibrium.

The levels of polymerised T. have also been measured under various

hormonal conditions, in the medial basal hypothalamus (MBH) and adeno-hypophysis (AH) of adult rats (Valenti et al,1980). LH, FSH or prolactin treatment depressed the level of total MBH. T. in orchidectomized rats while FSH and prolactin augmented it in the AH. Post-castration there was a negative correlation between serum LH and total or polymerised MBH. T, whereas in the AH the correlation was positive. Oestradiol-progesterone treatment of oophorectomized rats produced a significant positive correlation between serum LH and polymerised T. in the AH but not the MBH.

This and other experiments discussed above indicate that the MT-free T. equilibrium is considerably altered by the secretory state of its cellular environment. Such alterations seem to be readily detectable by using a ^{3}H-colchicine binding assay for the two T. pools. However, in measuring these pools in mammary glands, Pencek and Loizzi (1980) have detected a considerable residue of free colchicine after charcoal treatment during the T. assay. Between 40% and 75% residue was present in the MT-stabilising buffer while 15% to 30% was present in the depolymerisation buffer. Removal of this free colchicine decreased the estimated total T. levels while doubling the levels of polymerised T. This and other problems associated with the ^{3}H-colchicine binding assay have led a number of workers towards an alternative method for detecting changes in cellular T. levels. In particular the radioimmuno-assay method has become prominent. Le Gu ern et al (1979) have described a method capable of detecting quantities of T. as low as 50pg. Using such a radioimmunoassay, Hiller and Weber (1978) have demonstrated that tissue culture cells (mouse L cells, mouse 3T3 cells and chick embryo fibroblasts) contain T. corresponding to between 2.5% and 3.3% of their total protein. These cells treated with virus particles showed no change in their T. content while some transformed brain cells (glia

tumour cells and neuroblastoma cell lines) had lower T. levels than normal brain tissue. The radioimmunoassay method not only has advantages of sensitivity for T. detection but can readily be adapted for the detection of other MT. proteins such as M.A.P's.

Isolation of exocrine pancreas zymogen granules.

Zymogen granules were isolated from whole rodent pancreas (usually 20 - 30 mice were used since rat pancreas was found to be too high in collagen for E.M.studies) by two main methods.

Preparations for E.M.studies were made using an adaption and combination of the methods of Vandermeers-Piret et al (1971) and Kirshner et al (1973). The pancreas was removed from freshly killed (by suffocation in ether or cervical dislocation) animals and placed in buffer Tris-maleate-10mM; Sucrose-0.3M; pH 6.5 with HCl) on ice at $4^{o}C$; zymogen granules were extracted within an hour of pancreas removal. The combined pancreas material was homogenised in fresh, 20% weight to volume, buffer using an M.S.E. motor-driven, steel homogeniser followed by a Jencons teflon-glass, hand-operated homogeniser.

The zymogen granules were separated from the homogenate by differential centrifugation in an M.S.E. 'Mistral 4L' centrifuge cooled to $4^{o}C$. An initial 10 minute spin at 400g sedimented out the cell debris and nuclei, the supernatant was then centrifuged at 1,000g for 10 minutes to collect the zymogen granules. This granule pellet was resuspended in fresh buffer and placed on ice while the 400g pellet and 1,000 supernatant were recombined, rehomogenised and recentrifuged to extract more granules; this cycle being repeated up to three times or until the yield of granules became minimal. The resultant crude suspension of zymogen granules were combined and washed several times.

Further purification was carried out using a Urografin solution. Urografin is an X-ray contrast medium containing a mixture of the sodium and methylglucamine salts of N-N'-diacetyl-3-5-diamino-2,4,6--triodobenzoic acid in the ratio of 10 : 66 in aqueous solution. The

zymogen granule suspension was layered over a 30% Urografin solution (Schering Chemicals Ltd), containing 0.15m sucrose, in the proportion 2 : 1, granule suspension : Urografin solution. The tubes containing the two layers were then centrifuged at 6,000g for 15 minutes and 64,000g for 45 minutes in an M.S.E.'Superspeed 50' ultracentrifuge using the 10 x 10ml or 8 x 50ml angle rotors and cooling to 4°C. After centrifugation the upper layer was carefully removed using a pasteur pipette until the granule layer was reached. This was also removed and given several washes in fresh buffer. The clean zymogen granule suspension was then stored in buffer on ice for experimental use.

A more rapid method of isolation of zymogen granules was also used on freshly killed pancreas material (modified from Ermak and Rothman (1978)). Collected pancreas were homogenised in ten times weight to volume of ice-cold buffer (Trismaleate-10mM; Sucrose-0.3M; pH 5.5 with Tris base) with five strokes of a Jencons teflon-glass, hand-operated homogeniser. The homogenate was centrifuged at 320g for 10 minutes in an M.S.E.'Mistral 4L' centrifuge cooled to 4°C. The resultant supernatant was then removed and recentrifuged at 900g for 10 minutes at 4°C. The pellet produced by the second centrifugation consisted of an upper tan layer of mitochondria and a lower white layer of zymogen granules. The mitochondria were removed by two to three swirls of one to two ml of buffer, finally swabbing the pellet with cotton wool to remove all the tan layer. The zymogen granules were then resuspended in 20% of the original volume of buffer and recentrifuged at 600g for 10 minutes at 4°C. Any remaining mitochondria were swabbed off the pellet. The extraction process was finally repeated with the retained 320g pellet and 900g supernatant and the washed pellets combined in fresh buffer, again about 20% of

the original volume.

All zymogen granule suspensions were used within 12 hours, storage being at $4^{\circ}C$ in clean buffer. On some occasions zymogen granule 'ghosts' were prepared by resuspending the granules in MT. isolation buffer. This transfer from isotonic sucrose buffer to a buffer of greater osmotic pressure caused the granules to lose their contents leaving 'ghost' membranes behind. These 'ghosts' were particularly useful for electron microscopy.

Isolation of MT. proteins.

MT. protein was purified from mammalian brain by a modification of the assembly-disassembly method of Shelanski et al (1973). This entails the disassembly of MT. and the release of their constituent proteins into solution so that they can be separated from other insoluble brain components by ultracentrifugation. The resultant MT. protein solution can then be further purified by reassembling the MT. proteins into MT. which are collected by ultracentrifugation leaving soluble contaminant proteins in the supernatant. Further purification can be achieved using several cycles of assembly-disassembly in fresh buffer.

The material used for MT. protein isolation was in the main slaughter-house porcine brain although murine brain was also used in some cases, using similar procedures. Pigs were killed by electrical stunning combined with throat slitting, mice and rats either by ether suffocuation or more usually cervical dislocation. In all cases brains were placed on ice within 30 minutes of animal death and treated within one hour. Two porcine (30 - 40 murine or 4 - 5 rat brains) were homogenised in an equal weight to volume of MT. isolation buffer (2 (N-morpholino) ethane sulphonic acid (MES)-100mM; Ethylene glycol bis (B-aminoethyl ether) N, N, N', N',-tetracetic acid (EGTA)-1mM; 0.1mM Ethylene-diamine tetracetic acid (EDTA); 0.5mM-$MgCl_2$; pH 6.4 with HCl) to which had been added 1mM ATP. Both ATP and GTP were stored at -20°C in a 50mM stock solution, suitable volumes being added to buffers as required. Homogenisation was carried out using an M.S.E. motor-driven, steel homogeniser and/or Jencons teflon-glass, hand-operated homogeniser. The homogenate was placed on ice for about one hour, reaching a temperature of 4°C, and then centrifuged at 75,000g for 60 minutes at 4°C.

Ultracentrifugation was carried out on an M.S.E. 'Superspeed 50' ultracentrifuge with either a 10 x 10ml or 8 x 50ml angle rotor using polypropylene tubes. Rotors were always pre-cooled or pre-warmed to the centrifuge running temperature.

After the initial centrifugation the supernatant was treated in one of two ways: in earlier preparations an equal volume of MT. glycerol isolation buffer was added to the supernatant, this was identical to isolation buffer with the addition of 8M glycerol; later preparation supernatants were not diluted. In each case 1mM GTP was added to the supernatants and they were incubated at 37°C in a water bath for 20 to 30 minutes. It was later found that the GTP concentration could be reduced to 0.1mM without any significant reduction in MT. protein yield. Pellets were discarded. The warmed supernatant was centrifuged at 75,000g for 45 minutes at 20 to 30°C (the rotor was warmed to 37°C and the centrifuge run without cooling).

The resultant supernatant was removed and discarded. The pellets consisting of impure MT. were resuspended in MT. isolation buffer with added GTP (1mM or 0.1mM). The amount of buffer used for resuspension depended upon the size of the pellets but was normally in the region of 10% of the original volume of the first supernatant. The pellets in buffer were homogenised in a hand-operated, teflon-glass homogeniser and left on ice at 4°C for one hour. The resultant MT. protein solution was then again purified to remove insoluble contaminant proteins by centrifugation at 75,000g for one hour at 4°C. These 'one-cycle' MT. proteins were then normally stored overnight in 8M glycerol at -20°C.

MT. were reclaimed for further purification by adding an equal volume of MT. isolation buffer with GTP (1mM or 0.2M), warming to 37°C for 30 minutes and centrifuging at 75,000g for 45 minutes at 20 to 30°C. The resultant pellets of MT. were resuspended in an approximately

equal weight to volume of MT. isolation buffer (with 1mM or 0.1mM GTP), homogenised and placed on ice at $4^{o}C$ for one hour. This solution was then centrifuged at 75,000g for one hour at $4^{o}C$ to remove contaminant insoluble proteins. These 'two-cycle' MT. proteins were stored for experimental use in 8M glycerol at $-20^{o}C$ for between one day and one month.

Typical yields of total 'two-cycle' MT. proteins were in the region of 105mg in 3ml of buffer from two 75g brains.

Separation of microtubule proteins.

Purified T. was separated from gross MT. proteins isolated from porcine brain using the method of Weingarten et al (1975). This involved the use of a cellulose phosphate cation exchange chromatography column.

Prior to use phosphocellulose (Whatman) was washed sequentially with 50% ethanol, 0.5M NaOH, distilled water, 0.5M HCl, and distilled water again. The phosphocellulose was stirred for 30 minutes in each washing solution, with the exception of distilled water which was used in excess until its pH remained constant, each solution then being removed by filtration on a porcelain vacuum filter. Care was taken at each stage to avoid allowing the phosphocellulose to dry out. After washing, the phosphocellulose was equilibrated in ten times normal strength MES-EDTA buffer, pH 6.4 (see below), and then packed into either an LKB column (16mm x 600mm) or a disposable Pharmacia column (80mm x 15mm). In each case the columns were then re-equilibrated with normal strength MES-EDTA buffer (MES-25mM; $MgCl_2$-0.5mM; 2-mercaptoethanol-1mM; EDTA-0.1mM; pH 6.4 with HCl) using at least three column void volumes.

'Two-cycle' MT. proteins, isolated from porcine brain, usually in one to two ml of MES-EDTA buffer, were placed on the top of the cation exchange column. In the case of the larger LKB column the void volume was found to be about 30 x 1.5ml fractions using blue dextran, so 40 such fractions were collected. Measuring the light absorbance of these fractions at a wavelength of 280nm. (on a Cecil Instrument 'CE 272 Linear Readout' spectrophotometer), showed the separated T. peak to typically occur around fractions 31 to 37. These fractions were pooled and dialysed against the required buffer using Scientific Instruments

Centre Ltd. Visking dialysis tubing. The M.A.P's retained by the cation exchange column were finally eluted by changing the elution buffer to MES-EDTA buffer with 1M NaCl. Absorption spectrophotometry at a wavelength of 280nm showed the M.A.P's to come off the column typically in fractions 30 to 38 after changing buffers. Once again these fractions were pooled and dialysed against the requisite buffer for experimental use.

To purify M.A.P's in larger amounts than was possible with the method outlined above, the procedure of Kuznetsov et al (1978) was adopted. This involved the heat-denaturation of porcine brain proteins leaving the thermostable M.A.P's available for straightforward isolation. Typically 3 pig brains were homogenised in an equal weight to volume of Imidazole-EDTA buffer (Imidazole-50mM; KCl-50mM; $MgCl_2$-0.5mM; EDTA-0.1mM; 2-mercaptoethanol-1mM; pH 6.7 with HCl) using an M.S.E. motor-driven, steel homogeniser. This homogenate was then cooled to $4^{\circ}C$ and clarified by ultracentrifugation at 11,000g for 30 minutes at $4^{\circ}C$. To the supernatant was added an equal volume of Imidazole-EDTA buffer containing 2M KCl; a final KCl concentration of 1M being used to prevent the co-precipitation of M.A.P's during thermoprecipitation of the bulk of the brain proteins. The diluted supernatant was then split into less than 150ml portions and placed in a water bath at $95^{\circ}C$ for 8 to 10 minutes stirring occasionally. During this time the solution reached 82 to $87^{\circ}C$; the resultant aggregate protein was cooled to $4^{\circ}C$, pelleted by ultracentrifugation at 11,000g for 10 minutes and discarded.

To the clear supernatant was added saturated ammonium sulphate solution up to a final concentration of 41% saturation. This was then left for 30 minutes and the precipitated protein collected by centrifugation at 3,000g for 5 minutes at $4^{\circ}C$. The resultant pellet was

resuspended in Imidazole-EDTA buffer, dialysed against the same buffer (three times for 12 hours at 4°C) and clarified by ultracentrifugation at 31,000g for 30 minutes at 4°C. This clear solution was then put onto a cellulose phosphate cation exchange column, prepared as before and equilibrated with Imidazole-EDTA buffer. The column was washed overnight with Imidazole-EDTA buffer and then changed to Imidazole-EDTA buffer containing 0.5M NaCl collecting M.A.P's in fractions 34 to 42 (as shown by absorption spectrophotometry at a wavelength of 280nm). These fractions were pooled and dialysed against Imidazole-EDTA buffer (three times for 12 hours at 4°C) and then put onto a small volume (~10ml.) phosphocellulose column to concentrate the protein. This small column was washed with about five void volumes of Imidazole-EDTA buffer and then the M.A.P's were collected in 0.5M NaCl in Imidazole-EDTA buffer.

Three porcine brains typically yielded about 2ml of concentrated M.A.P. solution at about 3 to 4 mg/ml. This M.A.P. was finally dialysed against the requisite buffer for experimental use. Purified T. solution from one porcine brain was typically 2ml at a concentration between 1 to 2 mg/ml. All purified proteins in buffer were rapidly frozen in liquid nitrogen and stored at -20°C.

Separation and estimation of assembled and disassembled Tubulin from microtubules of mouse exocrine pancreas.

Both polymerised and depolymerised T. from murine exocrine pancreas were isolated using methods based on those of Pipeleers et al (1977). This involved the stabilisation of assembled MT. using a buffer, containing both glycerol and Dimethylsulphoxide (DMSO), and the separation of these from unpolymerised T. by ultracentrifugation.

Into the requisite number of ultracentrifuge tubes were pipetted 500µl of MT. stabilising buffer (MTS) (Glycerol-50%; DMSO-5%; $MgCl_2$-0.5mM; EGTA-1mM; EDTA-0.1mM; MES-100mM; GTP-0.5mM; pH-6.4 with HCl) the whole then being weighed. Whole pancreas samples were removed from freshly killed mice and placed, one per tube, into the MTS buffer kept at 4°C on ice. The tubes were then reweighed, the weight of pancreas determined and MTS buffer added in the proportion one to ten (tissue weight : volume). The tissue and buffer were decanted into a Jencons teflon-glass, hand-operated homogeniser and homogenised until no tissue pieces were visible (normally 10 - 15 strokes). The pancreas suspensions were then transferred to their centrifuge tubes and within an hour of homogenisation spun at 100,000g, for 45 minutes at 4°C. The resultant supernatants (supernatant 1) containing depolymerised T. were saved for T. estimation and the pellets washed each with 200µl of MTS buffer.

After washing the pellets were resuspended in one to ten tissue weight to volume of MT. depolymerising buffer (TS) (Sucrose-0.25M; $MgCl_2$-0.5mM; MES-100mM; GTP-0.5mM; Bovine serum albumin (BSA)-2%; pH-6.4 with HCl). These suspensions were left for one hour at 4°C and then centrifuged at 100,000g for 30 minutes at 4°C. The resultant supernatants (supernatant 2) containing T. derived from assembled MT. were saved for T. estimation and the pellets again resuspended in

1,000µl of TS buffer. These were recentrifuged as previously to yield supernatant 3.

The three supernatants derived from each pancreas sample were all diluted with an equal volume of the other requisite buffer to give a 50 : 50 mixture of MTS and TS buffers with a final pancreas concentration of one to twenty (tissue weight to volume). In some cases the supernatants were cloudy with fat deposits and were cleared by passing through an 0.8um pore Millipore filter. Clear supernatants were then assayed for T. using the ^{3}H-colchicine binding assay; the blank value for this being the mean d.p.m. derived from the third supernatants. All sample T. values were given as µg/ml of either polymerised (supernatant 2) or depolymerised (supernatant 1) pancreas T.

Estimation of protein concentrations.

The total protein concentrations of MT. protein solutions and other samples were estimated using a modification of the method of Lowry et al (1957). This involves two main reactions, copper binding to the protein in alkaline solution and reduction of a phosphomolybdic-phosphotungstic reagent (Foline-Ciocalteu phenol reagent) by the copper-treated protein to produce a blue colour which is proportional to the protein concentration, at least at low concentrations.

Initially a set of serial dilutions were prepared from a stock solution of 10mg/ml crystalline bovine serum albumin (Sigma). This stock was kept at -20°C and used within 2 weeks to prepare such sets of standards. Eight standards were prepared in a dilution series from 10mg/ml to 0.08mg/ml BSA in either distilled water or the relevant buffer together with a blank containing no protein. For the assay 200µl of each of these standards and the blank were placed in separate tubes alongside similar tubes containing 200µl of unknown samples.

Before the assay an alkaline copper solution was prepared from aqueous stock solutions of 2% Na_2CO_3 in 0.1N NaOH, 1% $CuSO_4\ 5H_2O$ and 2% potassium or sodium tartrate in the proportions 100 : 1 : 1 respectively. This alkaline copper solution was used for a day then discarded. 1ml of this solution was added to each standard, blank and sample, mixed thoroughly and allowed to stand for 10 minutes at room temperature. The Folin-Ciocalteu phenol reagent was diluted to a stock solution 1N in acid (~2 fold) on the basis of a titration against NaOH to the phenolphthalein indicator end point. Addition of 100µl of this stock phenol solution was carried out, rapidly with mixing, to each standard, blank and sample. After being left for 30 minutes at room temperature for the blue colour to develop, the tubes were all diluted with 5ml of

distilled water, mixed and their absorbance at a wavelength of 740nm against the blank, measured using a Beckman 'DB' spectrophotometer or a Cecil Instrument 'CE 272 Linear Readout' spectrophotometer.

These absorption readings for the standards were plotted against their known protein concentrations and the resultant curve used to find the protein concentrations of the unknown samples. In some cases the assays were redone more accurately by diluting the unknown samples to ensure that their absorption values lay on the early linear part of the curve.

The ^{3}H-colchicine binding assay for tubulin.

The property of colchicine to bind specifically to T. was used as the basis for this radioassay based on the method of Sherline et al (1974). Initially a stock solution of ^{3}H-colchicine was prepared using a 250μCi batch in 0.5ml of ethanol supplied by the Radiochemical Centre, Amersham. 250μCi of ring-A-4-^{3}H-colchicine was made up to 5ml with ethanol and stored at -20°C for T. assays.

A fresh 0.121mM solution of cold colchicine was prepared for each assay, to which was added (in the proportion 1 to 9, ^{3}H to cold) stock ^{3}H-colchicine, to produce a labelled, 0.11mM, aqueous colchicine solution. Sufficient volume being prepared for the required number of assay samples, blanks and standards usually in triplicate. 100μl of sample were pipetted into plastic, conical-based centrifuge tubes. Blanks and standards were prepared containing 100μl of the relevant buffer. To each tube was added 10μl of the labelled colchicine solution; all were thoroughly mixed and placed in a water bath at 37°C for 90 to 120 minutes, all tubes being similarly incubated. After this time the binding was stopped by adding 1ml of a 2mg/ml distilled water-activated charcoal (Sigma) suspension to each tube, with the exception of the standard to which only 1ml of distilled water was added. The whole was then mixed, allowed to stand for 10 minutes and centrifuged at 2,200g for 10 minutes at room temperature. A 500μl aliquot of the resultant supernatant for each tube was added to 7ml of Biofluor scintillation cocktail (New England Nuclear) ready for liquid scintillation β-counting.

The amount of colchicine bound to T. was calculated from the β-counter results by the following formula :-

$$\frac{\text{Sample (dpm)} - \text{Blank (dpm)}}{\text{Standard (dpm)} - \text{Blank (dpm)}} \times 1.1\text{nmoles}$$

By assuming that at saturation 1mole of colchicine was bound per mole of tubulin and that the molecular weight of T. was 110,000 daltons, the concentration of T. in each sample was calculated in µg/ml. Direct T. concentrations were obtained from β-counter d.p.m's by the use of a computer programme (see the Appendix). Initial testing of the ^{3}H-colchicine binding assay was carried out prior to use as experimental samples (see the Appendix); optimum parameters were thus selected for sample estimation.

Liquid scintillation counting.

To ascertain the amount of β-radiation emitting ^{3}H-colchicine in samples for T. estimation, liquid scintillation counting was carried out using the Packard 'Tri-Carb 2660' Liquid Scintillation System and 'Biofluor' High Efficiency Emulsifier Cocktail (New England Nuclear).

Before samples could be β-counted a quench correction curve was prepared using 'Biofluor' scintillant and stock ^{3}H-colchicine prepared for the T. assay. This curve was necessary to evaluate the efficiency of counting of individual samples and therefore to calculate their disintegration rate from their count rate. The stock ^{3}H-colchicine solution contained 250μCi in 5ml of ethanol; this provided us with an internal standard of 1.11×10^{8} d.p.m/ml on the date of analysis (since 1μCi produces 2.22×10^{6} d.p.m.). 100ul of this stock solution was added to 100ml of Biofluor scintillation cocktail, thoroughly mixed and apportioned into 10ml lots in glass scintillation vials. These 'quench standards' were then pre-counted to ensure that they each contained similar activity. Each standard was then quenched using differing amounts of formic acid from 0ml to 1.5ml, made up to equal volume and counted to estimate their counting efficiency and external standard channels ratio. (The ratio of the sample c.p.m. to the c.p.m. produced by an external gamma source - ^{226}Ra). The curve resulting from the plot of these values for each standard was later used to estimate the counting efficiency of unknown samples (from the ^{3}H-colchicine binding assay) and hence determine their d.p.m. from counted c.p.m. The values for a typical curve are shown overleaf

Standard No.	External standard ratio	%Efficiency
1	.2322	9.53
2	.3256	18.11
3	.3844	22.21
4	.4545	27.55
5	.5193	32.64
6	.5588	36.78
7	.6096	47.53
8	.6644	46.16
9	.7335	49.83
10	.7944	54.18

Each quench standard contained 1,068,930 d.p.m. (1.11×10^6 multiplied by 0.963 - the correction for radioactive decay of the standard).

Using the Packard 'Tri-Carb 2660' Liquid Scintillation System the above curve was stored in its in-built computer and used to automatically determine sample d.p.m's.

Radioimmunoassays.

Radioimmunoassays were used to determine the concentrations of T. and M.A.P's derived from both isolated brain MT. and tissue supernatants (exocrine pancreas). The basic methodology was based on that of Le Guern et al (1977).

Initially a set of serially diluted, replicated standards were prepared from a purified protein solution (of T. or M.A.P.) of known concentration. Three sets of standards were prepared each consisting of 13 serial dilutions of 100µl starting with the samefirst standard protein concentration. Typically a purified stock solution with a protein concentration of 2mg/ml was diluted 1 to 49 to give an initial standard concentration of 4,000ng/100µl and a final standard concentration, after serial dilution, of 1ng/100µl. All unknown samples were then suitably diluted (usually 1 : 9) and 100µl of each, in triplicate, placed in L.P.3 plastic radioimmunoassay tubes (Luckham Ltd.). Dilution of samples and standards was always with phosphate buffered saline (PBS) (NaH_2PO_4-10mM; NaCl-0.876%; Na azide-0.02%; pH 7.6 with HCl) containing 0.1% gelatine. In addition to the samples and standards in L.P.3 tubes, triplicate blank, background and total tubes were prepared; the blanks and backgrounds containing only 100µl of PBS.

Into each tube sample, standard, blank, background and total was added 50µl of diluted (usually 1 : 9) iodinated protein (^{125}I-M.A.P. or ^{125}I-T.) to compete for antibody binding with the 'cold' protein. The antibody solution (anti-M.A.P. or anti-T.) as rabbit antiserum was suitably diluted (usually 1 : 249) and 50µl of this diluted antiserum added to each tube with the exception of the total tubes and background tubes, the latter receiving 50µl of PBS. This antigen-antibody combination was capped thoroughly mixed and left for 24 hours at room

temperature.

After this period the second diluted antiserum (usually 1 : 19) was added, this was either goat anti-rabbit IgG or goat anti-rabbit whole serum (Miles-Yeda). This was diluted in EDTA-PBS (PBS + 0.05mM EDTA), EDTA being included to prevent complement interference with immunoprecipitation. 200µl of this second dilute antiserum was added to each tube with the exception of the total tubes. After a second incubation period of 24 hours at room temperature all the tubes were centrifuged at full speed in an M.S.E.'Mistral 4L' centrifuge for one hour at room temperature. The supernatant was drained off the resultant pellets and the insides of the tubes blotted dry with tissue paper. Finally all the tubes were counted for bound ^{125}I activity in counts per minute (c.p.m.) on an I.C.N. 'Gamma Set 500' gamma counter.

The percentage binding of ^{125}I was calculated for each standard and sample using the following formula :-

$$\frac{\text{Sample or standard (c.p.m.)} - \text{Background (c.p.m.)}}{\text{Blank (c.p.m.)} - \text{Background (c.p.m.)}} \times 100$$

The formulae of the lines for each of the three standard curves and the mean standard curve were computed using the plot of the logarithm of the standard protein (M.A.P. or T.) concentration against the percentage bound ^{125}I. The formula of the curve with the greatest correlation between these two parameters was then used to calculate the sample protein (M.A.P. or T.) concentration. All calculations were carried out using a computer programme. When it was found that background counts were excessively high a detergent, Triton X-100 (0.1%), was incorporated in the PBS used for dilution in the first incubation. This

in turn led to the final pellets having a tendency to break up, this was countered by incorporating potato starch (5mg/ml) in the EDTA-PBS used in the final incubation.

Initial, trial standard curves not used to assay samples are included in the Appendix.

Preparation of samples for the electron microscope.

1. Fixation

For routine study of MT. and zymogen granule material during experiments fixation and simultaneous negative staining were carried out using 1% uranyl acetate. A drop of the material for examination, in suspension, was placed on prepared Formvar or Nitrocellulose coated, copper 300 mesh E.M.grids and left for a few minutes to allow material to settle on the surface. Excess moisture was removed with a torn wedge of filter paper and the grid was then stained by running three to four drops of a 1% aqueous uranyl acetate solution over it. These stained grids were then left to dry in a dust free petri dish prior to examination in a T.E.M.

Conventionally sectioned material was fixed in one of two ways, prior to centrifugation or post-centrifugation. Pelleted material was fixed in 1% or 2% glutaraldehyde in either 0.05M phosphate buffer (pH 6.8) or MT. isolation buffer containing 8% tannic acid. Fixation prior to centrifugation was accoding to the method of Burton and Himes (1978). 5µl of unbuffered glutaraldehyde solution (25%) were added to each 0.5ml sample (final glutaraldehyde concentration - 0.25%) which were left for 5 minutes. After this time 1.5ml of 8% tannic acid in 0.05M phosphate buffer (pH 6.8) were added and the whole left for one hour at room temperature. Both pre- and post-centrifuged fixed material was washed in the appropriate buffer (MT. isolation or phosphate) for at least one hour and then post-fixed in 1% Osmium tetroxide as outlined below.

Fixed pellets which proved difficult to handle were embedded in 1.5% agarose. Molten agarose solution was mixed with the soft pellet and drawn up into a pasteur pipette, this was then forced out into

ice-cold buffer where it set into long ribbons which were cut into pieces $1mm^3$ for post-fixation.

2. Post-fixation

This was always carried out using 1% osmium tetroxide in the appropriate buffer. A glass vial containing 0.1g of OsO_4 crystals was cleaned in chromic acid to remove grease, scored with a diamond marker pen at one end and dropped into a grease-free 10ml measuring cylinder containing 2ml of buffer. The top of the measuring cylinder was sealed with Parafilm and shaken to break the vial. 8ml of buffer were then added to the cylinder and the whole left overnight for the crystals to dissolve. The resultant 1% OsO_4 solution, once filtered using grease-free glassware, was used for post-fixation; all steps being carried out in a fume cupboard.

Gluteraldehyde-fixed and washed material was placed in small capped jars and covered with the 1% OsO_4 solution for one hour; finally it was washed for a further hour in buffer ready for dehydration.

3. Dehydration

Post-fixed material was dehydrated by passing through a series of increasingly concentrated aqueous ehtanol. The drained material was covered with alcohol solution for five to twenty minutes then transferred to a more concentrated solution. The solutions used were 50%, 60%, 70%, 80%, 90%, 95% ethanol with two final changes of 100% ethanol for 30 minutes prior to embedding.

4. Embedding

All material was embedded in Spurr (1969) low viscosity epoxy resin:-

Ingredients (by weight)		Firm	Hard	Soft	Rapid cure	Long-life low viscosity
Epoxy resin	ERL-4206 :	10.0g	10.0	10.0	10.0	10.0
Flexibiliser	DER- 736 :	6.0	4.0	8.0	6.0	6.0
Anhydride hardener	NSA :	26.0	26.0	26.0	26.0	26.0
Accelerator	S-1 :	0.4	0.4	0.4	1.0	0.2
Cure time (70°C), hrs minimum :		8	8	8	3	16
Pot life, days :		3-4	3-4	3-4	2	7

Usually either 'firm' or 'long-life' mixtures were used, the quantity of DER-736 being increased to 7.0g to give a softer resin where needed. Resin components were mixed on a magnetic stirrer and left before use until all the air bubbles had left the mix.

The previously dehydrated material was transferred from the final alcohol to a 50/50 mixture of Spurr resin and 100% ethanol and left for more than two hours, after this period the material was put into pure resin and again left for over two hours. Two to three small ($< 1mm^3$) pieces of each sample were then put into a gelatine capsule filled with resin and left until they had completely absorbed resin and sunk to the base of the capsule. Each piece was then transferred to the base of a fresh gelatine capsule filled with fresh resin and labelled with a pencil-written square of paper in the top of the capsule. The resin was finally cured in an oven for at least 12 hours at 60 to 70°C.

Prior to sectioning the gelatine capsule was removed from the now hard resin blocks by placing in hot water for 10 to 15 minutes.

5. Sectioning

Resin blocks were trimmed before sectioning to remove superfluous resin around the embedded material. Under a dissecting microscope a razor blade was used to cut a trapezoidal shape to the sample end of

the blocks. Glass knives were prepared on an LKB knife maker with insulating tape boats sealed with dental wax. Thin sections were then cut on either an LKB ultramicrotome or a Cambridge 'Huxley' ultramicrotome. The sections when spread with xylene were between 90 and 60nm with corresponding interference colours of silver to grey.

6. Staining

All sections were collected on copper 300 mesh E.M.grids and double-stained with magnesium uranyl acetate and lead citrate stains. Drops of 1% magnesium uranyl acetate in 50% aqueous ethanol were placed in a covered cavity slide together with two to three section-bearing grids and left for 30 minutes at 60°C. The grids were then transferred to three changes of distilled water before lead citrate staining.

Lead citrate stain was prepared by mixing 1.33g of lead nitrate and 1.76g of sodium citrate in 30ml of distilled water for 30 minutes. The white precipitate of lead citrate was then dissolved by adding 8ml of 1N sodium hydroxide and making the whole up to 50ml. Drops of this stain were then placed into a wax-lined petri dish, surrounded by sodium hydroxide pellets to absorb CO_2 and prevent the formation of insoluble lead carbonate precipitates. Uranyl acetate stained grids were placed on the stain drops and the covered petri dish left at 60°C for 20 minutes. Upon removal the grids were washed in 0.02N sodium hydroxide, to remove any lead carbonate precipitates, in three changes of distilled water and left covered to dry on filter paper.

7. Electron microscopy

All negatively stained grids and conventional sections were examined in either an A.E.I. E.M.6B or J.E.O.L. JEM 100B electron microscope.

8. Critical-point drying of samples for S.E.M.

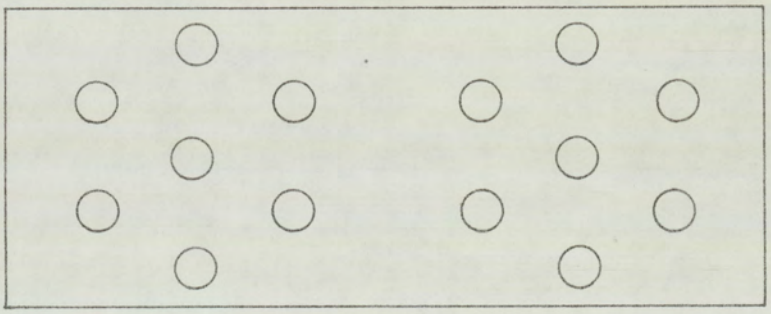
Material for examination in the scanning electron microscope was critical-point dried in a Polaron critical-point dryer. Conventionally dehydrated material in 100% ethanol was placed into two changes of amyl acetate for at least one hour. The critical-point dryer was cooled to below 20°C, by passing iced water through its outer casing, and the samples were then placed in wire-mesh receptacles within the sealed dryer. Liquid CO_2 was passed into the body of the dryer until it was almost full, the CO_2 was then flushed through for several minutes maintaining the level of liquid CO_2 at a constant. When the amyl acetate vapour ceased to pass out of the dryer in the exit vapour the inlet and outlet valves were closed and the whole left for 30 minutes. After this time a second liquid CO_2 flushing was carried out and the dryer resealed. The water in the jacket of the dryer was then heated until the meniscus of the liquid CO_2 disappeared (at about 48°C) leaving only CO_2 vapour. The outlet valve was finally opened to allow the CO_2 to slowly escape; once at atmospheric pressure the samples were removed.

Small broken pieces of critical-point dried sample were stuck to S.E.M. stubs and kept dry under vacuum. Before examination they were sputter coated with gold using an Edwards 'Speedivac' coating unit. All scanning electron microscopy was carried out on a Cambridge S.E.M. 2a scanner.

Immuniprecipitation testing using Ouchterlony plates.

In order to examine the specificity and strength of antibody solutions before using them in radioimmunoassays it was necessary to test their reaction with the required antigens. This was done using Ouchterlony plates for immunoprecipitation.

Ouchterlony plates were prepared by placing clean microscope slides on a levelled surface, usually a Shandon Southern plate pourer. Molten, 2% Noble Agar (Difco Laboratories) was then poured onto these slides to a depth of 3mm and allowed to set. A pattern of wells was then cut out of the agar using a template beneath the slides and a sharpened micropipette tip. The pattern used was as shown below :-



The agar solution was prepared in the buffer to be used for the future radioimmunoassays (usually PBS). Prepared Ouchterlony plates were stored at $4^{\circ}C$ in a sealed humidity chamber ready for use.

To estimate the optimum concentration, for radioimmunoassay, of an antibody solution (usually an antiserum), a set of serial dilutions was made from the stock and 5µl of each placed in the outer wells of the Ouchterlony plates. The antigen solution for comparison was then

placed in the central well and the plates left at a constant temperature in a humidity chamber, usually overnight. After this time the presence of a precipitin line between any of the outer wells and the inner well indicated that, after immunodiffusion of the antibodies and antigens, immunoprecipitation had taken place. Hence the range of antibody or antigen concentrations over which immunoprecipitation could be expected to occur in a radioimmunoassay was determined.

Where the presence of precipitin lines was indistinct the Ouchterlony plates were fixed and stained using 0.025% Coomassie Brilliant Blue R (Sigma) in aqueous 25% propan-2-ol, 10% acetic acid. These were then destained in 0.0025% Coomassie Blue in aqueous 10% propan-2-ol, 10% acetic acid and finally cleared in aqueous 10% acetic acid. Where a permanent record of the precipitin lines was necessary the plates were air dried and kept.

Iodination of proteins.

Purified and separated MT. proteins were iodinated using the chloramine T. method of Greenwood et al (1963).

Initially a fresh (no more than three days old) batch of 2mCi of Iodine-125, as sodium iodide in NaOH solution (Radiochemical Centre, Amersham), was diluted by adding 30µl of phosphate buffered saline (PBS) ($Na H_2 PO_4$-10mM; HaCl-0.876%; Na azide-0.02%; pH-7.6) to the initial 20ul. 1mCi (25µl) of this stock was then added to about 20mg of protein in 10ml of PBS. To the mixture of protein and iodine-125 was added 10µl of a 0.1mg/ml solution of chloramine-T. (N-chloro-p-toluene sulphonamide) in PBS. The whole was then mixed and left for two minutes; the reaction being stopped after this time by the addition of 50µl of a 2mg/ml sodium metabisulphite solution in PBS closely followed by 100µl of a 10mg/ml solution of potassium iodide in PBS. After mixing, iodinated protein was separated from free iodine on a Sephadex-G25 disposable chromatography column (Pharmacia). Iodinated protein was isolated, by γ-counting the 0.5ml fractions, typically in fractions 5, 6 and 7 of the void volume. Free iodine did not begin to appear in any amount until fraction 10.

The iodination method typically produced a 1.5ml stock of iodinated protein at a concentration less than 13µg/ml and with a γ-count rate of between 50,000 and 1,000,000cpm/ml depending on the initial amount of protein used. This stock was rapidly frozen in liquid nitrogen and stored at -20°C for use in radioimmunoassays.

EXPERIMENTAL PROCEDURES

The in vitro combination of microtubules and zymogen granules.

MT. protein was isolated, normally from porcine brain, in the normal way and stored at -20°C in 8M glycerol in isolation buffer. Zymogen granules from mouse exocrine pancreas were also isolated and stored in granule buffer at 4°C, no longer than overnight. Prior to combination with MT. these granules were pelleted by centrifugation and resuspended in a minimal volume of MT. isolation buffer (MES-100mM; EGTA-1mM; EDTA-0.1mM; $MgCl_2$-0.5mM; pH 6.4 with HCl) and kept on ice at 4°C. Alternatively 'granule ghosts' were prepared in the place of whole granules. Equal portions of MT. protein solution and granule suspension were then combined according to the following experimental regime :-

MT. + G (4°C) : MT. protein solution + Zymogen granule solution
MT. + G (37°C) : " + "
MT. only (37°C) : " + MT. isolation buffer
G only (37°C) :8M glycerol MT.buffer + Zymogen granule suspension

Each of the above combinations, usually replicated, was made 1mM in GTP and incubated for 30 minutes at the indicated temperature. After this period all the incubation tubes were centrifuged at 1,000g for 10 minutes at room temperature in the M.S.E. 'Mistral 4L' centrifuge.

After centrifugation the supernatants were removed for protein estimation (^{3}H-colchicine binding assay or R.I.A.) and the pellets were either prepared for electron microscopy or resuspended in MT. isolation buffer. To estimate bound MT. protein to zymogen granules the pellets were resuspended in equal, minimal volumes of MT. isolation buffer containing 1mM GTP and incubated at 4°C for one hour to disassemble the MT. These suspensions were then recentrifuged at 1,000g

for 10 minutes at 4°C and the supernatants again removed for protein estimation. The remaining pellets were either discarded, prepared for electron microscopy or again resuspended in buffer and cooled to 4°C. Protein supernatants were obtained often through three cycles of resuspension and centrifugation of the zymogen granules in fresh, cold buffer. These supernatants were stored at -20°C for estimation of their T. content within two days. The formation of MT. during initial incubation was monitored using negatively stained E.M. grids.

Macroscopic examination of interaction between microtubules and zymogen granules.

MT. protein and isolated zymogen granules were combined according to the in vitro regime outlined in the previous section. After the initial centrifugation the pellets were fixed for electron microscopy. Both before and after centrifugation four of the different types of MT.-granule combination were observed and compared carefully. The post-incubation mixtures were compared morphologically for both viscosity and clarity, by eye as were the post-centrifugation supernatants. Pellets produced by centrifugation were compared for size both before and after fixation and also when fixed prior to centrifugation.

Scanning electron microscopy study of the interaction between microtubules and zymogen granules.

As in the previous procedure MT. protein was combined with isolated zymogen granules *in vitro* at two incubation temperatures. These mixtures when first centrifuged produced pellets which were fixed for S.E.M. Pellets were also produced from material fixed before centrifugation. These fixed pellets were processed for scanning electron microscopy and examined in the S.E.M.

Transmission electron microscope study of the interaction between microtubules and zymogen granules.

Pelleted, combined MT. and zymogen granules were fixed either before or after centrifugation. These pellets were then prepared for T.E.M. and examined in the electron microscope. Both first incubation and cold secondary incubation pellets were examined and compared for the presence of any forms of interaction between the MT. and zymogen granule membranes.

The estimation of the effect of exocrine pancreatic stimulation on the microtubule-free tubulin equilibrium.

In order to examine the alteration in the proportions of T. found in MT. and as 'free-pool' T. in the exocrine pancreas during secretion the assay described previously to separate and estimate pancreas T. was used. Initially a comparison was made between the pancreas T. levels of both fed and fasted mice. However, since the differences in both polymerised T. (MT.) and depolymerised T. ('free-pool') proved to be insignificant a chemical pancreatic stimulant was used to replace feeding.

In all cases a set of mice selected to be as similar as possible (in terms of age, sex and live weight) were divided into two groups at random. These two groups were then fasted overnight in preparation for experimentation the next morning. Each group of mice was differently treated, one group to stimulate exocrine pancreas secretion and the other to leave pancreatic secretion at basal (fasted) level. Early experiments involved the comparison of fed and fasted animals; pre-fasted mice were split into two groups and one group allowed to feed on pellet food. In later experiments fasted mice were compared with Pilocarpine treated mice. Pre-fasted mice were split again into two groups; one group was given 3.99mg of Pilocarpine HCl (Sigma) in 100µl of PBS per 30g (approx.) mouse to stimulate exocrine secretion, the other group being given 100µl of PBS per mouse as a control. Saline and Pilocarpine in saline were both administered by injection (25 gauge needle subcutaneously).

All mice were left for 30 to 60 minutes, each group being left for the same period. After this time signs of the effect of Pilocarpine (where given) on exocrine secretion were obvious, these included

increased lacrimation, over excretion, and general stress. Saline treated animals showed none of these symptoms. Mice were killed by cervical dislocation and their pancreas removed. The removed pancreas were then assayed for both assembled and disassembled T. using the methods outlined previously. The two treatment groups' results were then compared statistically by computer (see the Appendix).

Examination of the microtubule-free tubulin equilibrium during the stimulated exocrine pancreas time course.

The effect of Pilocarpine-stimulated exocrine pancreas secretion on the MT. - T. equilibrium was investigated over the time period after stimulation. Two types of experiment were carried out; one where pilocarpine treatment alone was given to a group of mice and their pancreas T. levels measured, and a second type where both pilocarpine-stimulated and non-stimulated levels were investigated.

To evaluate the effect of pilocarpine on exocrine pancreas T. levels a group of similar mice (on the basis of age, sex and live weight) were fasted overnight before next morning's experiment. These mice were then split into five groups, at random, each group consisting of 3 or 4 replicate mice. Each group of mice was designated one of five time periods after Pilocarpine injection, either 10, 20, 30, 45 and 60 minutes or in other experiments 0, 2.5, 5, 10 and 20 minutes. In all cases an injection (25 gauge needle subcutaneously) of 3.99mg of Pilocarpine in 100µl of saline was given per 30g (approx.) mouse. A randomised plan was used to decide the order of treatment of mice; they were selected for injection, incubation for the designated time period, killing and pancreas removal at random both with respect to time period group and replicate number. Each randomly selected mouse was injected, left for its required incubation period, then killed and its pancreas removed. The pancreas was homogenised in MTS buffer as described previously and left on ice at $4^{o}C$. All the mice were so treated before any other steps were carried out to separate assembled and disassembled T. Once all the mice had had their pancreas removed and homogenised the final steps in separation of assembled and disassembled T. were carried out. Finally the amount of T. in each fraction

was assayed by ^{3}H-colchicine binding assay or R.I.A. The resultant values were then analysed statistically using a computer programme (see the Appendix).

The second type of experiment was arranged similarly to the previous type except that both Pilocarpine injected (3.99mg in 100µl PBS per mouse) and saline-only injected animals (100µl per mouse) were included. In this case similar mice were split at random into two groups, a Pilocarpine-treated group and a saline-treated group. Each group was then randomly divided into five time period (after injection) groups of 10, 20, 30, 45 and 60 minutes. Mice were then treated at random with respect to replicate number time period group and injection type. Homogenised pancreas were assayed as before for assembled and disassembled T. and the results compared statistically using a computer programme (see the Appendix).

Macroscopic examination of the interaction between isolated zymogen granules and MT. proteins.

Photographs P1 to P4 show the results of careful macroscopic observations of the interaction between isolated zymogen granules and MT. proteins in a typical in vitro experiment. P1 shows the experimental tubes after incubation at 37°C or 4°C and prior to centrifugation. From left to right they were α: MT. protein and granules incubated at 4°C; and β: MT. protein and granules, γ: MT. protein alone and Δ: granules alone, all incubated at 37°C. It can be seen that the second experimental mixture (β) was much more turbid than the other two containing granules (α and Δ) which were very similar in this respect.

The second photograph, P2, shows the supernatants collected, after centrifugation at 1,000g for 10 minutes, from the experimental tubes in the first photograph. The supernatants from all but the second tube (β: MT. proteins with granules incubated at 37°C) were of similar volume, the second tube's supernatant being considerably reduced in volume.

Photographs P3 and P4 show the pellets collected, after centrifugation, from the experimental tubes in photograph P1. P3 shows the pellets immediately after centrifugation covered by tannic acid/gluteraldehyde fixative and P4 shows these same pellets removed from the experimental tubes. The pellets labelled α, B and Δ correspond to the experimental tubes α, B, Δ in the first photograph. However, those labelled α_F and β_F were derived from experimental tubes corresponding to α_F and B_F in photograph P1, but they were tannic acid/gluteraldehyde fixed before centrifugation. Both P3 and P4 show that whereas pellets labelled α and Δ (α: MT. protein and granules incubated at 0°C, Δ: granules alone incubated at 37°C) were of similar volume, pellet β (MT. protein and granules

incubated at 37°C showed a three-fold increase in volume. This difference in size was also shown even if the samples were fixed before centrifugation (α_F and β_F).

The fixed pellets shown in photograph P4 were those which were dehydrated, critical point dried and prepared for S.E.M.

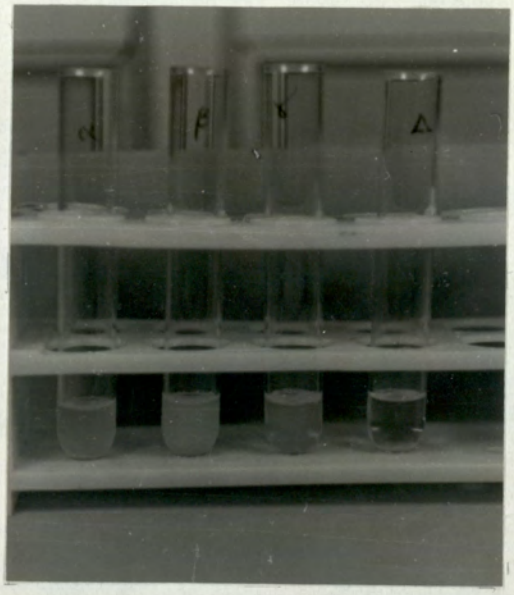
Photographs P1 to P4 : Macroscopic examination of the MT.-zymogen granule interaction.

P1 : Experimental tubes after incubation and before centrifugation.

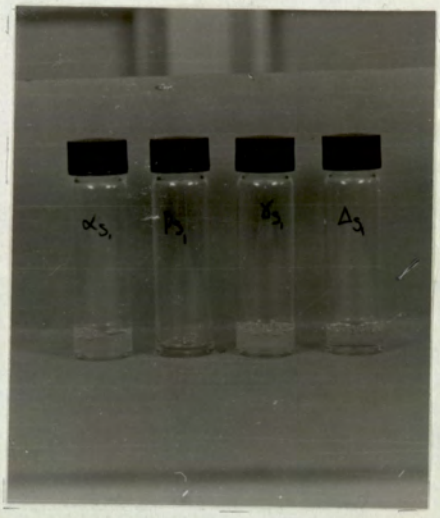
P2 : Supernatants after centrifugation.

P3 : Granule pellets during E.M. fixation.

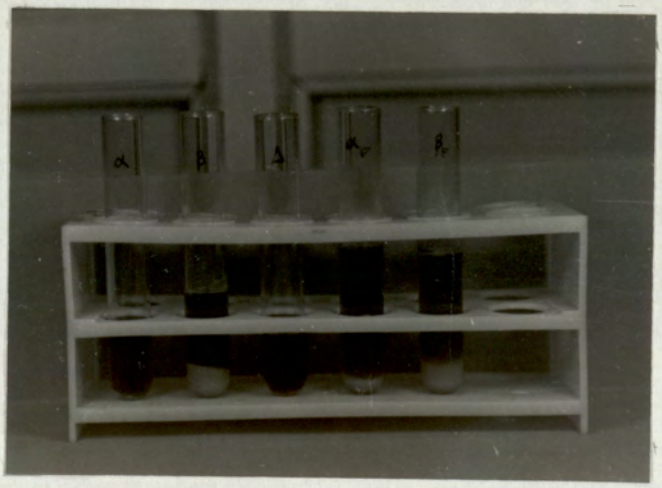
P4 : Granule pellets after fixation.



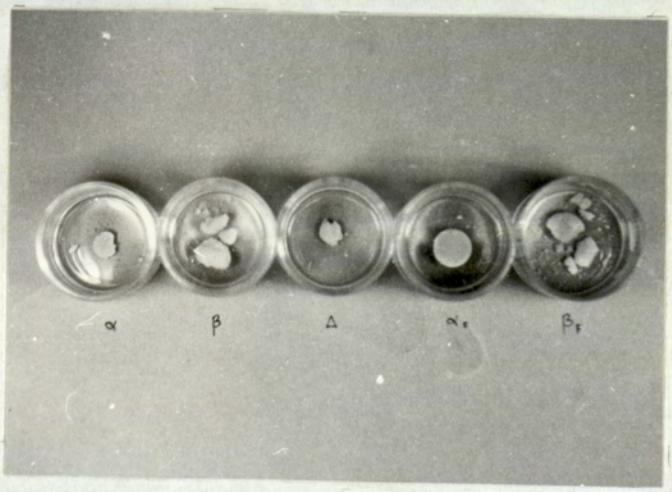
P1.



P2.



P3.



P4.

Scanning electron microscope study of the interaction between isolated zymogen granules and MT. proteins.

The pellets shown in photograph P4 of the previous section were looked at in more detail by scanning electron microscopy. Photographs P5 to P9 each show overall S.E.M. views of these pellets. Photographs P10 to P12 are closer details of some parts of the β_r (MT. protein and granules combined at 37°C and fixed before centrifugation) derived pellet.

The pellet derived from MT. protein and granules kept at 4°C (P5) and granules incubated alone at 37°C (P7) should be contrasted with the pellet from the MT. protein/granule mixture warmed to 37°C (P8). The 'granular' appearance of the former two pellets is markedly different to the complex meshwork viewed in the latter. These contrasts are maintained even when the mixtures were tannic acid/gluteraldehyde fixed before centrifugation. Photograph P6 shows such a view of the pellet derived from the cold MT. protein/granule mixture and photograph P9 such a view of the warmed mixture pellet.

Of the detailed views of the pellets derived from the warmed MT. protein/granule mixture, pre-centrifugation fixed, photograph P10 shows a close up of the MT. meshwork while P11 and P12 show zymogen granules on the surface of this mesh. Note that the former granule is uncoated while the latter is coated by the MT. web.

Photographs P5 to P12 : S.E.M. examination of the MT.-zymogen granule interaction.

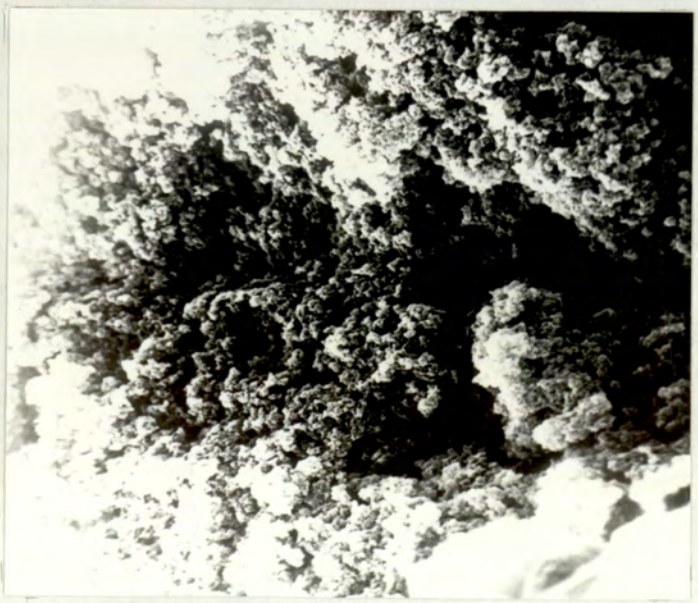
P5 : Overall view of pellet from tube containing MT. proteins and zymogen granules incubated at 4°C; x 3.5K.

P6 : Overall view of pellet from tube containing MT. proteins and zymogen granules incubated at 4°C but fixed before centrifugation; x 3.5K.

P7 : Overall view of pellet from tube containing only zymogen granules incubated at 37°C; x 3.6K.

P5.

P6.



P7.

P8 : Overall view of pellet from tube containing MT. proteins and zymogen granules incubated at 37°C; x 7.1K.

P9 : Overal view of pellet from tube containing MT. proteins and zymogen granules incubated at 37°C but fixed before centrifugation; x 7.0K.

P10: Close up view of pellet in photograph P9; x 17.7K.

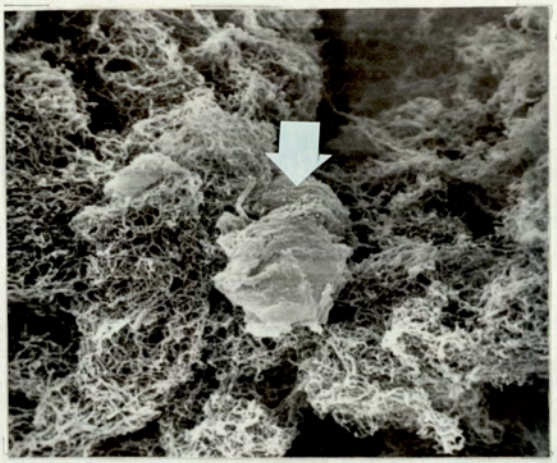
P8.

P9.

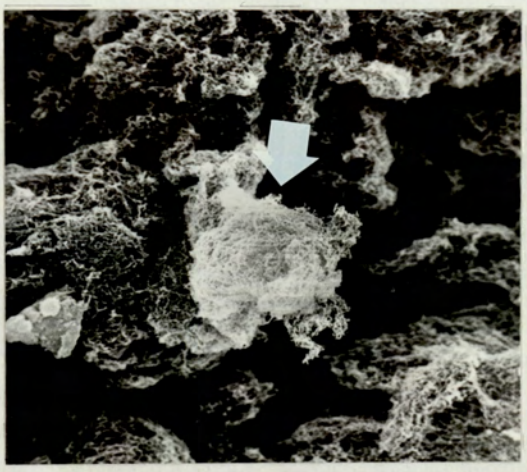
P10.

P11: Close up view of pellet in photograph P9 showing zymogen granule; x 15.0K.

P12: As above; x 5.6K.



P11.



P12.

Transmission electron micrographs of MT. assembled in vitro and isolated zymogen granules.

When porcine brain MT. proteins and isolated murine exocrine pancreas zymogen granules were combined in the *in vitro* experiments, described previously, photographs P13 to P16 show how samples of the pellets appeared in transmission electron micrographs.

Photograph P13 shows various MT. fragments in both transverse and longitudinal section (t.s. and l.s.). The sectioned MT. in the centre of the picture shows both views of the typical *in vitro* structure. Notice that in the t.s. at left centre the individual tubular subunits are apparent. These subunits can also be seen in the l.s. of this MT., the individual protofilaments going up to the top right-hand corner of the photograph. Other fragments in this photograph also show the tubular nature of the MT., the dark stained side walls are surrounded by a lighter stained central core. Of particular note is the apparent presence of side arms on the sides of the l.s. of the long MT. fragment. Although not of any great size, there appears to be a periodic attachment of side arm material throughout the MT. length.

A representative view of a sectioned exocrine pancreatic granule is shown in photograph P14. Here we can see a dense central core of zymogen surrounded by an outer membrane. The core of zymogen appears less dense than it would in *in vivo* sectioned material. This was probably the result of a steady loss of material under the influence of osmotic shock during and after isolation. Material surrounding the granule may represent such material lost from other granules. In fact the removal of the internal zymogen core by alteration in the osmolarity of the isolation buffer became necessary in order to visualise the

phospholipid bilayer membrane of the isolated granule or 'granule ghosts' as they became.

Photographs P15 and P16 show such 'granule ghosts' in the process of losing their contents. Photograph P15 shows a rather fuzzy membrane outline, since the section did not cut the membrane normally in all places, however the bilayer is visible in the upper left part of the 'granule ghost'. Zymogen material is still apparent adhering to the inner membrane surface although the greater part of this material was being lost to the surrounding medium. Notice the MT. t.s. above the granule and the l.s. above this. Photograph P16 shows a much clearer granule membrane the last of its zymogen contents being lost from a break in the membrane. The typical bilayer membrane structure can be seen all around the section and an MT. l.s. fragment can be seen close to the membrane.

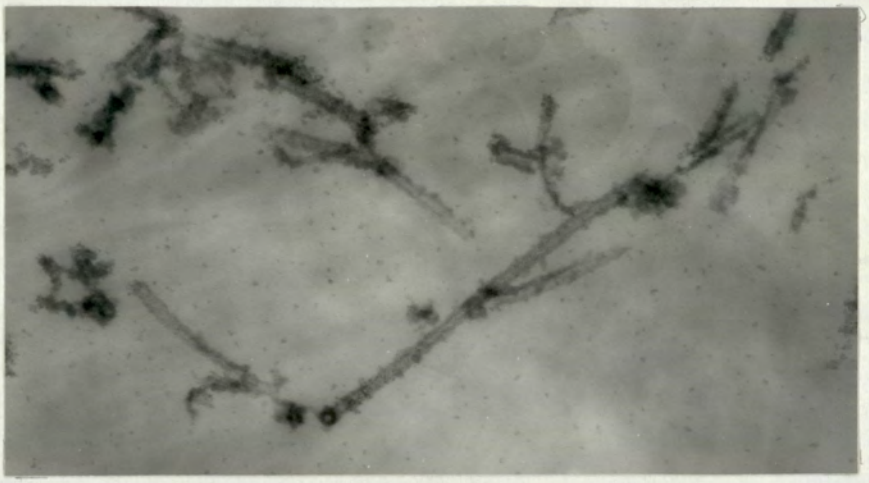
Photographs P13 to P16 : T.E.M. views of MT. and zymogen granules.

P13: MT. proteins incubated alone at 37°C showing assembled MT. seen in t.s. and l.s.; x 69.8K.

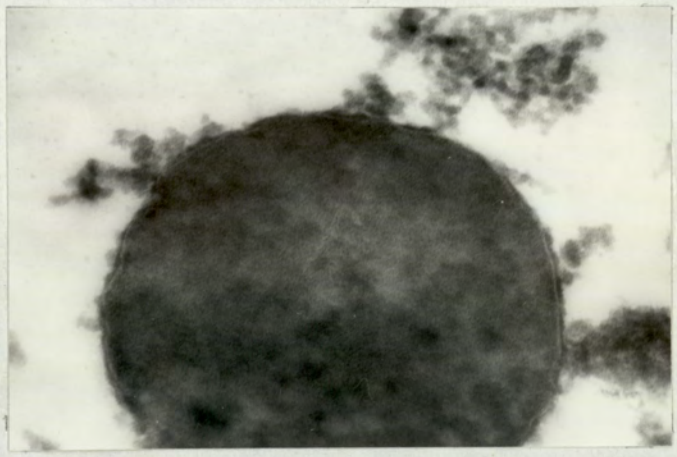
P14: Zymogen granules incubated with MT. proteins at 4°C, showing a granule complete with its contents; x 93.2K.

P15: MT. proteins and zymogen granules incubated at 37°C showing a granule losing its contents; x 71.5K.

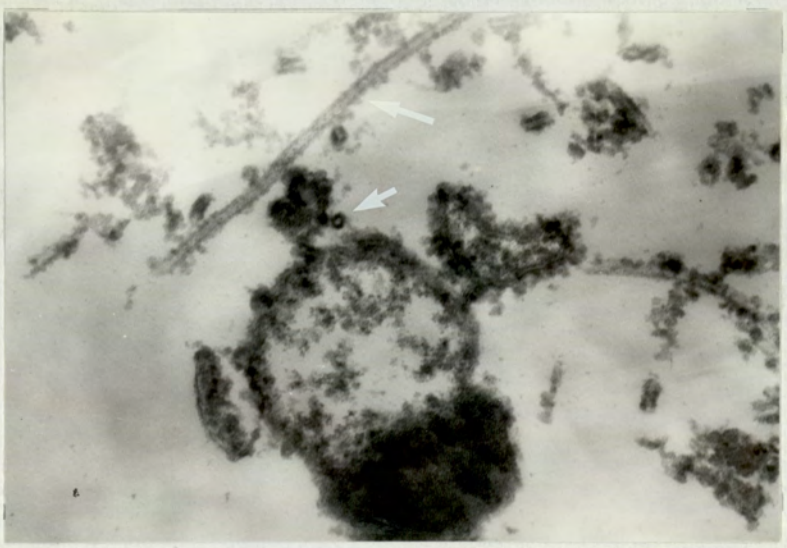
P16: MT. proteins and zymogen granules incubated at 37°C showing a granule 'ghost' losing its contents; x 38.9K.



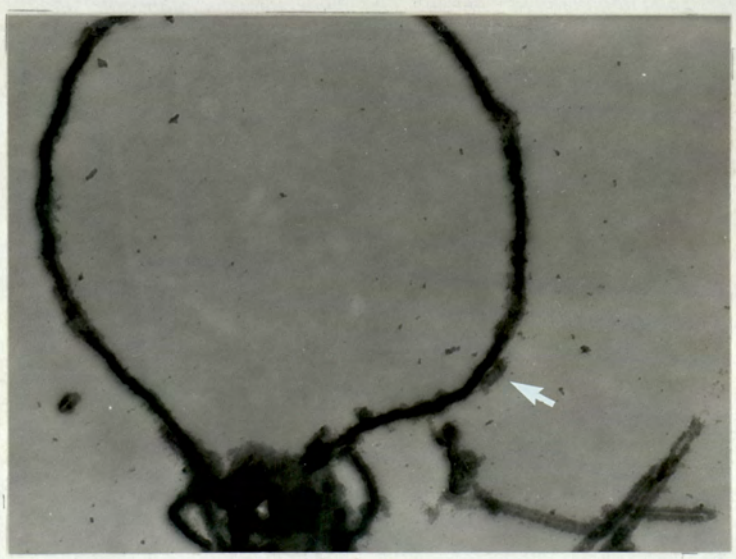
P13.



P14.



P15.



P16.

Transmission electron microscope study of the interaction between isolated zymogen granules and MT. proteins.

Over a considerable period experiments were carried out to examine the effect of assembled MT. on the sedimentation of exocrine pancreatic secretory granules or their ghosts. These experiments were carried out, controlled as was outlined in the experimental procedures section, with T.E.M. monitoring at all stages. Pellets collected in the complete absence of MT. proteins or in the presence of disassembled MT. proteins (kept at 4°C) showed no association between MT. and 'granule ghosts'. Control samples containing only assembled MT. produced no pellets since the sedimentation rates used (1,000g for 10 minutes) were far too low to bring them down, indeed if they had pelleted under these conditions the experiments would have been inconclusive since we were trying to detect if the MT. pelleted in association with the zymogen granules. T.E.M. monitoring of pellets formed by the granules, previously in association with MT., by cold treatment (incubated at 4°C for 1 hour) and centrifugation at 1,000g for 10 minutes showed a complete absence of MT. - granule associations; assembled MT. could never be found in such pellets.

Photographs in this section, P17 to P23, show the interaction of assembled MT. with pelleted zymogen 'granule ghosts'. In each case the granules were devoid of contents after being placed in several washes of MT. isolation buffer. Photographs P17 to P20 show the l.s. of MT. in close association with the 'granule ghosts' and photographs P21 to P23 show the same association with the MT. in t.s. The area shown in photograph P17 has an l.s. of an MT. fragment of some length remaining in the plane of section. It shows not only the tubular nature of the MT. but also there is some indication of the striations associated with

the protofilaments. These two characteristic morphologies are the result of the variation in the plane of section through the MT. tube. More importantly the photograph shows the MT. in close juxtaposition to the granule membrane. Although no structural association is apparent here, there is certainly some sign of side arm presence on the MT. fragment.

The MT. l.s. in photograph P18 is once again particularly long showing both the tubular nature of the MT. and the decoration indicative of side arm presence. The distance between the MT. and the granule membrane is greater than in the previous photograph although there is some indication of a side arm connection between the two. The area of granule membrane indicated does not show the typical bilayer morphology since the plane of section here was more tangential, cutting along a single part of the bilayer. This effect was also apparent on parts of the granule membrane shown in photograph P19. Here the MT. l.s. is once again in close juxtaposition to the granule membrane but unlike the previous pair of photographs we can see definite periodic association between the two, gaps being apparent between the points of attachment. This photograph and photograph P20 also show the close association of an MT. t.s. with the 'granule ghost'. The MT. l.s. in photograph P20 may not be associated with the granule membrane although there is certainly some material connecting the two.

MT. in t.s. associated with 'granule ghosts' are more clearly shown in photographs P21, P22 and P23. In photograph P21 numerous MT. t.s. are visible although only the two indicated are actually in contact with the granule membrane. They both appear to be in close contact with no real sign of any structural association. Similarly no side arms are apparent on the lower MT. t.s. indicated on photograph P22. The upper t.s. however does appear to have material between it and the granule

membrane, although since the section appears to be more oblique than a true t.s. it is probably just part of the MT. going out of the plane of section. The final photograph, P23, showing MT. in t.s. in association with a 'granule ghost', not only has individual sectioned MT. associated with the granule membrane, but also a small group of MT. fragments as indicated. Two of the MT. are in close contact with the granule membrane while the others are grouped against this pair. An obliquely cut MT. to the right of the group also has a thin strand of material joining it to the granule membrane.

Photographs P17 to P23 : T.E.M. examination of the MT.-zymogen granule interaction.

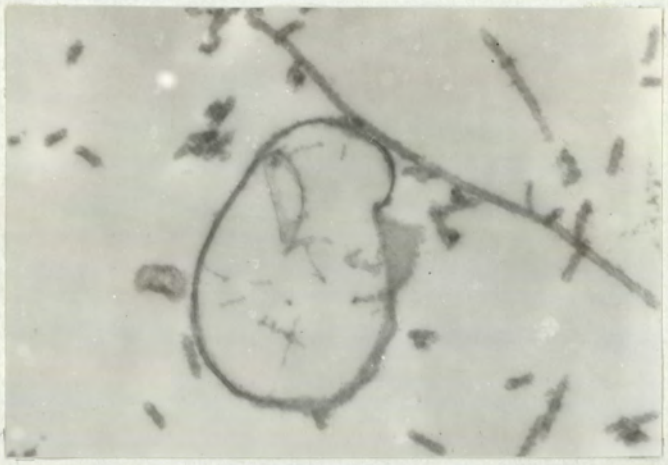
All photographs show T.E.M. views of the MT.-zymogen granule pellet from the tube incubated at 37°C.

P17: Zymogen granule'ghost'associated with MT. seen in l.s.;x 40.5K.

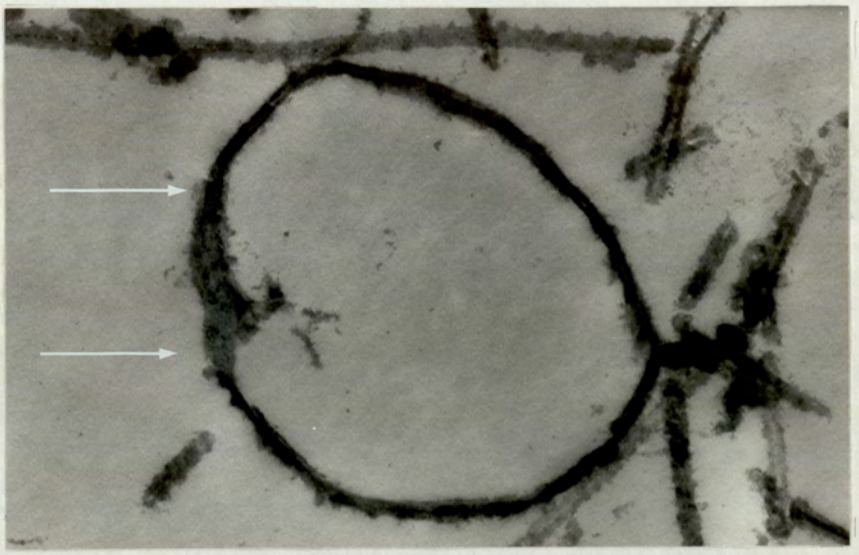
P18: As above;x 58.2K.

P19: As above;x 70.9K.

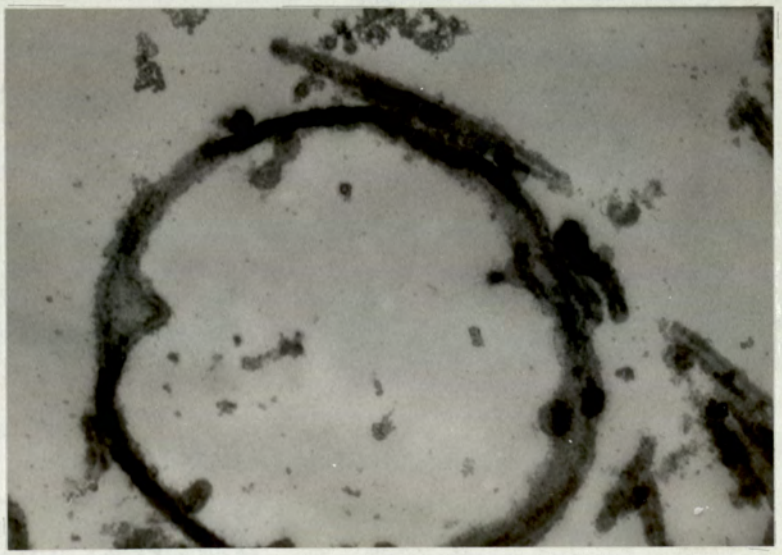
P20: Zymogen granule'ghost'associated with MT. seen in both t.s. and l.s.;x 36.4K.



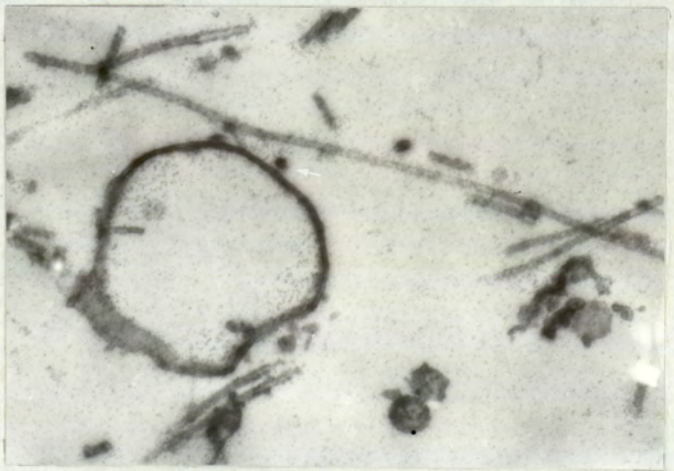
P17.



P18.



P19.

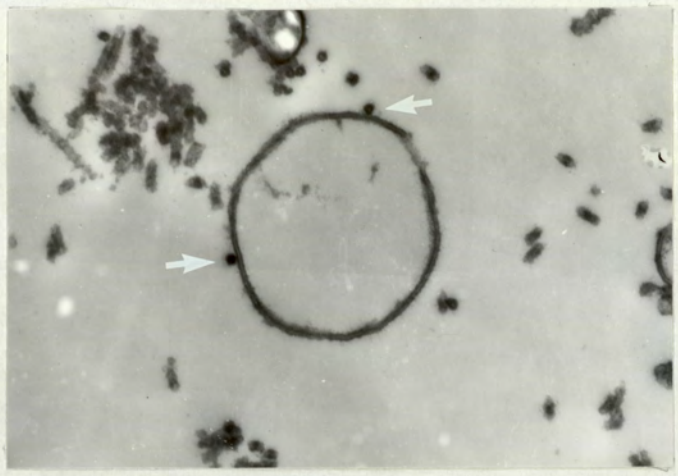


P20.

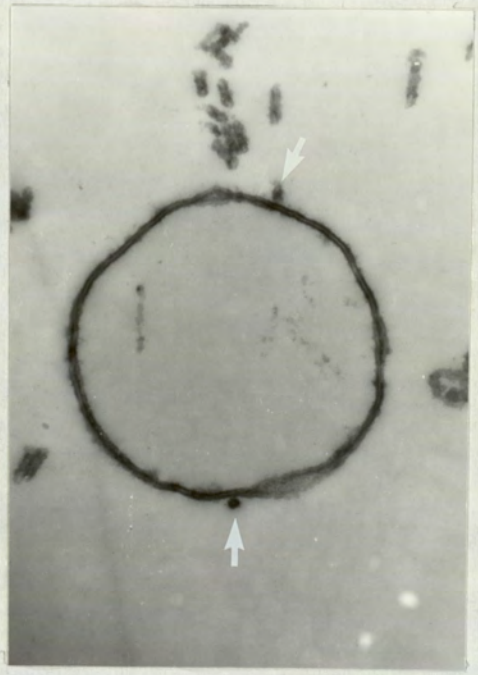
P21: Zymogen granule'ghost'associated with MT. seen in t.s.;x 36.8K.

P22: As above;x 36.4K.

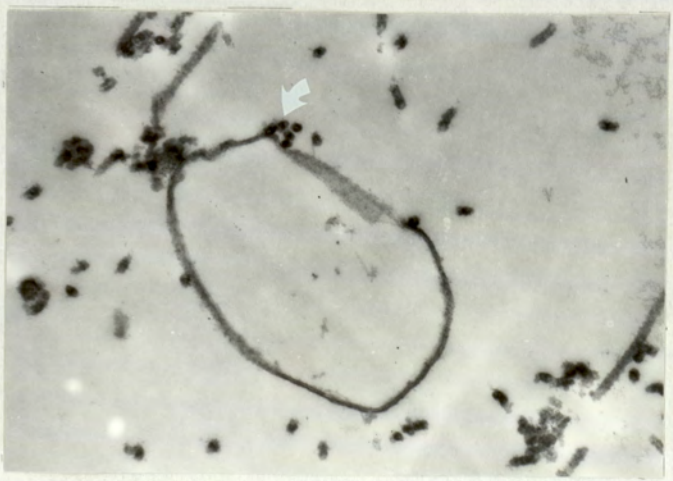
P23: As above;x 36.2K.



P21.



P22.



P23.

High magnification, transmission electron microscope study of the interaction between isolated zymogen granules and MT. proteins.

The photographs of zymogen 'granule ghosts' in close association with MT. fragments in the previous section provides evidence for an interaction between the two organelles. However the physical nature of the connection, if present, between the two was not discernible. The photographs in this section show the interaction at higher magnifications, small regions of granule membrane in close proximity to the MT. being examined much more carefully. The views presented of this arrangement are representative of what was observed over a long period, although obviously when working at the limits of magnification of the instrument used it was necessary to eliminate a number of the photographs collected on the basis of quality.

The first group of photographs, P24 to P29, show MT. in l.s. in close association with the granule membrane. The second group, P30 to P33, show the same association only with the MT. in t.s. In each case the MT. show their characteristic structure, either in l.s. as a stained outer tube with a clearer central core or in t.s. as a circle of subunits arranged in a perfect circle. The individual subunits are often apparent as tannic acid, negatively stained clear areas in a dark matrix. The granule membrane displays its characteristic bilayer structure.

Photograph P24 shows a 'granule ghost' membrane encompassing the top right-hand corner of the print with three MT. fragments in close proximity. The lower left fragment shows not only the tubular nature of the MT. but also exhibits in parts periodic striations indicative of the individual protofilaments making up the MT. Both the larger fragments exhibit wall decoration representing the possible presence of side arms. However, the most noticeable feature is the wispy material connecting the right-hand

MT. fragment to the granule membrane. This wispy material was present in the area of the membrane which has been sectioned more tangentially and therefore did not display the more typical bilayer structure represented by the other parts of the membrane which are shown.

Much closer contact between the granule membrane and the MT. is shown in photograph P25. The granule membrane in the right-hand corner of the picture lies right against the MT. running from left to lower right. This MT. does not display a tubular structure but has indications of the striations produced by the individual protofilaments. There is no clear indication of any structural relationship between the two organelles, although there may well be some connection at the point where the MT. leaves the granule membrane to the left. The membrane and MT. are certainly in very intimate association throughout their areas of contact.

More distant association is represented in photographs P26 and P27. P26 shows a length of MT. in l.s. running from top right to bottom almost paralleled by the granule membrane to its left. Where the pair approach one another most closely there is some indication of periodic interconnections. Whether these arise from the membrane, the MT. side wall or both is uncertain; certainly the decoration on the MT. wall coincides with ridges on the outer part of the granule membrane. More distinct, regular connections between a granule membrane and an MT. l.s. are displayed in photograph P27. This shows a granule membrane enclosing the upper right-hand third of the picture and an MT. fragment below and to its left. Both in the areas of close contact and more distant association there is some indication of interconnections between the two organelles. At the right-hand end of the MT. piece are two areas where material joins the granule to the MT. and at the far left-hand end is another joined area. In between there are also indications of

coinciding MT. wall decoration and granule membrane ridges.

The presence of definite connections between these two organelles in vitro is shown in photographs P28 and P29. The portion of granule membrane to the left of the picture is joined to a piece of MT., to its right, by a complete interconnecting arm. The position of this arm on the MT. fits in well with the periodic decoration indicative of side arms above it on the wall. Also it coincides with a bulge in the granule membrane at the point of attachment. Unfortunately the plane of section has not presented the membrane as a bilayer in this area so it is difficult to detect any definite structural outgrowths. The second photograph, P29, showing a definite MT.-'granule ghost' interconnection, also encounters this problem of delineating any structural outgrowths from the membrane. The granule membrane encompassing the upper right-hand corner of the photograph has, once again, been sectioned too tangentially to decide where the arm connecting it to the MT., running from upper left to lower right, originates. Even so there is a definite structural connection between the two organelles and some indication of connections in three places further along the MT. to the left; the farthest link being produced by large adjacent expansions of the MT. wall and granule membrane.

The last group of photographs in this section shows granule membranes in close association with MT. in t.s. In the first photograph, P30, the granule membrane running from upper left to right lies alongside an MT. seen in t.s. at lower centre. To the left of this t.s. is an MT. sectioned between t.s. and l.s. appearing in the plane of the photograph at two points. In the t.s. of the MT. we can discern the individual subunits which lie in a circle close to the granule membrane. Surrounding the MT. there appears to be some coating material and it is this coating which appears to join the MT. to the membrane. The normally perfectly

circular profile of the MT. also seems somewhat flattened against the membrane. Coating around the MT. is also evident in photograph P31. Once again the MT. in t.s. in the centre of the photograph displays its individual constituent subunits and hollow central core. Indicated to the left side of the MT. can be seen coating material which appears to extend into an arm pointing out from the MT. wall.

A feature of this photograph and the next one, P32, is that the point in the granule membrane where the MT. contact is somewhat indented and contact is much more intimate than in the previous photograph, P30. In photograph P31 no real structural features are visible coming from the membrane bilayer although there does seem to be some coating around the MT. adjacent to the point of contact and continuing part way around the MT. arc. This coating is restricted to the point of contact of the MT. and membrane in photograph P32.

In the final photograph of this group, P33, the MT. in the centre of the picture has been cut in a plane oblique to the MT. axis. Consequently the individual subunits are not clearly visible. This view does, however, show material protruding from the side wall of the MT. and connecting it to the granule membrane.

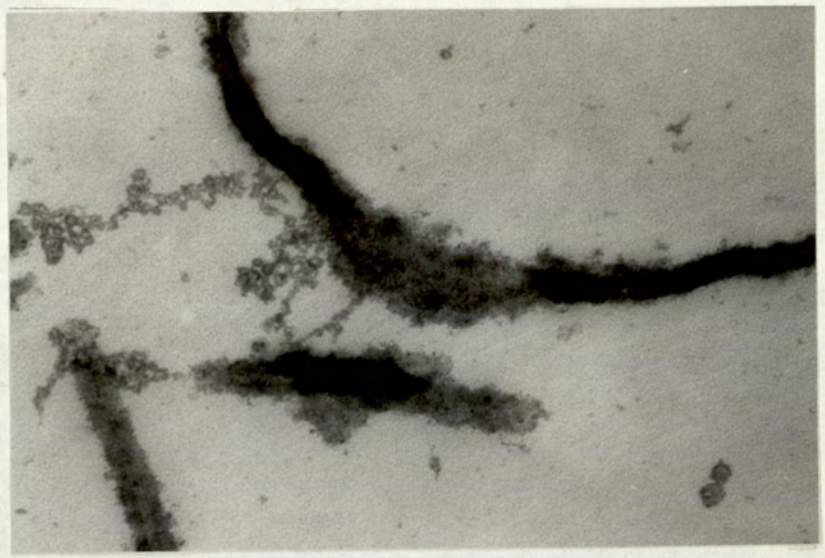
Photographs P24 to P33 : High power T.E.M. examination of the MT.-zymogen granule interaction.

All photographs show T.E.M. views of the MT.-zymogen granule pellet from the tube incubated at 37°C.

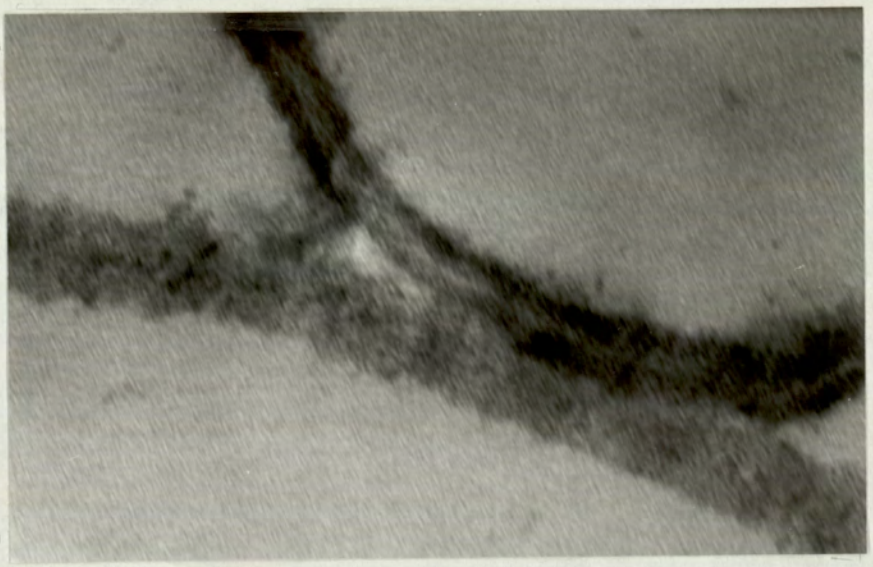
P24: Portion of granule'ghost'membrane connected to a MT. seen in l.s. by wisps of material;x 117.0K.

P25: Granule membrane closely associated with a MT. seen in l.s.;x 232.2K

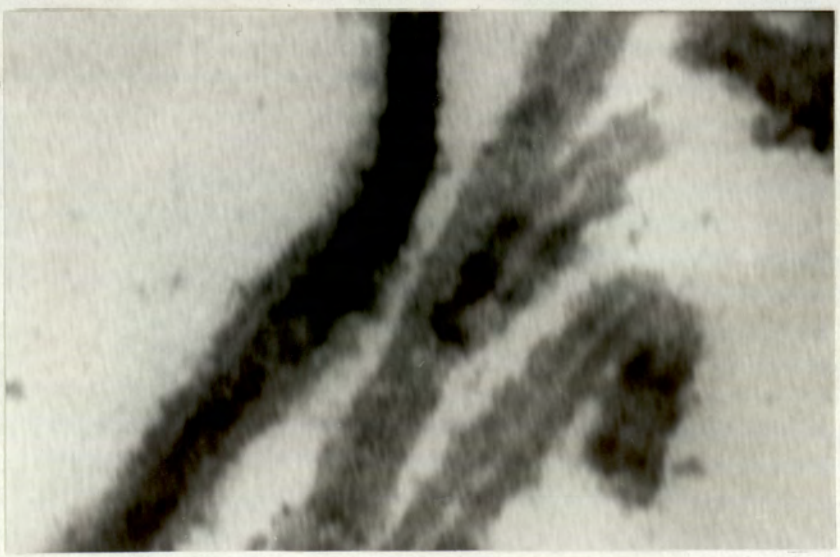
P26: Granule membrane in close alignment with MT. fragments in l.s.;x 235.3K.



P24.



P25.

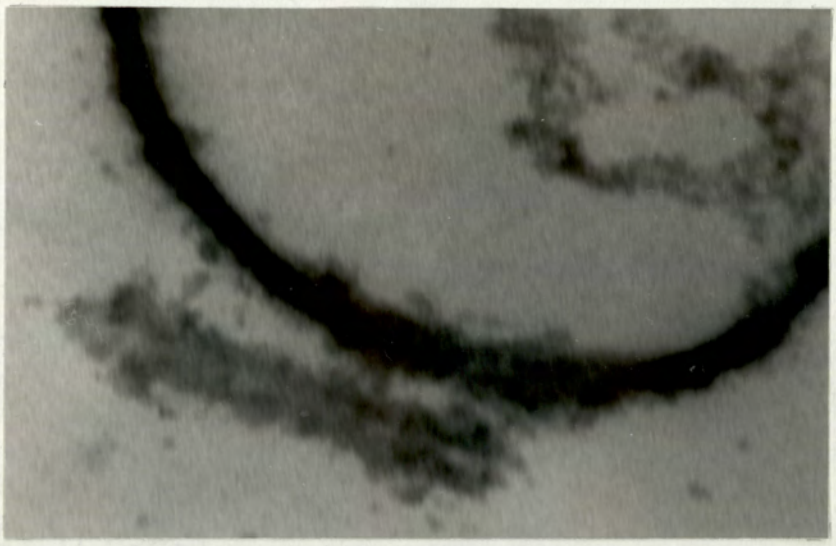


P26.

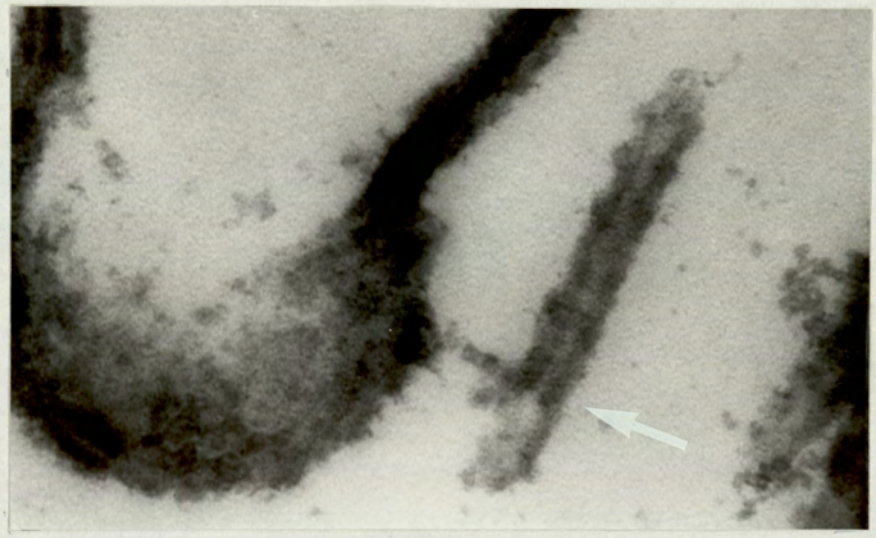
P27: Granule membrane closely connected to a MT. seen in l.s.;x 233.8K.

P28: A MT. side arm connecting granule'ghost'membrane and a MT. seen in l.s.;x 189.6K.

P29: Side arm connection of granule membrane and a MT. seen in l.s.;x 229.8K.



P27.



P28.

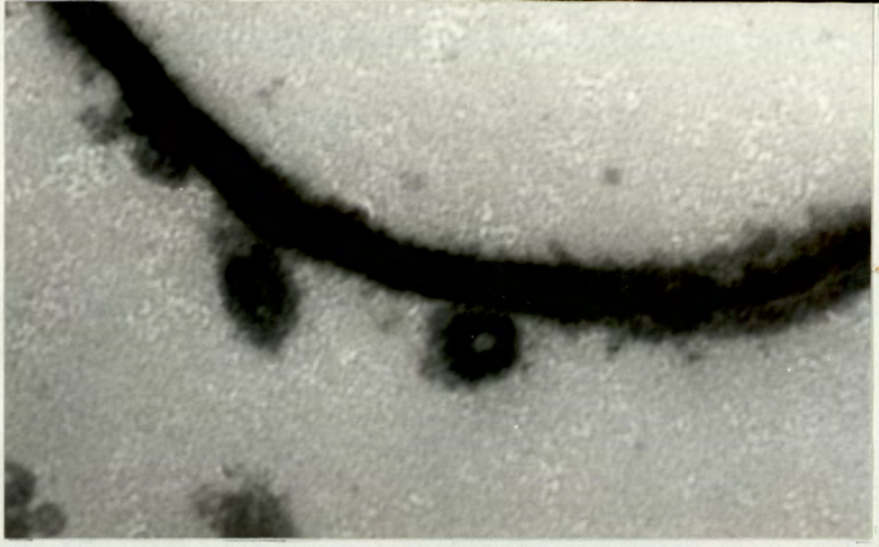
P29.

P30: A MT. seen in t.s. lying in close proximity to a granule'ghost' membrane;x 234.3K.

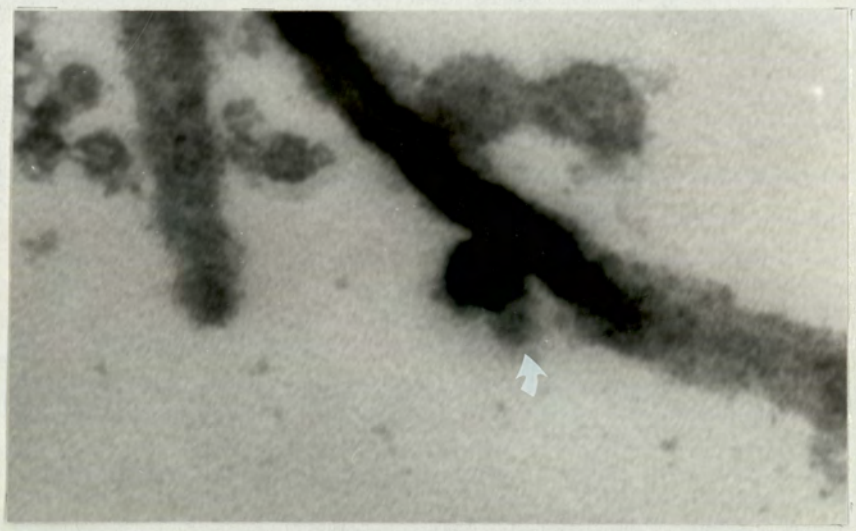
P31: As above;x 236.0K.

P32: As above;x 139.9K.

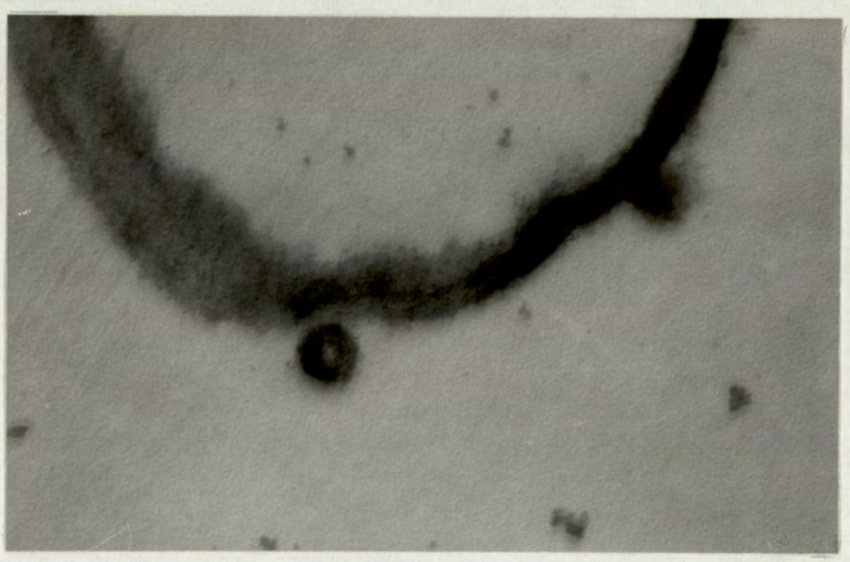
P33: As above;x 234.7K.



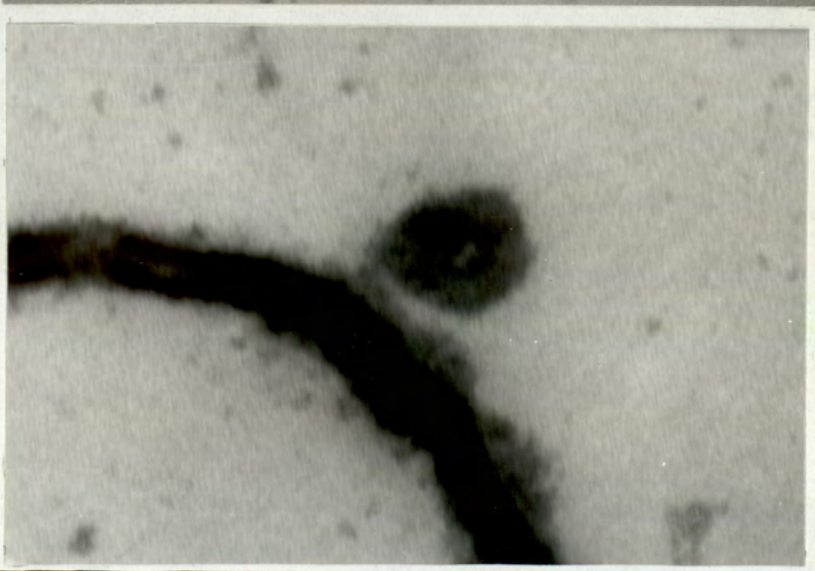
P30.



P31.



P32.



P33.

^{3}H-colchicine binding assay studies of the interaction between isolated zymogen granules and MT. proteins.

In this first assay section the experimental combination of isolated zymogen granules and MT. proteins was examined biochemically. The supernatants derived from the pellets previously described in the electron microscope sections were assayed for T. by the ^{3}H-colchicine binding assay. The assay values were then compared by one way analysis of variance comparing the results on the basis of both the presence and absence of granules and the temperature of incubation.

Eight experiments were carried out in all each with basically the same design. However, in experiments 04 and 05 MT. protein with M.A.P. removed was used in addition to whole MT. proteins. Also in addition to measuring the T. levels of first and second supernatants experiments 04, 06, 07 and 08 all had further supernatants which were assayed. At the end of this section is a summary of the experimental results.

Microtubule protein-zymogen granule experiment 01.

In this experiment the MT. proteins were purified from pig brain and the zymogen granules isolated from 45 mouse pancreas. 1.5ml of the 'two cycle' MT. protein solution were used in combination with 1.5ml of zymogen granule suspension for each sample. The procedures were as described in the methods except that the supernatants were assayed for T. by ^{3}H-colchicine binding assay diluted. First supernatants were diluted 1:0, 1:1, 1:2 and 1:3 and second supernatants 1:1, both with MT. isolation buffer. The final concentrations of T. were corrected for dilution before analysis.

Analysis was by one way analysis of variance the treatment being partitioned into the effect of temperature (4°C or 37°C) and the effect of addition of zymogen granules by using orthogonal contrasts (see the Appendix). The temperature effect was found by comparing 'MT.+ G' at 4°C and 'MT.+ G' at 37°C. The effect of addition of granules was found by comparing 'MT. ONLY' with this latter pair combined.

MT.-G : 01.

One-way Anovar using orthogonal linear contrasts.

Table 3.1 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	423.8	139.9	515.8	11.1
Replicates	8	8	6	8
Means	52.975	17.4875	85.9667	1.3876
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	3	3	-8	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	29607.4	9869.12	264.508	***
Temperature	1	5037.45	5037.45	135.011	***
+or- granules	1	11232.4	11232.4	301.045	***
Error	26	970.094	37.3113	-	
Total	29	30577.5	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	24.4332	50.2346	35.4875	6.27933
+or- granules	140.358	288.576	50.7354	6.012

Table 3.2 : Supernatant 2. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	12.4	125.5	28.4	.6
Replicates	2	2	2	2
Means	6.2	62.75	14.2	.3
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	4873.51	1624.5	744.792	***
Temperature	1	3197.9	3197.9	1466.15	***
+or- granules	1	548.101	548.101	251.29	***
Error	4	8.72461	2.18115	-	
Total	7	4882.24	-	-	

	TOTAL CONTRASTS - Standard error	Confidence interva;	- MEAN CONTRASTS - Mean effect	Confidence interval
Temperature	2.95374	8.1996	56.55	4.0998
+or- granules	5.11604	14.2021	20.275	3.55053

In the case of both supernatants the overall effect of treatments was very highly significant ($P < 0.001$) and when the treatment was partitioned both parts were also very highly significant ($P < 0.001$).

Microtubule protein-zymogen granule experiment 02.

MT. proteins purified from pig brain were combined with zymogen granules isolated from 25 mouse pancreas, 1ml of each solution being used per sample. The ^{3}H-colchicine binding assay for T. was carried out on diluted supernatants. After correction for dilution the results were analysed by one way analysis of variance using orthogonal contrasts to partition the treatments.

MT.-G : 02.

One way Anovar using orthogonal linear contrasts.

Table 3.3 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	5488.4	1208.3	4929.3	15.9
Replicates	12	10	13	15
Means	392.029	120.83	379.177	1.06
LINEAR CONTRAST COEFFICIENTS :				
Temperature	5	-7	0	0
+or- granules	13	13	-24	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	1.56028E+06	520094	319.752	***
Temperature	1	429034	429034	263.769	***
+or- granules	1	84573.7	84573.7	51.9957	***
Error	48	78074.5	1626.55	-	
Total	51	1.63836E+06	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	1168.89	2350.64	271.199	33.5805
+or- granules	4333.23	8714.13	100.148	27.9299

Table 3.4 : Supernatant 2. results and analysis, all values derived from measured total T. in μg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	590.9	3039.3	140.1	5.3
Replicates	2	6	2	2
Means	295.45	506.55	70.05	2.65
LINEAR CONTRAST COEFFICIENTS :				
Temperature	3	-1	0	0
+or- granules	1	1	-4	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	536037	178679	157.418	***
Temperature	1	66844.8	66844.8	68.8909	***
+or- granules	1	235592	235592	207.558	***
Error	8	9080.5	1135.06	-	
Total	11	545118	-	-	

	- TOTAL CONTRASTS -		MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	165.05	380.605	211.1	63.4342
+or- granules	213.079	491.359	383.725	61.4199

Both supernatants had treatment effects and partitioned treatment effects which were very highly significant ($P < 0.001$).

Microtubule protein-zymogen granule experiment 03.

In this experiment MT. proteins prepared from porcine brain were separated into two portions. One portion was placed on a cellulose phosphate cation exchange column to remove M.A.P's, as described in the methods section, the other portion being left complete. Each type of MT. protein both with and without M.A.P's was combined with a partitioned zymogen granule suspension prepared from 30 mice pancreas. One way analysis of variance was used to partition the effect of treatments during analysis.

MT.-G : 03.

One way Anovar using orthogonal linear contrasts.

Table 3.5 : Supernatant 1. using T. with M.A.P's; results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	1218.9	464.9	1269.7	83.1
Replicates	5	3	5	5
Means	243.78	154.967	253.94	16.62
LINEAR CONTRAST COEFFICIENTS :				
Temperature	3	-5	0	0
+or- granules	5	5	-8	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	180722	60240.6	55.7635	***
Temperature	1	14789.6	14789.6	13.6905	**
+or- granules	1	5812.94	5812.94	5.38093	*
Error	14	15124	1080.29	-	
Total	17	195846	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	360.048	772.302	88.8133	51.4868
+or- granules	749.499	1607.68	43.465	40.1919

Table 3.6 : Supernatant 2. using T. with M.A.P's; results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	694.8	2903.1	268.3	3.5
Replicates	5	5	5	5
Means	138.96	580.62	53.66	.7

LINEAR CONTRAST COEFFICIENTS :

Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	1.04782E+06	349273	53.6818	***
Temperature	1	487659	487659	74.9513	***
+or- granules	1	312385	312385	48.0124	***
Error	16	104101	6506.34	-	
Total	19	1.15192E+06	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	255.075	540.76	441.66	108.152
+or- granules	441.803	936.623	306.13	93.6623

Table 3.7 : Supernatant 1. using T. without M.A.P's; results and analysis, all values derived from measured T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	137.2	110.3	239	18.7
Replicates	5	4	5	5
Means	27.44	27.575	47.8	3.74

LINEAR CONTRAST COEFFICIENTS :

Temperature	4	-5	0	0
+or- granules	5	5	-9	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	4867.43	1622.48	136.813 ***
Temperature	1	4.05022E-02	4.05022E-02	3.41528E-03NS
+or- granules	1	1324.58	1324.58	111.693 ***
Error	15	177.887	11.8591	-
Total	18	5045.31	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	46.2022	98.4568	.135004	4.92284
+or- granules	86.4363	184.196	20.3	4.09324

Table 3.8 : Supernatant 2. using T. without M.A.P's; results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	8.5	18	5.7	7
Replicates	5	5	5	5
Means	1.7	3.6	1.14	1.4

LINEAR CONTRAST COEFFICIENTS :

Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	18.716	6.23867	2.19324 NS.
Temperature	1	9.025	9.025	3.17279 NS.
+or- granules	1	7.60033	7.60033	2.67194 NS.
Error	16	45.512	2.8445	-
Total	19	64.228	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	5.33339	11.3068	1.9	2.26136
+or- granules	9.2377	19.5839	1.51	1.95839

Whole MT. proteins combined with zymogen granules produced significant effects of treatments even when partitioned in both supernatants; although the significance of the effect of addition of granules in the first supernatant was somewhat reduced ($P<0.05$). When M.A.P's were removed from the MT. proteins used to combine with the zymogen granules a significant effect of treatment ($P<0.001$) was produced only in the first supernatant and then only by the effect of addition of granules, no temperature effect was shown.

Microtubule protein-zymogen granule experiment 04.

Here porcine MT. protein was combined with a zymogen granule suspension isolated from 2 rat pancreas. Four supernatants were collected each of 1,200ul, the first consisting of 600ul each of MT. protein solution and granule suspension. One way analysis of variance using orthogonal linear contrasts was used to analyse the results of the ^{3}H-colchicine binding assay for T.

MT.-G : 04.

One way Anovar using orthogonal linear contrasts.

Table 3.9 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	25.6	1.1	8.13	0
Replicates	2	2	2	2
Means	12.8	.55	40.65	0
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	2175.13	725.043	85.9821	***
Temperature	1	150.062	150.062	17.7957	*
+or- granules	1	1539.07	1530.07	182.516	***
Error	4	33.73	8.4325	-	
Total	7	2208.86	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	5.80775	16.1223	12.25	8.06116
+or- granules	10.0593	27.9247	33.975	6.98117

All values for supernatants 2 - 4 zero T.

A significant effect of treatment could only be demonstrated in the first supernatant results since the other supernatant produced T. assay values of zero. This was mainly due to the addition of granules ($P<0.001$) having a greater effect than the temperature alteration ($P<0.05$).

Microtubule protein-zymogen granule experiment 05.

For thisexperiment isolated zymogen granules from 7 rat pancreas were combined with both M.A.P.-free MT. proteins and whole MT. proteins; M.A.P.-free protein was also used with added DMSO (10%). The T. ^{3}H-colchicine binding assay results were once again analysed by partitioned, one way analysis of variance.

MT.-G : 05.

One way Anovar using orthogonal linear contrasts.

Table 3.10 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	224.9	91.6	193.2	0
Replicates	2	2	2	2
Means	112.45	45.8	96.6	0
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	15674.1	5224.71	282.03	***
Temperature	1	4442.22	4442.22	239.791	***
+or- granules	1	407.168	407.168	21.9789	**
Error	4	74.1016	18.5254	-	
Total	7	15748.2	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	8.60823	23.8964	66.65	11.9482
+or- granules	14.9099	41.3898	17.475	10.3475

Table 3.11 : Supernatant 2. results and analysis, all values derived from measured total T. in μg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	0	164.6	0	0
Replicates	2	2	2	2
Means	0	82.3	0	0
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	10159.9	3386.64	2646.26	***
Temperature	1	6773.29	6773.29	5292.52	***
+or- granules	1	2257.76	2257.76	1764.17	***
Error	4	5.11914	1.27979	-	
Total	7	10165.1	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	2.26255	6.28084	82.3	3.14042
+or- granules	3.91885	10.8787	41.15	2.71969

All supernatants derived from samples without M.A.P's contained zero T.

Only supernatants derived from experimental samples including M.A.P. had a significant effect of treatment since the other samples gave T. assay values of zero. The partitioned effect of treatment, addition of granules, was of less significance ($P < 0.01$) than all other treatment effects ($P < 0.001$).

Microtubule protein-zymogen granule experiment 06.

Here porcine MT. proteins were combined with zymogen granules from 4 rat pancreas. Three supernatants were collected and their ^{3}H-colchicine binding assayed T. concentrations analysed by one way analysis of variance.

MT.-G : 06.

One way Anovar using orthogonal linear contrasts.

Table 3.12 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	1266.4	943.4	713.3	99.4
Replicates	2	2	2	2
Means	633.2	471.7	356.65	49.7

LINEAR CONTRAST COEFFICIENTS :

Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	364287	121429	20.9871	**
Temperature	1	26082.2	26082.2	4.50791	NS.
+or- granules	1	51116.8	51116.8	8.83476	*
Error	4	23143.5	5785.87	-	
Total	7	387430	-	-	

	- TOTAL CONTRASTS -		MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	152.13	422.313	161.5	211.156
+or- granules	263.497	731.467	195.8	182.867

Table 3.13 : Supernatant 2. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	0	161	0	0
Replicates	2	2	2	2
Means	0	80.5	0	0
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	9720.37	3240.12	19.5636	**
Temperature	1	6480.25	6480.25	39.1272	**
+or- granules	1	2160.08	2160.08	13.0424	*
Error	4	662.48	165.62	-	
Total	7	10382.9	-	-	

	- TOTAL CONTRASTS -		MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	25.7387	71.4506	80.5	35.7253
+or- granules	44.5807	123.756	40.25	30.939

All supernatant 3. values zero T.

Significant effects of treatment ($P < 0.01$) were shown only in first and second supernatants, the third supernatants all having T.concentrations of zero. In the first supernatant, the effect of temperature was not significant although it was highly so ($P < 0.01$) in the second supernatant. The effect of addition of the granules remained significant ($P < 0.05$) in both supernatants.

Microtubule protein-zymogen granule experiment 07.

In this experiment porcine MT. protein was combined with zymogen granules prepared from 4 rat pancreas and three supernatants were collected. Assayed T. concentrations were compared by one way analysis of variance.

MT.-G : 07.

One way Anovar using orthogonal linear contrasts.

Table 3.14 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	114.4	17.9	552.2	7.6
Replicates	2	1	2	2
Means	57.2	17.9	276.1	3.8
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-2	0	0
+or- granules	2	2	-3	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	90926.5	30308.8	1680.91	***
Temperature	1	1029.66	1029.66	57.1042	**
+or- granules	1	64588.8	64588.8	3582.05	***
Error	3	54.0937	18.0312	-	
Total	6	90980.6	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	10.4013	33.097	39.3	16.5485
+or- granules	23.2581	74.0072	232	12.3345

Table 3.15 : Supernatant 2. results and analysis, all values derived from measured total T. in μg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	.2	21.5	1.3	0
Replicates	1	2	1	1
Means	.2	10.75	1.3	0
LINEAR CONTRAST COEFFICIENTS :				
Temperature	2	-1	0	0
+or- granules	1	1	-3	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	127.055	42.3517	338.813 *
Temperature	1	74.2017	74.2017	593.613 *
+or- granules	1	26.4033	26.4033	211.227 *
Error	1	.125	.125	-
Total	4	127.18	-	-

	- TOTAL CONTRASTS - Standard error	Confidence interval	MEAN CONTRASTS - Mean effect	Confidence interval
Temperature	.866025	11.0037	10.55	5.50186
+or- granules	1.22475	15.5616	5.93333	5.1872

All values for supernatant 3. zero T.

Here the zero third supernatant results were not significant, however the effect of treatment in the first supernatant was very highly significant ($P < 0.001$) although the second supernatant was less so ($P < 0.05$). Partitioning the treatment effects showed a less significant effect of temperature ($P < 0.01$) than granule addition ($P < 0.001$) in the first supernatant while there was no such difference in the second supernatant.

Microtubule protein-zymogen granule experiment 08.

Porcine MT. proteins were combined with zymogen granule isolated from 50 mice pancreas. The three resultant supernatants were assayed by ^{3}H-colchicine binding assay for T. and for M.A.P. using RIA (see experiment RIA-MTG : 04). The assay results were treated by one way analysis of variance.

MT.-G : 08.

On way Anovar using orthogonal linear contrasts.

Table 3.16 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	397	129.3	243.1	15.6
Replicates	3	3	3	3
Means	132.333	43.1	81.0333	5.2
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	26537.4	8845.8	14.7259	**
Temperature	1	11943.9	11943.9	19.8834	**
+or- granules	1	89.334	89.334	.148717	NS.
Error	8	4805.58	600.697	-	
Total	11	31343	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	60.0349	138.44	89.2333	46.1468
+or- granules	103.983	239.786	6.68334	39.9643

Table 3.17 : Supernatant 2. results and analysis, all values derived from measured total T. in μg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	.5	150.2	0	0
Replicates	3	3	3	3
Means	.166667	50.0667	0	0

LINEAR CONTRAST COEFFICIENTS :

Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	5627.56	1875.85	3041.76	***
Temperature	1	3735.02	3735.02	6056.46	***
+or- granules	1	1261.69	1261.69	2045.88	***
Error	8	4.93359	.616699	-	
Total	11	5632.49	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	1.92359	4.4358	49.9	1.4786
+or- granules	3.33175	7.68303	25.1167	1.2805

Table 3.18 : Supernatant 3. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	90.8	91.9	0	73.8
Replicates	3	3	3	3
Means	30.2667	30.6333	0	24.6
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	1896.21	632.07	97.5548 ***
Temperature	1	.201663	.201663	3.11251E-2 NS.
+or- granules	1	1854.41	1854.41	286.212 ***
Error	8	51.833	6.47913	-
Total	11	1948.04	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	6.23496	14.3778	.366664	4.79261
+or- granules	10.7993	24.9031	30.45	4.15052

The treatment effect overall was significant in all the supernatants, the first being less so ($P < 0.01$) than the second and third ($P < 0.001$). In only the second supernatant were both partitioned treatments significant ($P < 0.001$), in the first supernatant the effect of addition of granules was insignificant and in the third supernatant the effect of temperature was insignificant.

Microtubule protein-zymogen granule experiments 01 to 08 - summary of results.

The table below shows a summary of all the experiments which assayed the supernatants collected using the in vitro granule-MT. procedure. The mean effects are shown for the temperature of incubation and addition or exclusion of granule in ug/ml of T. In the former case 37°C incubated values are given with respect to 4 °C and in the latter case those without granules are given with respect to the mean of those with granules.

Table 3.19 : Summary of mean effects, confidence intervals and significances for experiments MT.-G 01 to Mt.-G 08.

Expt No	SN. No	Mean temp. effect	±C.I.	Sig^{n} P.<	Mean granule effect	±C.I.	Sig^{n} P.<
01	1	-35.5	6.3	.001	+50.7	6.0	.001
	2	+56.6	4.1	.001	-20.3	3.6	.001
02	1	-271.2	33.6	.001	+100.1	27.9	.001
	2	+211.1	63.4	.001	-383.7	61.4	.001
03	1	-88.8	51.5	.01	+43.5	40.2	.05
(+ MAP's)	2	+441.7	108.2	.001	-306.1	93.7	.001
03	1	+0.1	4.9	NS.	+20.3	4.1	.001
(-MAP's)	2	+1.9	2.3	NS.	-1.5	2.0	NS.
04	1	-12.3	8.1	.05	+34.0	7.0	.001
All SN-2 to 4 values zero T.							
05	1	-66.7	11.9	.001	+17.5	10.3	.01
(+ MAP's)	2	+82.3	3.1	.001	-41.2	2.7	.001
All samples -MAP's zero T.							
06	1	-161.5	211.2	NS.	-195.8	182.9	.05
	2	+80.5	35.7	.01	-40.3	30.9	.05
All SN-3 values zero T.							
07	1	-39.3	16.5	.01	+232.0	12.3	.001
	2	+10.6	5.5	.05	-5.9	5.2	.05
All Sn-3 values zero T.							
08	1	-89.2	46.1	.01	-6.7	40.0	NS.
	2	+49.9	1.5	.001	-25.1	1.3	.001
	3	+0.4	4.8	NS.	-30.5	4.2	.001

The overall effect of incubation temperature in the first supernatant was in the main to cause a significant reduction in measured T. when

using the higher temperature as against the lower one. This reduction was corresponded by an overall increase in T. in the second supernatant originating from the originally warmed sample as against the one kept cold. The only experiment where the warm first supernatant T. levels were greater with respect to the cold was 'MT-G : 03' where the absence of M.A.P's produced a small insignificant increase.

In general the absence of granules produced an increase in the levels of T. in the first supernatant when such supernatants derived from samples containing only MT. protein were compared with the mean values for supernatants with granules and MT. proteins. The corresponding effect on the second supernatant, of leaving out granules, was to produce a decrease in the levels of supernatant T. The only experiments with exceptions to these granule induced alterations were 'MT-G : 06' and 'MT-G : 08'; the latter had an insignificant first supernatant fall in the T. level produced by leaving out the granules while the former had a similar significant fall ($P<0.05$) in the first supernatant.

In some experiments, ('MT-G : 04, 06, 07 and 08') further supernatants were collected from the resuspended second pellets using cold depolymerisation conditions. Most of the supernatant had insignificant alterations in T. levels as a result of prior differences in incubation temperatures or the addition of granules. Indeed in three cases ('MT-G : 04, 06, 07') the levels of T. in these later supernatants were all zero. In only MT-G : 08 were there measurable levels of T. in the third supernatants; the effect of raising the incubation temperature was to produce an insignificant increase in the T. levels while absence of granules produced a significant ($P<0.001$) reduction in these levels.

In experiments where the MT. protein used had had M.A.P's removed before incubation with the granule suspension, i.e., 'MT-G : 03 and 05', the M.A.P-free supernatants had zero T. levels in the latter while the

former had insignificant alterations in these levels as a result of differences in incubation temperatures and presence of granules.

The effects of exocrine pancreas stimulation on the MT-free T. equilibrium.

In order to establish the importance of MT. to the secretory process *in vivo* the levels of assembled MT-derived T. and free depolymerised T. were determined in the exocrine pancreas. These levels were assayed whilst the pancreas was under two different secretory states, resting and actively secreting. Initially mice in the two comparative treatment groups had their secretory states altered by altering their feeding regimes, i.e., by comparing free feeding animals with fasting animals. However, later experiments compared animals treated with the chemical secretagogue, Pilocarpine, with fasting animals.

Of the early experiments 'PANC : 01' compared the pancreas T. levels of fed and starved mice, 'PANC : 02' measured the levels in free feeding mice and 'PANC : 03' those of Pilocarpine-treated and fed mice. Most of the other experiments in this section were separated into two main groups on the basis of the time at which they were carried out. Both groups 'PANC : 04 to 13' and 'PANC : 14 to 18' compared starved mice with Pilocarpine-treated mice. The methodology used for each group was identical apart from the time for which the mice were left after injection (with either Pilocarpine or saline). In the earlier group mice were left for 25 to 30 minutes after injection before killing, in the later group the time elapsed was 45 to 60 minutes. In each case the mice always exhibited external signs of exocrine secretion and the mice within each experiment had identical incubation times.

All the T. pool results were determined from the ^{3}H-colchicine assay derived B-counter values by a computer programme. These results were then computer analysed to provide information about the effects of treatment on the mice as well as the variations between the mice. In

addition the variations between the experiments were studied by combining the results of the experiments within each group. The individual experimental results are given in this section initially with the combined results summarised at the end.

Exocrine pancreas microtubule experiment 01.

Here the levels of pancreas assembled and dis assembled T. were measured in two groups each of five mice. One group consisted of free feeding mice (F01 to F05) and the other of mice fasted for 24 hours (S01' to S05'). The pancreas polymerised T. and depolymerised T. were seperated for each mouse and assayed by ^{3}H-colchicine binding assay. The results were compared using one way analysis of variance incorporating a hierarchical partitioning of the variation to estimate the effects of treatment, mice variation and assay determinations (see the Appendix for details).

PANC : 01

One way Anovar using a hierarchical design.

Table 3.20 : Supernatant 1. results and analysis, allvalues derived from measured depolymerised pancreas T. in µg/ml.

Mouse No.	- Determinations - 1	2	3
F01	14.6	12.8	14.8
F02	6.4	6.0	9.5
F03	9.1	12.8	12.5
F04	3.3	8.8	3.3
F05	6.5	6.8	8.8
S01'	14.3	9.4	6.9
S02'	15.7	10.5	5.1
S03'	6.5	3.5	6.3
S04'	4.5	4.9	4.6
S05'	1.8	2.3	1.7

Source of variation		D.F.	S.S	M.S.	V.R.	
Treatments	:	1	47.8	47.8	1.19 NS	$\sigma_D^2 + 3\sigma_M^2 + 15T^2$
Mice within treatments	:	8	320.1	40.0	6.10***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	20	131.1	6.6	-	σ_D^2

Mice within treatment variance (P=0.05) : $5.09 \leqslant \sigma_M^2 \leqslant 49.92$

Table 3.21 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No.	1	2	3
F01	-	2.4	2.1
F02	-	-	0.5
F03	-	0.3	-
F04	0.3	-	0.5
F05	3.4	1.6	1.9
S01′	2.8	1.7	3.6
S02′	0.9	0.1	-
S03′	-	-	0.5
S04′	6.5	11.0	14.5
S05′	0.7	0.1	0.9

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	30.4	30.4	0.97 NS	$\sigma_D^2 + 3\sigma_M^2 + 15T^2$
Mice within treatments	:	8	250.1	31.3	15.38***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	20	40.7	2.0	-	σ_D^2

Mice within treatments variance (P=0.05) : $4.44 \leqslant \sigma_M^2 \leqslant 35.74$

In the case of both assayed polymerised and depolymerised T. there was not shown to be any significant effect of treatment (feeding or fasting) on the mice pancreas T. levels. However, in each case there was a very highly significant ($P < 0.001$) difference between the mice within each treatment group.

Exocrine pancreas microtubule experiment 02.

In this case the polymerised and depolymerised T. from the pancreas of 6 mice was determined. These mice were all free feeding, no fasting period was included. The MT. and 'free pool' T. components were isolated and assayed as before by ^{3}H-colchicine binding assay, three determinations being made per mouse supernatant.

PANC : 02

Table 3.22 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
F06	4.2	5.3	13.2
F07	1.4	2.9	3.3
F08	1.7	2.4	1.3
F09	4.7	7.3	5.3
F10	S a m p l e	l o s t	
F11	3.9	4.8	4.2

Mean ± confidence interval (P=0.05) = 4.4 ± 1.60

Table 3.23 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
F06	0.6	0.5	0.5
F07	0.8	23.8	0.6
F08	0.9	7.6	1.5
F09	0.8	-	0.4
F10	0.6	0.2	0.3
F11	0.4	1.2	0.7

Mean $\pm$ confidence interval = 1.0 ± 0.89

The determined 'free pool' T. gave an overall mean of 4.4µg/ml while MT. derived T. had a mean value of 1.0µg/ml.

Exocrine pancreas microtubule experiment 03.

Two groups each of three mice were treated and their MT- derived T. and 'free pool' T. pancreas content determined. All mice were free feeding before injection; one group (P01' to P03') with 3.99mg Pilocarpine in 100ul PBS per mouse the other group (F12 to F14) with 100ul of PBS. ^{3}H-colchicine binding assay determined T. concentrations were compared by a hierarchical design of one way analysis of variance.

PANC : 03

One way Anovar using a hierarchical design.

Table 3.24 : Supernatant 1. results and anylysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
P01'	4.4	6.2	6.1
P02'	5.3	7.0	5.1
P03'	3.5	5.2	5.0
F12	4.5	4.1	4.8
F13	3.2	3.6	8.3
F14	0.9	4.5	2.8

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	6.5	6.5	2.34 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	:	4	11.1	2.8	1.16 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	12	28.7	2.4	-	σ_D^2

Table 3.25 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
P01'	1.4	2.1	2.5
P02'	1.0	1.1	1.3
P03'	3.3	3.1	2.8
F12	2.3	4.7	3.3
F13	0.6	1.2	2.4
F14	1.1	-	0.4

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	0.4	0.4	0.09 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	:	4	19.2	4.8	9.38**	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	12	6.1	0.5	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.51 \leqslant \sigma_M^2 \leqslant 11.92$

In the case of both polymerised and depolymerised T. there was no significant effect of treatment on mouse pancreas T. level. However, the within treatment mouse values for polymerised T. were highly significantly ($P < 0.01$) different from one another.

Exocrine pancreas microtubule experiments 04 to 13.

This set of experiments were all carried out using the same methodology. In each case two groups each of three mice were treated to alter the secretory state of their exocrine pancreas. All the mice were pre-fasted overnight and then each group was injected; the 'P' group with 3.99mg of Pilocarpine in 100ul of PBS per mouse and the 'S' group with 100ul of PBS. Separated pancreas T. pools were then estimated by ^{3}H-colchicine binding assay and the results analysed by the hierarchical design of one way analysis of variance (see the Appendix for details). All calculations were by a computer programme.

PANC : 04

One way Anovar using a hierarchical design.

Table 3.26 : Supernatant 1. results and discussion, all values derived from measured depolymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No.	1	2	3
P01	8.0	8.4	8.8
P02	13.1	14.0	12.2
P03	5.1	7.0	6.7
S01	13.6	11.5	16.7
S02	12.2	9.6	5.5
S03	0.5	0.4	0.5

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	9.3	9.3	0.11 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	:	4	351.8	88.0	25.84 ***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	12	40.8	3.4	-	σ_D^2

Mice within treatments variance (P = 0.05) : $10.12 \leqslant \sigma_M^2 \leqslant 234.87$

Table 3.27 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
P01	-	0.4	0.2
P02	0.5	20.6	0.6
P03	0.2	0.4	0.1
S01	1.1	1.7	0.7
S02	0.4	0.4	1.1
S03	1.0	0.3	0.5

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	13.9	13.9	0.56 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	99.3	24.8	1.11 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	269.5	22.5	-	σ_D^2

PANC : 05

Table 3.28 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No.	1	2	3
P04	10.5	9.3	10.7
P05	8.8	8.4	8.9
P06	8.3	10.3	7.8
S04	7.7	9.8	7.8
S05	7.4	7.1	7.6
S06	10.5	9.7	11.2

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	0.9	0.9	0.2 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	18.7	4.7	6.5 **	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	8.7.	0.7	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.47 \leqslant \sigma_M^2 \leqslant 10.97$

Table 3.29 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P04	0.6	1.8	1.0
P05	1.6	1.0	1.7
P06	1.3	1.1	1.2
S04	1.3	4.9	1.5
S05	1.5	1.6	1.2
S06	1.4	1.1	1.4

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	1.2	1.2	1.58 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	3.0	0.8	0.98 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	9.3	0.8	-	σ_D^2

PANC : 06

One way Anovar using a hierarchical design.

Table 3.30 : Supernatant 1. Results and analysis, all values derived from measured depolymerised pancreas T.in µg/ml

	- Determinations -		
Mouse No	1	2	3
P07	3.5	3.6	5.7
P08	12.0	15.2	2.9
P09	16.6	19.4	26.4
S07	22.9	3.5	3.4
S08	5.3	15.2	8.3
S09	4.2	3.8	6.6

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	56.2	56.2	0.48 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	477.2	117.8	3.18 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	445.0	37.1	-	σ_D^2

Table 3.31 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P07	-	0.5	3.7
P08	0.4	0.2	-
P09	0.9	1.8	1.5
S07	2.5	0.3	0.3
S08	1.1	0.5	0.7
S09	0.4	0.3	-

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	0.4	0.4	0.5 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	3.7	0.8	1.0 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	11.8	1.0	-	σ_D^2

PANC : 07

One way Anovar using a hierarchical design.

Table 3.32 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P10	8.5	5.4	15.4
P11	10.3	9.2	10.4
P12	26.2	31.1	48.8
S10	7.4	11.8	7.3
S11	11.9	13.2	7.0
S12	7.6	8.4	6.8

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	390.9	390.9	1.19 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	1313.0	328.2	10.55 ***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	373.2	31.1	-	σ_D^2

Mice within treatments variance (P = 0.05) : $35.56 \leqslant \sigma_M^2 \leqslant 825.40$

Table 3.33 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P10	1.5	3.5	0.9
P11	0.9	1.5	9.6
P12	2.5	3.3	3.6
S10	2.6	0.7	5.8
S11	172.2	1.4	-
S12	1.5	1.8	1.2

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	1419.3	1419.3	0.92 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	6295.2	1548.8	0.95 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	19665.7	1638.8	-	σ_D^2

PANC : 08

One way Anovar using a hierarchical design.

Table 3.34 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P13	7.1	6.4	6.4
P14	8.9	8.9	9.3
P15	10.1	10.8	12.8
S13	10.8	8.1	9.9
S14	10.5	10.3	20.0
S15	15.3	16.3	14.8

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	1	68.9	68.9	3.2 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	4	85.8	21.4	3.6 *	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	12	71.0	5.9	-	σ_D^2

Mice within treatments variance (P - 0.05) : $1.86 \leqslant \sigma_M^2 \leqslant 43.13$

Table 3.35 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P13	14.3	1.3	15.6
P14	1.5	0.5	2.6
P15	2.0	3.4	1.6
S13	1.9	1.2	0.9
S14	2.1	6.7	4.3
S15	2.8	-	1.3

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	26.9	26.9	0.66 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	163.4	40.8	3.41 *	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	143.9	12.0	-	σ_D^2

Mice within treatments variance (P = 0.05) : $3.45 \leqslant \sigma_M^2 \leqslant 80.16$

PANC : 09

One way Anovar using a hierarchical design.

Table 3.36 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P16	4.7	4.9	5.5
P17	4.5	3.9	4.2
P18	6.1	7.0	4.9
S16	6.2	6.2	6.2
S17	7.7	8.1	7.7
S18	10.9	7.3	7.4

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	26.8	26.8	7.8 *	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	13.7	3.4	3.7 *	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	11.1	0.9	-	σ_D^2

Treatment difference ± confidence interval (P = 0.05) = 2.44 ± 2.42

Mice within treatment variance (P = 0.05) : $0.01 \leqslant \sigma_M^2 \leqslant 0.22$

Table 3.37 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P16	1.4	1.6	1.4
P17	2.4	1.3	2.1
P18	1.4	1.4	1.3
S16	1.1	0.9	1.1
S17	1.4	0.8	0.9
S18	1.3	1.2	1.3

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	1.0	1.0	6.4 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	:	4	0.6	0.2	2.0 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	12	0.9	0.1	-	σ_D^2

PANC : 10

One way Anovar using a hierarchical design.

Table 3.38 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P19	3.4	2.9	3.9
P20	4.9	2.9	4.8
P21	2.7	3.0	3.2
S19	3.1	3.7	3.6
S20	7.1	8.1	7.2
S21	4.5	3.5	5.7

Source of variation		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	11.3	11.3	1.56 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	:	4	28.9	7.2	16.74***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	:	12	5.2	0.4	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.81 \leqslant \sigma_M^2 \leqslant 18.87$

Table 3.39 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P19	2.1	0.2	1.2
P20	0.2	-	0.6
P21	2.6	0.6	1.2
S19	2.0	0.3	1.8
S20	2.6	0.6	1.6
S21	2.2	1.5	1.7

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	1.8	1.8	2.76 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	2.6	0.6	0.93 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	8.3.	0.7	-	σ_D^2

PANC : 11

One way Anovar using a hierarchical design.

Table 3.40 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P22	4.8	6.3	5.2
P23	4.2	4.3	3.3
P24	4.4	3.8	4.1
S22	3.2	4.2	5.1
S23	6.2	3.7	4.0
S24	3.5	4.6	3.2

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	0.4	0.4	0.32 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	5.2	1.3	1.83 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	8.5	0.7	-	σ_D^2

Table 3.41 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P22	1.4	1.0	4.2
P23	3.9	2.3	3.9
P24	1.0	0.6	0.7
S22	0.9	1.1	0.9
S23	2.5	2.2	2.3
S24	2.7	3.3	3.0

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	1	1.1×10^{-5}	1.1×10^{-5}	2.5×10^{-5} NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	4	16.8	4.2	6.18 **	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	12	8.1	0.7	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.42 \leqslant \sigma_M^2 \leqslant 9.76$

PANC : 12

One way Anovar using a hierarchical design.

Table 3.42 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P25	4.8	4.7	5.2
P26	4.8	5.0	2.7
P27	4.4	6.7	3.8
S25	4.1	3.6	5.8
S26	3.5	1.9	2.9
S27	3.9	7.2	4.1

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	1.6	1.6	0.65 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	9.9	2.5	1.56 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	19.1	1.6	-	σ_D^2

Table 3.43 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P25	-	1.0	-
P26	1.8	-	-
P27	0.2	-	-
S25	0.01	1.1	-
S26	0.5	0.7	0.9
S27	-	-	0.3

Source of variation	D.F.	S.S.	M.S.	V.R.		Parameters estimated
Treatments	: 1	1.1×10^{-2}	1.1×10^{-2}	4.6×10^{-2}	NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	0.9	0.2	0.74	NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	3.7	0.3	-		σ_D^2

PANC : 13

One way Anovar using a hierarchical design.

Table 3.44 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P28	6.9	5.2	6.5
P29	9.3	3.5	4.2
P30	10.0	5.1	5.8
S28	6.2	5.4	5.7
S29	5.3	5.6	14.6
S30	6.7	11.9	25.3

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	51.0	51.0	1.61 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	126.4	31.6	1.37 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	276.0	23.0	-	σ_D^2

Table 3.45 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P28	5.0	1.1	3.3
P29	4.1	2.3	2.2
P30	2.3	2.8	1.8
S28	3.8	1.3	1.9
S29	2.9	3.2	3.2
S30	2.0	1.3	1.4

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	0.8	0.8	0.73 NS	$\sigma_D^2 + 3\sigma_M^2$
Mice within treatments	: 4	4.5	1.1	0.94 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	14.4	1.2	-	σ_D^2

In all the experiments except experiment 09 there was no significant ($P > 0.05$) effect of treatment on the levels of T. in either the polymerised or the depolymerised pools. Experiment 09 produced a significant ($P < 0.05$) treatment effect of 2.44µg/ml in the depolymerised pool but none in the polymerised one. The within treatment mice T. levels had significant variation for the depolymerised pool in all experiments except experiments 06, 11, 12 and 13. However, the within treatment polymerised T. pool had no significant mouse variation overall except in experiments 08 and 11.

Exocrine pancreas microtubule experiments 14 to 18.

This series of experiments was carried out using a similar methodology to the previous groups. However, there was a difference in the number of mice included in the experiment in one case. Experiment 14 had two treatment groups; 'P' and 'S', each consisting of five mice rather than the normal three.

PANC : 14

One way Anovar using a hierarchical design.

Table 3.46 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P31	5.0	4.1	4.9
P32	13.2	15.7	3.7
P33	9.8	19.3	7.0
P34	11.6	20.6	5.5
P35	3.8	5.1	7.7
S32	7.6	5.6	5.5
S33	6.8	6.7	15.0
S34	6.7	7.5	8.2
S35	6.4	10.9	5.6
S36	9.6	8.8	16.1

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	3.1	3.1	0.11 NS	$\sigma_D^2 + 3\sigma_M^2 + 15T^2$
Mice within treatments	: 8	220.3	27.5	1.43 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 20	385.1	19.3	-	σ_D^2

Table 3.47 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P31	3.7	2.7	2.5
P32	5.1	2.9	3.9
P33	0.3	0.5	0.3
P34	0.8	0.8	1.5
P35	-	-	0.7
S32	0.2	0.1	0.4
S33	1.9	0.8	0.4
S34	0.5	-	0.6
S35	0.7	0.8	0.5
S36	0.9	1.3	0.7

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	8.4	8.4	1.93 NS	$\sigma_D^2 + 3\sigma_M^2 + 15\,T^2$
Mice within treatments	: 8	34.8	4.3	15.36 ***	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 20	5.7	0.3	-	σ_D^2

Mice within treatment variance (P = 0.05) : $0.62 \leqslant \sigma_M^2 \leqslant 4.97$

PANC : 15

One way Anovar using a hierarchical design.

Table 3.48 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P36	17.0	17.4	6.7
P37	13.4	8.9	80.7
P38	12.6	6.0	23.5
S37	47.2	46.1	31.3
S38	14.4	33.5	38.6
S39	9.3	5.1	9.8

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	102.8	102.8	0.17 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	2375.6	593.9	1.82 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	3922.3	326.9	-	σ_D^2

Table 3.49 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P36	16.7	3.0	6.8
P37	2.4	13.7	-
P38	5.4	1.0	0.2
S37	3.9	0.3	5.5
S38	3.5	1.7	10.0
S39	0.8	5.2	0.5

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	17.6	17.6	0.89 NS	$\sigma_D^2 + 3\sigma_M^2 + 9T^2$
Mice within treatments	: 4	79.3	19.8	0.82 NS	$\sigma_D^2 + 3\sigma_M^2$
Determinations within mice	: 12	288.7	24.1	-	σ_D^2

PANC : 16

One way Anovar using a hierarchical design.

Table 3.50 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P39	2.8	2.1	2.3
P40	3.7	4.4	2.1
P41	0.8	1.8	1.8
S41	2.8	2.2	1.9
S42	4.0	4.0	3.1
S43	3.1	1.9	2.1

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	0.6	0.6	0.26 NS	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	: 4	9.3	2.3	4.97 *	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	: 12	5.6	0.5	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.22 \leqslant \sigma_m^2 \leqslant 5.18$

Table 3.51 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in μg/ml.

	- Determinations -		
Mouse No	1	2	3
P39	0.6	1.6	4.3×10^{-2}
P40	0.4	0.6	1.0
P41	0.6	-	-
S41	-	0.4	0.5
S42	0.7	0.9	1.0
S43	1.9	1.1	1.1

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	1	0.4	0.4	0.76 NS	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	4	2.2	0.6	2.94 NS	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	12	2.3	0.2	-	σ_D^2

PANC : 17

One way Anovar using a hierarchical design.

Table 3.52 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P42	2.3	2.7	6.6
P43	7.7	6.0	6.3
P44	6.0	6.5	7.0
S44	4.3	5.4	7.7
S45	3.4	3.1	3.3
S46	8.3	7.5	8.6

Source of variation	D.F.	S.S.	M.S.	V.R.		Parameters estimated
Treatments	: 1	4.9×10^{-4}	4.9×10^{-4}	3.9×10^{-5}	NS	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	: 4	50.2	12.5	8.36	**	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	: 12	18.0	1.5	-		σ_D^2

Mice within treatments variance (P = 0.05) : $1.32 \leqslant \sigma_m^2 \leqslant 30.67$

Table 3.53 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P42	0.4	1.9	2.4
P43	2.0	3.2	3.1
P44	0.9	0.8	0.6
S44	1.1	2.0	1.1
S45	0.4	1.1	-
S46	-	-	0.2

Source of variations		D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	:	1	4.0	4.0	1.83 NS	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	:	4	8.7	2.2	6.73 **	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	:	12	3.9	0.3	-	σ_D^2

Mice within treatments variance (P = 0.05) : $0.22 \leqslant \sigma_m^2 \leqslant 5.13$

PANC : 18

One way Anovar using a hierarchical design.

Table 3.54 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P45	2.2	2.7	2.5
P46	4.5	3.5	2.9
P47	3.8	4.0	3.5
S47	5.4	4.2	5.2
S48	8.0	8.6	8.6
S49	7.3	6.4	7.3

Source of variation	D.F.	S.S.	M.S.	V.R.	Parameters estimated
Treatments	: 1	55.0	55.0	10.17 *	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	: 4	27.6	5.4	20.12 ***	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	: 12	3.2	0.3	-	σ_d^2

Treatment difference $\pm$ confidence interval (P = 0.05) = 3.50 $\pm$ 3.04

Mice within treatments variance (P = 0.05) : $0.62 \leqslant \sigma_m^2 \leqslant 14.28$

Table 3.55 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

	- Determinations -		
Mouse No	1	2	3
P45	3.2	2.7	2.7
P46	12.4	12.7	12.3
P47	3.3	3.3	2.7
S47	2.4	1.8	2.1
S48	0.6	1.8	0.9
S49	2.5	2.9	3.1

Source of variation	D.F.	S.S.	M.S.	V.R.		Parameters estimated
Treatments	: 1	76.2	76.2	1.65	NS	$\sigma_D^2 + 3\sigma_m^2 + 9T^2$
Mice within treatments	: 4	184.7	46.2	352.06	***	$\sigma_D^2 + 3\sigma_m^2$
Determinations within mice	: 12	1.6	0.1	-		σ_D^2

Mice within treatments variance (P = 0.05) : $5.51 \leqslant \sigma_m^2 \leqslant 127.87$

A significant ($P < 0.05$) effect of treatment was only produced in the depolymerised T. pool of the mice in experiment 18, the difference being in the region 3.5µg/ml. No other treatments were significant, although the mice within treatment variation was significant in three out of five experiments for both the depolymerised and polymerised pools.

Exocrine pancreas microtubule experiemtns 04 to 18 combined.

By combining the results of experiments 04 to 13 and 16 to 18 for analysis the variation between individual experiments was determined. This was done using a hierarchical design for a one way analysis of variance together with a block design for a two way analysis of variance. (See the Appendix for details). All calculations were once again done by a computer programme.

PANC : 04 to PANC : 13

One way Anovar using a hierarchical design, for combined results of experiments PANC : 04 to PANC : 13.

Table 3.56 : Supernatant 1. analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Source of variation		D.F.	S.S.	M.S.	V.R.
1. Experiments	:	9	1604.33	178.26	2.89 NS
2. Treatments within experiments	:	10	617.44	61.74	1.02 NS
3. Mice within treatments	:	40	242.55	60.61	5.78 ***
4. Determinations within mice	:	120	1258.58	10.49	-

Parameters estimated

1. $\sigma_D^2 + 3\sigma_m^2 + 9T^2 + 18\sigma_E^2$
2. $\sigma_D^2 + 3\sigma_m^2 + 9T^2$
3. $\sigma_D^2 + 3\sigma_m^2$
4. σ_D^2

Mice within treatments variance (P = 0.05) : $11.26 \leqslant \sigma_m^2 \leqslant 27.35$

Table 3.57 : Supernatant 2. analysis, all values derived from measured polymerised pancreas T. in μg/ml.

Source of variation	D.F.	S.S.	M.S.	V.R.
1. Experiments	9	1826.36	202.93	1.38 NS
2. Treatments within experiments	20	1465.32	146.53	0.90 NS
3. Mice within treatments	40	6490.01	162.25	0.97 NS
4. Determinations within mice	120	20135.6	167.80	-

Parameters estimated

1. $\sigma_D^2 + 3\sigma_m^2 + 9T^2 + 18\,\sigma_E^2$
2. $\sigma_D^2 + 3\sigma_m^2 + 9T^2$
3. $\sigma_D^2 + 3\sigma_m^2$
4. σ_D^2

Two way Anovar for mean treatment values of the combined experiments PANC : 04 to PANC : 13.

Table 3.58 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Experiment No	Pilocarpine Treatment Mean	Saline Treatment Mean
PANC : 04	9.27	7.83
PANC : 05	9.22	8.76
PANC : 06	11.68	8.15
PANC : 07	18.37	9.05
PANC : 08	8.98	12.89
PANC : 09	5.09	7.53
PANC : 10	3.51	5.10
PANC : 11	4.49	4.18
PANC : 12	4.69	4.09
PANC : 13	6.29	9.65

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatments	: 1	0.94	0.94	0.13	NS
Experiments	: 9	178.26	19.81	2.63	NS
Error	: 9	67.66	7.52	-	
Total	: 19	246.86	-	-	

Table 3.59 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Experiment No	Pilocarpine Treatment Mean	Saline Treatment Mean
PANC : 04	2.56	0.80
PANC : 05	1.26	1.78
PANC : 06	0.99	0.68
PANC : 07	3.05	20.80
PANC : 08	4.78	2.33
PANC : 09	1.58	1.10
PANC : 10	0.97	1.59
PANC : 11	2.12	2.12
PANC : 12	0.33	0.38
PANC : 13	2.76	2.33

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatments	: 1	9.16	9.16	0.54	NS
Experiments	: 9	202.93	22.55	1.32	NS
Error	: 9	153.65	17.07	-	
Total	: 19	365.75	-	-	

PANC : 16 to PANC : 18

One way Anovar using a hierarchical design for combined results of experiments PANC : 16 to PANC : 18.

Table 3.60 : Supernatant 1. analysis, all values derived from measured depolymerised pancreas T. in µg/ml

Source of variation	D.F.	S.S.	M.S.	V.R.	
1. Experiments	: 2	94.13	47.06	2.54	NS
2. Treatments within experiments	: 3	55.61	18.54	2.74	NS
3. Mice within treatments	: 12	81.15	6.76	9.06	***
4. Determinations within mice	: 36	26.86	0.75	-	

Parameters estimated

1. $\sigma_D^2 + 3\sigma_m^2 + 9T^2 + 18\,\sigma_E^2$
2. $\sigma_D^2 + 3\sigma_m^2 + 9T^2$
3. $\sigma_D^2 + 3\sigma_m^2$
4. σ_D^2

Mice within treatment variance (P=0.05) : $1.03 \leqslant \sigma_m^2 \leqslant 5.46$

Table 3.61 : Supernatant 2. analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Source of variation	D.F.	S.S.	M.S.	V.R.	
1. Experiments	: 2	118.82	59.41	2.21	NS
2. Treatments within experiments	: 3	80.55	26.85	1.65	NS
3. Mice within treatments	: 12	195.55	16.30	76.09	***
4. Determinations within mice	: 36	7.71	0.21	-	

Parameters estimated

1. $\sigma_D^2 + 3\sigma_m^2 + 9T^2 + 18\,\sigma_E^2$
2. $\sigma_D^2 + 3\,\sigma_m^3 + 9T^2$
3. $\sigma_D^2 + 3\,\sigma_m^2$
4. σ_D^2

Mice within treatments variance (P=0.05) : $2.76 \leqslant \sigma_m^2 \leqslant 14.61$

Two way Anovar for mean treatment values of the combined experiments PANC : 16 to PANC : 18.

Table 3.62 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Experiment No	Pilocarpine Treatment Mean	Saline Treatment Mean
PANC : 16	2.42	2.79
PANC : 17	5.67	5.66
PANC : 18	3.28	6.78

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatments	: 1	2.48	2.48	1.34	NS
Experiments	: 2	10.46	5.23	2.82	NS
Error	: 2	3.70	1.85	-	
Total	: 5	16.64	-	-	

Table 3.63 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Experiment No	Pilocarpine Treatment Mean	Saline Treatment Mean
PANC : 16	0.54	0.85
PANC : 17	1.71	0.77
PANC : 18	6.14	2.02

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatments	: 1	3.75	3.75	1.45	NS
Experiments	: 2	13.20	6.60	2.54	NS
Error	: 2	5.20	2.60	-	
Total	: 5	22.15	-	-	

No significant variations could be demonstrated between the experiments in either of the two combined sets, nor was there any demonstrable treatment effect using either the one or two way analysis of variance. However, the one way analysis did show a very highly significant ($P<0.001$) variation between the mice within treatment results for measured depolymerised T. in both sets of experiments and for polymerised T. in the second set (16 to 18).

Exocrine pancreas microtubule experiments 01 to 18
- summary of the results and analyses.

The experiments in this section all attempted to compare the MT. and T. pools in the actively secreting and resting pancreas of mice. The results obtained from the ^{3}H-colchicine binding assay for T. were analysed using a hierarchical design for analysis of variance. In the tables below the experimental results for the two pancreas T. pools are presented. Each table shows the significance of the effects of treatment of the mice together with the treatment difference when significant. In addition the significance of the 'Mice within treatment' variation is shown with the estimated variance if significant. Finally the 'Determinations within mice' estimated variances are also presented. At the end of each table are the relevant values for the pooled experimental results.

Table 3.64 : Supernatant 1. results and analyses summary, all values derived from measured depolymerised pancreas T. in µg/ml.

Experiment No.	-Treatments- Significance	Difference	-Mice within Treatments- Significance	Variance	Determinations Variance
01	NS	-	.001	11.15	6.6
02	-	-	-	-	-
03	NS	-	NS	-	2.4
04	NS	-	.001	28.18	3.4
05	NS	-	.01	1.32	0.7
06	NS	-	NS	-	37.1
07	NS	-	.001	99.05	31.1
08	NS	-	.05	5.18	5.9
09	.05	2.44	.05	0.03	0.9
10	NS	-	.001	2.26	0.4
11	NS	-	NS	-	0.7
12	NS	-	NS	-	1.6
13	NS	-	NS	-	23.0
14	NS	-	NS	-	19.3
15	NS	-	NS	-	326.9
16	NS	-	.05	0.62	0.5
17	NS	-	.01	3.68	1.5
18	.05	3.50	.001	1.71	0.3
04-13	NS	-	.001	16.71	10.5
16-18	NS	-	.001	2.01	0.8

Table 3.65 : Supernatant 2. results and analyses summary, all values derived from measured polymerised pancreas T. in µg/ml.

Experiment No.	-Treatments- Significance	Difference	-Mice within Treatments- Significance	Variance	Determinations Variance
01	NS	-	.001	9.74	2.0
02	-	-	-	-	-
03	NS	-	.01	1.43	0.5
04	NS	-	NS	-	22.5
05	NS	-	NS	-	0.8
06	NS	-	NS	-	1.0
07	NS	-	NS	-	1638.8
08	NS	-	.05	9.62	12.0
09	NS	-	NS	-	0.1
10	NS	-	NS	-	0.7
11	NS	-	.01	1.17	0.7
12	NS	-	NS	-	0.3
13	NS	-	NS	-	1.2
14	NS	-	.001	1.35	0.3
15	NS	-	NS	-	24.1
16	NS	-	NS	-	0.2
17	NS	-	.01	0.62	0.3
18	NS	-	.05	15.34	0.1
04-13	NS	-	NS	-	167.8
16-18	NS	-	.001	5.36	0.2

The effect of treatment for pancreatic secretion had a significant ($P<0.05$) effect in only two experiments, 'PANC : 09 and 18'. In both cases the levels of depolymerised T. were higher as a whole in the saline-treated mice than in the Pilocarpine-treated mice. In experiment 'PANC : 09' the difference between the two groups of mice was 2.4µg/ml and in experiment 'PANC : 18' 3.5µg/ml. No concomitant effects of treatment for pancreatic secretion could be demonstrated in the second supernatant corresponding to changes in the levels of polymerised T.

Throughout the experiments the 'within treatment groups' mice variation was considerable. Significant variation was demonstrated in ten out of eighteen of the first supernatant results and seven out of eighteen of the second supernatant results. The levels of this significance varied from insignificant ($P>0.05$) to very highly significant ($P<0.001$) with variances estimated at between 0.03 and 99.1 for the first supernatant and between 0.6 and 15.3 for the second. Similarly a great deal of variation was present in the 'within mice' determinations, estimated variances lay between 0.3 and 326.9 for the first supernatant and between 0.1 and 1638.0 for the second.

So far as the pooled experimental results were concerend once again no significant effect of treatment, to induce secretion, was shown. The overall 'within treatment group' mice variation was significant in the first supernatant for experiments 04 to 13 and 16 to 18 but only in 16 to 18 in the second supernatants. The 'within mice' determination variation was greater in the larger group of experiments than in the smaller group. The variation between individual experiments was also shown to be insignificant in all cases.

Examination of the microtubule, free tubulin equilibrium during the stimulated exocrine pancreas time course.

The previous group of experiments tried to examine the effect of Pilocarpine treatment on the two exocrine pancreas T. pools at a specific time point after injection. This second group of experiments was designed to look at the same T. pools only this time over a continuous period after injection. In this way it was hoped to compare the T. pools of the actively secreting and basal secreting pancreas over the period from commencement of secretion to cessation. Once again the ^{3}H-colchicine binding assay was used to measure the separated T. pools only this time the experimental results were analysed by a more complex statistical procedure.

Exocrine pancreas microtubule experiments 19 to 22.

In experiments 19, 20 and 22 the polymerised and depolymerised T. pools in the pancreas of mice injected with pilocarpine were examined over the time period after injection. Experiment 21 compared these same pools in two groups of mice over similar time periods; one group was injected with pilocarpine as before, the other was a control group given only PBS. Four mice were included in each time period group in all the experiments except number 22 where only three were used. For each supernatant obtained three determinations were carried out to obtain the mean T. concentrations shown. The time periods used for experiments 19, 21 and 22 were 10, 20, 30, 45 and 60 minutes after injection; experiment 20 used the time periods 1, 2.5, 5, 10 and 20 minutes after injection.

The ^{3}H-colchicine binding assayed results were analysed using a one way analysis of variance where the treatment effect was partitioned

into a linear, quadratic and cubic portion using orthogonal polynomials (see the Appendix for details). All calculations were carried out by computer programmes (see the Appendix). In addition the polymerised T. pool supernatants for experiment 22 were assayed by R.I.A., see RIA-PANC : 01 for results.

PANC : 19

One way Anovar using orthogonal polynomials.

Table 3.66 : Orthogonal polynomial coefficients for treatment time points, used to analyse results of both supernatant 1. and supernatant 2.

Treatment Levels	Reduced Levels	Orthogonal polynomial coefficients - Linear	Quadratic	Cubic
10	0	- 2.30	+ 3.15	- 2.71
20	1	- 1.30	- 0.89	+ 3.95
30	2	- 0.30	- 2.94	+ 1.76
45	3.5	+ 1.20	- 2.25	- 5.02
60	5	+ 2.70	+ 2.93	+ 2.02

Table 3.67 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in μg/ml.

Time (mins)	Mouse Means - 1	2	3	4	Overall Mean	Overall Total
10	2.89	1.34	3.21	6.47	3.48	13.91
20	3.11	6.11	4.15	4.15	4.38	17.53
30	14.74	20.48	5.59	3.25	11.01	44.05
45	17.15	11.57	2.31	4.68	8.93	35.72
60	3.69	6.43	3.93	3.81	4.47	17.87

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatment overall	: 4	176.05	44.01	1.87	NS
Linear effect	: 1	8.46	8.46	0.36	NS
Quadratic effect	: 1	126.54	126.54	5.37	*
Cubic effect	: 1	5.29	5.29	0.22	NS
Residual	: 1	35.76	35.76	7.52	NS
Error	: 15	353.47	23.56	-	
Total	: 19	529.52	-	-	

Formula of the response curve : $Y = 6.5 + 0.4X - 1.0X^2$

Curve points :-

X coordinates	Y coordinates	Standard error
10	2.53	2.22
20	6.85	1.40
30	9.22	1.66
45	9.10	1.62
60	4.57	2.33

Table 3.68 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Time (mins)	Mouse 1	2	Means 3	4	Overall Mean	Overall Total
10	1.93	1.98	1.76	2.48	2.04	8.16
20	1.16	0.94	1.05	0.00	0.79	3.16
30	0.45	0.63	2.11	0.81	1.00	4.00
45	0.36	0.58	1.72	0.54	0.80	3.20
60	0.22	0.82	0.00	0.67	0.43	1.71

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatment overall	: 4	5.97	1.49	5.02	**
Linear effect	: 1	3.86	3.86	12.98	**
Quadratic effect	: 1	0.60	0.60	2.03	NS
Cubic effect	: 1	1.05	1.05	3.51	NS
Residual	: 1	0.46	0.46	1.55	NS
Error	: 15	4.46	0.30	-	
Total	: 19	10.43	-	-	

Formula of the response curve : $Y = 1.0 - 0.2X$

Curve points :-

X coordinates	Y coordinates	Standard Error
10	1.58	0.20
20	1.33	0.15
30	1.09	0.12
45	0.71	0.15
60	0.34	0.22

PANC : 20

One way Anovar using orthogonal polynomials.

Table 3.69 : Orthogonal polynomial coefficients for treatment time points, used to analyse results of both supernatant 1. and supernatant 2.

Treatment Levels	Reduced Levels	Orthogonal polynomial coefficients - Linear	Quadratic	Cubic
0	0	- 3.00	+ 7.75	- 9.97
2.5	1	- 2.00	+ 0.50	+ 8.96
5	2	- 1.00	- 4.75	+10.91
10	4	+ 1.00	- 0.25	-12.10
20	8	+ 5.00	+ 5.75	+ 2.20

Table 3.70 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Time (mins)	Mouse Means - 1	2	3	4	Overall Mean	Overall Total
0	5.11	7.50	6.39	5.57	6.14	24.57
2.5	12.17	3.28	5.47	4.55	6.37	25.47
5	2.63	6.74	5.04	3.70	4.38	17.51
10	7.43	4.32	7.37	7.41	6.63	26.53
20	5.97	5.81	4.61	2.16	4.63	18.50

Source of variation	D.F.	S.S.	M.S.	V.R.	
Treatment overall	: 4	17.56	4.39	0.85	NS
Linear effect	: 1	3.34	3.34	0.65	NS
Quadratic effect	: 1	0.45	0.45	0.09	NS
Cubic effect	: 1	6.24	6.24	1.27	NS
Residual	: 1	7.52	7.52	1.46	NS
Error	: 15	77.54	5.17	-	
Total	: 19	95.09	-	-	

Formula of the response curve : Y = 5.63 (Standard error = 0.51)

Table 3.71 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Time (mins)	Mouse Means - 1	2	3	4	Overall Mean	Overall Total
0	3.69	4.79	5.11	3.48	4.72	16.48
2.5	2.08	3.92	6.90	3.72	4.16	16.63
5	3.72	3.98	3.00	3.44	3.38	13.53
10	3.66	4.79	2.85	6.75	4.51	18.05
20	1.10	6.10	3.67	5.42	4.07	16.29

Source of variation	D.F.	S.S.	M.S.	V.R.
Treatment overall	: 4	2.70	0.67	0.27 NS
Linear effect	: 1	0.07	0.07	0.03 NS
Quadratic effect	: 1	0.003	0.003	0.001 NS
Cubic effect	: 1	1.40	1.40	0.56 NS
Residual	: 1	1.23	1.23	0.59 NS
Error	: 15	37.97	2.52	-
Total	: 19	40.49	-	-

Formula of the response curve Y = 4.05 (Standard error = 0.35)

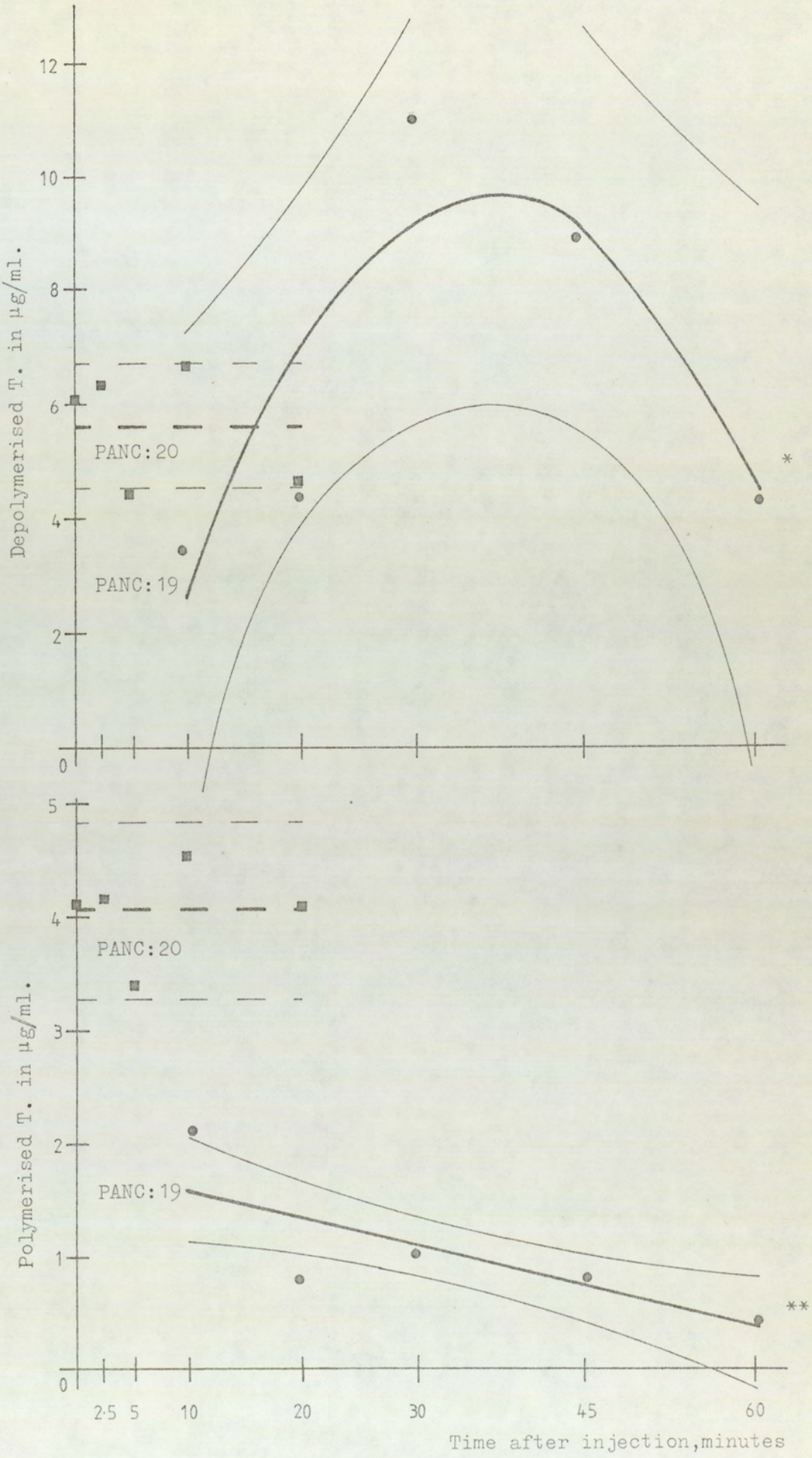
Table 3.72 : Reanalysis of supernatant 1. and supernatant 2. using normalised data (Log_n (Y + 1), where Y is the mean mouse pancreas T. value in µg/ml).

Supernatant 1 :-

Source of variation		D.F.	S.S.	M.S.	V.R.	
Treatment overall	:	4	0.53	0.13	1.23	NS
Linear effect	:	1	0.08	0.08	0.74	NS
Quadratic effect	:	1	0.01	0.01	0.09	NS
Cubic effect	:	1	0.25	0.25	2.31	NS
Residual	:	1	0.19	0.19	1.76	NS
Error	:	15	1.62	0.11	-	
Total	:	19	2.15	-	-	

Supernatant 2 :-

Source of variation		D.F.	S.S.	M.S.	V.R.	
Treatment overall	:	4	0.003	0.001	0.006	NS
Linear effect	:	1	0.005	0.005	0.04	NS
Quadratic effect	:	1	0.002	0.002	0.02	NS
Cubic effect	:	1	0.006	0.006	0.05	NS
Residual	:	1	-	-	-	NS
Error	:	15	1.823	0.122	-	
Total	:	19	1.83	-	-	



Shown are lines of best fit, observed values and 95% confidence limits.

PANC : 21

Two way Anovar using orthogonal polynomials.

Table 3.73 : Orthogonal polynomial coefficients for time points, used to analyse results of both supernatant 1. and supernatant 2.

Treatment Levels	Reduced Levels	Orthogonal polynomial coefficients - Linear	Quadratic	Cubic
10	0	- 2.30	+ 3.15	- 2.71
20	1	- 1.30	- 0.89	+ 3.95
30	2	- 0.30	- 2.94	+ 1.76
45	3.5	+ 1.20	- 2.25	- 5.2
60	5	+ 2.70	+ 2.93	+ 2.02

Coefficients for treatments :-

Saline : all - 1.00

Pilocarpine : all + 1.00

Table 3.74 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Treatments	Times (mins)	Replicate Mice 1	2	3
	10	5.52	4.58	4.12
	20	5.06	2.67	9.37
Saline	30	6.21	7.02	4.32
	45	0.73	7.47	4.89
	60	5.93	5.26	4.90
	10	6.44	4.08	9.16
	20	6.83	3.00	9.33
Pilocarpine	30	2.45	3.56	2.12
	45	7.15	8.67	5.85
	60	7.93	9.82	6.72

Source of variation	D.F.	S.S.	M.S.	V.R.	
Total Treatment	: 9	64.08	7.12	1.50	NS
Treatment	: 1	7.56	7.56	1.59	NS
Times overall	: 4	19.63	4.91	1.03	NS
Linear effect	: 1	3.59	3.59	0.76	NS
Quadratic effect	: 1	7.89	7.89	1.66	NS
Cubic effect	: 1	0.05	0.05	0.01	NS
Residual	: 1	8.10	8.10	1.71	NS
Treatment x Times	: 4	36.89	9.22	1.94	NS
Trt x T - L	: 1	4.45	4.45	0.94	NS
Trt x T - Q	: 1	11.76	11.76	2.48	NS
Trt x T - C	: 1	7.33	7.33	1.54	NS
Trt x T - R	: 1	13.35	13.35	2.81	NS
Error	: 20	94.89	4.74	-	
Total	: 9	158.97	-	-	

Table 3.75 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Treatments	Times (mins)	Replicate Mice 1	2	3
	10	6.60	0.33	-
	20	1.81	1.86	2.03
Saline	30	2.84	3.30	6.20
	45	1.50	3.57	4.27
	60	4.12	3.41	2.68
	10	1.94	3.27	6.72
	20	2.62	2.04	1.84
Pilocarpine	30	0.86	1.19	0.28
	45	4.08	2.61	2.53
	60	2.63	3.33	2.55

Source of variation	D.F.	S.S.	M.S.	V.R.	
Total Treatment	: 9	27.59	3.07	1.13	NS
Treatment	: 1	1.21	1.21	0.45	NS
Times overall	: 4	6.10	1.53	0.56	NS
Linear effect	: 1	0.88	0.88	0.33	NS
Quadratic effect	: 1	1.73	1.73	0.64	NS
Cubic effect	: 1	3.18	3.18	1.17	NS
Residual	: 1	0.30	0.30	0.11	NS
Treatment x Times	: 4	20.27	5.07	1.87	NS
Trt x T - L	: 1	2.16	2.16	0.79	NS
Trt x T - Q	: 1	7.96	7.96	2.93	NS
Trt x T - C	: 1	2.87	2.87	1.06	NS
Trt x T - R	: 1	7.28	7.28	2.68	NS
Error	: 20	54.29	2.71	-	
Total	: 29	81.87	-	-	

Table 3.76 : Reanalysis of supernatant 1. and supernatant 2. results using normalised data (Log_{10} (Y + 1), where Y is the mouse pancreas T. determination value in µg/ml).

Supernatant 1 :-

Source of variation	D.F.	S.S.	M.S.	V.R.
Total Treatment	: 9	0.38	0.04	1.42 NS
Treatment	: 1	0.02	0.02	0.80 NS
Times overall	: 4	0.11	0.03	0.90 NS
Linear effect	: 1	0.01	0.01	0.45 NS
Quadratic effect	: 1	0.07	0.07	2.33 NS
Cubic effect	: 1	0.001	0.001	0.02 NS
Residual	: 1	0.02	0.02	0.78 NS
Treatment x Times	: 4	0.25	0.06	2.10 NS
Trt x T - L	: 1	0.02	0.02	0.84 NS
Trt x T - Q	: 1	0.04	0.04	1.34 NS
Trt x T - C	: 1	0.08	0.08	2.69 NS
Trt x T - R	: 1	0.11	0.11	3.54 NS
Error	: 20	0.59	0.03	-
Total	: 29	0.97	-	-

Formula of the response curve : Y = 0.79 (Standard error = 0.03)

Supernatant 2 :-

Source of variation	D.F.	S.S.	M.S.	V.R.	
Total Treatment	: 0	0.60	0.07	1.99	NS
Treatment	: 1	0.004	0.004	0.11	NS
Times overall	: 4	0.11	0.03	0.80	NS
Linear effect	: 1	0.08	0.08	2.26	NS
Quadratic effect	: 1	0.005	0.005	0.14	NS
Cubic effect	: 1	0.02	0.02	0.51	NS
Residual	: 1	0.01	0.01	0.30	NS
Treatment x Times	: 4	0.49	0.12	3.65	*
Trt x T - L	: 1	0.05	0.05	1.47	NS
Trt x T - Q	: 1	0.23	0.23	6.87	*
Trt x T - C	: 1	0.08	0.08	2.37	NS
Trt x T - R	: 1	0.13	0.13	3.88	NS
Error	: 20	0.67	0.03	-	
Total	: 29	1.28	-	-	

Formula of the response curve :-

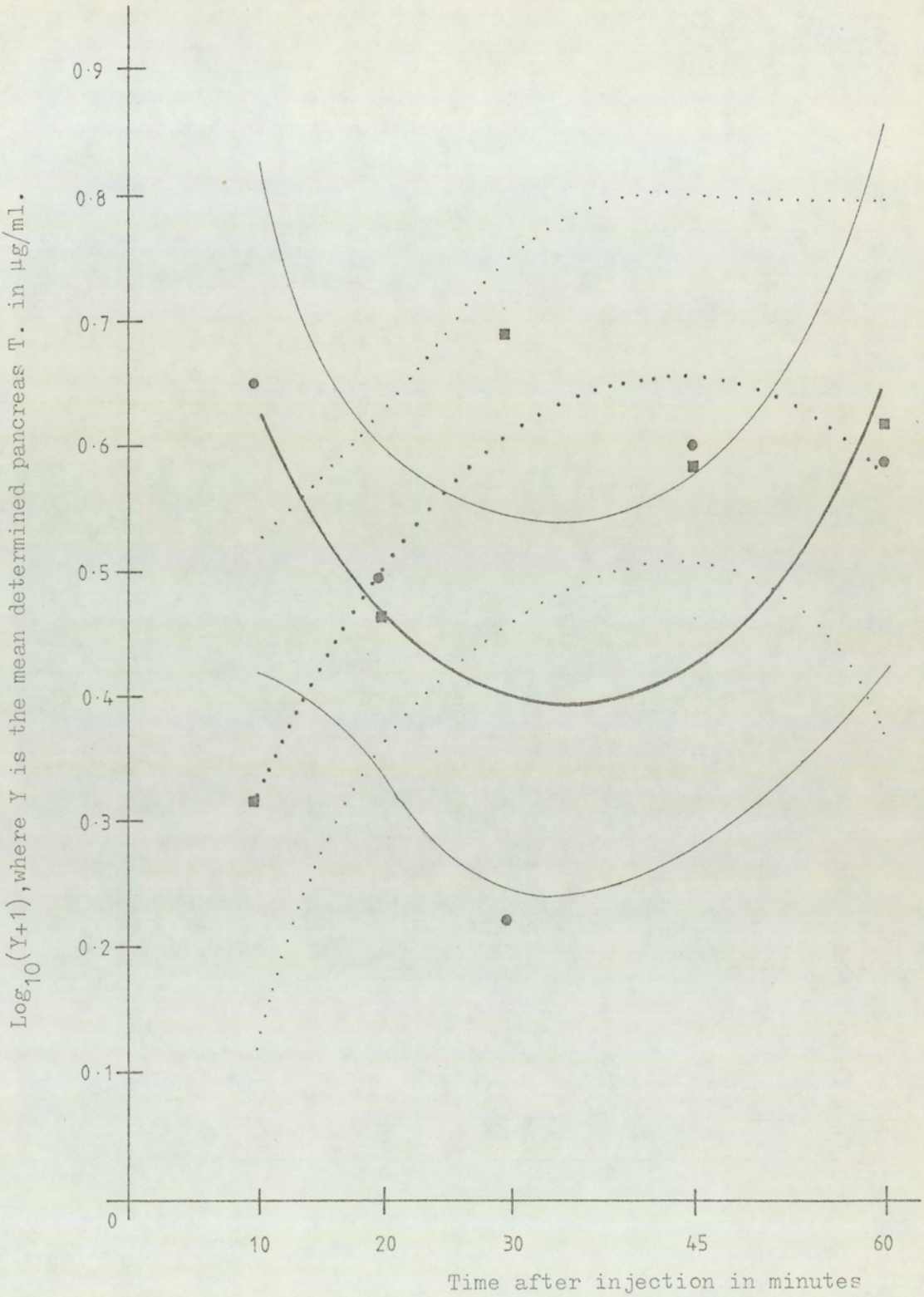
$$Y = 0.518 - 0.012 + 0.028X + 0.005\ X^2 - 0.023X + 0.034\ X^2$$

(from the model : Y = general mean + K_1.Trt + K_2.T-L + K_3.T-Q + K_5.Trt x T-L + K_6.Trt x T-Q)

Curve points :-

	X coordinates	Y coordinates	S.E.	- Confidence Limits -	
	10	0.32	0.10	0.12	0.52
	20	0.49	0.06	0.36	0.62
Saline	30	0.60	0.07	0.45	0.75
	45	0.66	0.07	0.51	0.80
	60	0.58	0.10	0.37	0.79
	10	0.62	0.10	0.42	0.82
	20	0.46	0.06	0.34	0.59
Pilocarpine	30	0.39	0.07	0.24	0.54
	45	0.42	0.07	0.28	0.57
	60	0.63	0.10	0.42	0.85

Figure 3.2 : Pancreas microtubules during Pilocarpine induced secretion.



——— Line of best fit for Pilocarpine injected mice, ● observed values.
····· Line of best fit for saline injected mice, ■ observed values.
Shown are 95% confidence limits for lines of best fit, interaction significant at the $P<0.005$ level.

PANC : 22

One way Anovar using orthogonal polynomials.

Table 3.77 : Orthogonal polynomial coefficients for treatment time points, used to analyse results of both supernatant 1. and supernatant 2.

Treatment Levels	Reduced Levels	Orthogonal polynomial coefficients - Linear	Quadratic	Cubic
10	0	- 2.30	+ 3.15	- 2.71
20	1	- 1.30	- 0.89	+ 3.95
30	2	- 0.30	- 2.94	+ 1.76
45	3.5	+ 1.20	- 2.25	- 5.02
60	5	+ 2.70	+ 2.93	+ 2.02

Table 3.78 : Supernatant 1. results and analysis, all values derived from measured depolymerised pancreas T. in µg/ml.

Time (mins)	Mouse Means 1	2	3	Overall Mean	Overall Total
10	6.02	7.40	13.02	8.81	26.44
20	7.72	1.46	3.69	4.29	12.87
30	3.47	13.96	8.53	8.56	25.68
45	5.39	5.60	4.92	5.31	15.92
60	12.17	14.17	3.65	10.00	29.99

Source of variation	D.F.	S.S.	M.S.	V.R.
Treatment overall	: 4	72.40	18.10	1.11 NS
Linear effect	: 1	4.64	4.64	0.29 NS
Quadratic effect	: 1	23.67	23.67	1.46 NS
Cubic effect	: 1	0.15	0.15	0.009 NS
Residual	: 1	43.94	43.94	2.70 NS
Error	: 10	162.56	16.26	-
Total	: 14	234.96	-	-

Table 3.79 : Supernatant 2. results and analysis, all values derived from measured polymerised pancreas T. in µg/ml.

Time (mins)	- Mouse Means - 1	2	3	Overall Mean	Overall Total
10	0.17	0.65	0.36	0.39	1.18
20	4.26	0.00	0.04	1.43	4.29
30	0.50	6.82	0.00	2.44	7.32
45	1.01	1.54	0.00	0.85	2.55
60	2.45	0.59	0.00	1.01	3.04

Source of variation	D.F.	S.S.	M.S.	V.R.
Treatment overall	: 4	7.18	1.79	0.39 NS
Linear effect	: 1	0.01	0.01	0.003 NS
Quadratic effect	: 1	3.43	3.43	0.75 NS
Cubic effect	: 1	2.40	2.40	0.53 NS
Residual	: 1	1.34	1.34	0.29 NS
Error	: 10	45.50	4.55	-
Total	: 14	52.68	-	-

Table 3.80 : Reanalysis of supernatant 1. and supernatant 2. using normalised data (Log_n (Y+ 1), where Y is the mean mouse pancreas T. value in µg/ml).

Supernatant 1 :-

Source of variation	D.F.	S.S.	M.S.	V.R.
Treatment overall	: 4	1.11	0.28	1.04 NS
Linear effect	: 1	0.06	0.06	0.22 NS
Quadratic effect	: 1	0.33	0.33	1.22 NS
Cubic effect	: 1	0.04	0.04	0.15 NS
Residual	: 1	0.68	0.68	2.52 NS
Error	: 10	2.70	2.27	-
Total	: 14	3.81	-	-

Supernatant 2 :-

Source of variation	D.F.	S.S.	M.S.	V.R.
Treatment overall	: 4	0.36	0.09	0.16 NS
Linear effect	: 1	0.04	0.04	0.07 NS
Quadratic effect	: 1	0.19	0.19	0.35 NS
Cubic effect	: 1	0.08	0.08	0.15 NS
Residual	: 1	0.05	0.05	0.09 NS
Error	: 10	5.50	0.55	-
Total	: 14	5.86	-	-

Experiments 19 and 21 were the only ones to show a significant effect of treatment on the levels of either polymerised or depolymerised pancreas T. in the mice. In experiment 19 a significant quadratic effect of treatment was demonstrated on the depolymerised pancreas T. pool and a significant linear effect on the polymerised T. pool. Curves of best fit were plotted to show these effects graphically. In the case of experiment 22 a significant effect was only shown once the data had been normalised. This effect was shown on the polymerised T. pool where a

significant quadratic interaction was demonstrated between the two treatments. Once again curves of best fit were plotted to illustrate this interaction graphically,

Measurement of MT. proteins using the radioimmunoassay technique.

The experiments in the two previous sections of the results used the ^{3}H-colchicine binding assay for T. to estimate both the amount of T. bound *in vitro* to zyomogen granules and also the alterations in T. pools during exocrine pancreatic secretion. Here we present the results of similar experiments using R.I.A's to measure both T. and M.A.P. Experiments 'RIA-MTG : 01' to 'RIA-MTG : 04' were *in vitro* granule binding procedures carried out as before while 'RIA-PANC : 01' was an *in vivo* exocrine pancreas experiment. In the case of 'RIA-MTG : 04' the samples assayed for M.A.P. had previously been assayed for T. by ^{3}H-colchicine binding. Also the samples assayed for T. in 'RIA - PANC : 01' had previously been assayed by ^{3}H-colchicine binding in 'PANC : 22'.

Radioimmunoassays for microtubule protein-zymogen granule experiments RIA-MTG : 01 to RIA-MTG : 04.

MT. protein prepared from porcine brain was combined with zymogen granules isolated from 20 mouse pancreas in the usual way, except that the third supernatant was collected after leaving the granules in buffer overnight at $4^{o}C$. The diluted (1 : 9) supernatants were assayed twice for T. (R-01 and R-03) and once for M.A.P. (R-02) using R.I.A's. In the case of R-01 the supernatants were assayed in three dilutions (1 : 9, 1 : 99 and 1 : 999) the results then being corrected to 1 : 9 before analysis. The final experiment, R-04, was carried out in the same way as above, using 50 mouse pancreas, but the supernatants were assayed for T. by ^{3}H-colchicine binding assay (see MT-G : 08) and for M.A.P. using the R.I.A. The results of all four experiments were computer analysed by one way analysis of variance using orthogonal linear contrasts to compare individual sample groups.

RIA-MTG : 01

Table 3.81 : R.I.A. standard curve values for measured T.

% Bound = ((STND - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 microl.	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
1200.	-194.904	-120.472	-101.242	-138.873
600.	-232.337	-259.939	-249.622	-247.299
300.	-255.131	-212.189	-262.855	-243.392
150.	-245.895	-27.1732	-231.905	-174.991
75.0001	-12.9816	87.3784	-12.9276	20.4897
37.5	15.5924	26.7195	14.08	18.7973
18.75	47.7314	42.0598	36.7123	42.1678
9.375	91.1055	87.7025	72.4163	83.7414
4.6875	100.612	157.436	113.63	123.893
2.34375	124.595	57.7782	102.395	94.9226
1.17187	96.0209	64.7461	131.833	97.5333
.585938	135.668	133.885	94.9946	121.516
.292969	130.699	128.7	128.7	129.366

Total mean : 163119. counts

BLK mean : 7119.67 counts

BKG mean : 5268.33 counts

Curve formulae :

Curve I : Y = -52.4159 X + 138.316 R = .901185

Curve II : Y = -40.3308 X + 129.497 R = .818523

Curve III : Y = -47.5284 X + 126.715 R = .860616

Mean : Y = -46.7584 X + 131.51 R = .883645

Curve with greatest correlation coefficient used to calculate sample protein concentrations : Curve I.

One way Anovar using orthogonal linear contrasts.

Table 3.82 : Supernatant 1. results and analysis all values derived from measured T. in μg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	61.29	30.53	12.58	20.42
Replicates	3	3	3	3
Means	20.43	10.18	4.79	6.81
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio - F
Treatment	3	456.67	152.22	0.92 NS
Temperature	1	157.70	157.70	0.96 NS
+or- granules	1	246.86	246.87	1.50 NS
Error	8	1318.28	164.79	-
Total	11	1774.95	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	31.44	72.51	10.25	24.17
+of- granules	54.46	125.59	11.11	20.93

Table 3.83 : Supernatant 2. results and analysis, all values derived from measured T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	12.92	20.50	9.35	19.34
Replicates	3	3	3	3
Means	4.31	6.84	3.12	6.45
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	28.07	9.36	0.20 NS
Temperature	1	9.58	9.58	0.20 NS
+or- granules	1	12.04	12.04	0.26 NS
Error	8	375.64	46.96	-
Total	11	403.71	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	16.79	38.71	2.53	12.90
+or- granules	29.07	67.04	2.45	11.17

Table 3.84 : Supernatant 3. results and analysis, all values derived from measured T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	2.57	21.72	1.39	6.24
Replicates	3	3	3	3
Means	0.86	7.24	0.46	2.08

LINEAR CONTRASTS COERFFICIENTS :

Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	88.06	29.35	4.73 *
Temperature	1	61.12	61.12	8.60 *
+or- granules	1	25.70	25.70	3.61 NS
Error	8	56.88	7.11	-
Total	11	144.94	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	6.53	15.06	6.38	5.02
+or- granules	11.31	26.09	3.59	4.35

RIA-MTG : 02

Table 3.85 : R.I.A. standard curve values for measured M.A.P.

% Bound = ((STND - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 microl.	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
8000.	639.834	532.78	548.962	573.859
4000.01	684.647	527.801	507.884	573.444
2000.	489.211	485.477	424.481	466.39
1000.	474.274	409.543	347.303	410.373
500.	379.668	454.357	298.755	377.593
250.	341.079	368.465	205.394	304.979
125.	392.116	321.162	326.141	346.473
62.5	471.784	251.452	1019.5	580.913
31.25	239.004	304.979	212.863	252.282
15.625	236.514	134.44	154.357	175.104
7.8125	199.17	158.091	241.494	199.585
3.90625	300.	231.535	968.464	500.
1.95313	265.145	278.838	205.394	249.792

Total mean : 17762 counts

BLK mean : 905.667 counts

BKG mean : 986 counts

Curve formulae :

Curve I : Y = 49.1105 X + 156.144 R = .865258

Curve II : Y = 44.0387 X + 130.361 R = .893386

Curve III : Y = 3.38451 X + 403.735 R = 3.24567E-02

Mean : Y = 32.1779 X + 230.08 R = .599017

Curve with greatest correlation coefficient used to calculate sample protein concentrations : Curve II

One way Anovar using orthogonal linear contrasts.

Table 3.86 : Supernatant 1. results and analysis all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	291782.	21831.4	19089.1	1200.58
Replicates	3	3	3	3
Means	97260.6	7277.15	6363.03	400.195
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	1.93688E+10	6.45625E+09	.968852	NS
Temperature	1	1.21455E+10	1.21455E+10	1.82261	NS
+or- granules	1	4.21469E+09	4.21469E+09	.632474	NS
Error	8	5.33105E+10	5.55382E+09	-	
Total	11	7.26793E+10	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	199957.	461102	89983.5	153701.
+or- granules	346336.	798651.	45905.8	133109.

Table 3.87 : Supernatant 2. results and analysis, all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	140877.	1.02432E+06	3879.14	11781.5
Replicates	3	3	3	3
Means	46958.9	341439.	1293.05	3927.16
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Treatment	3	2.40207E+11	8.00689E+10	.998484	NS
Temperature	1	1.30078E+11	1.30078E+11	1.62211	NS
+or- granules	1	7.44253E+10	7.44253E+10	.928107	NS
Error	8	6.41524E+11	8.01905E+10	-	
Total	11	8.81730E+11	-	-	

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	693645.	1.59954E+06	294480.	533182.
+or- granules	1.20143E+06	2.77049E+06	192906.	461749

Table 3.88 : Supernatant 3. results and analysis, all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	21436.6	42518.2	6956.66	248.461
Replicates	3	3	3	3
Means	7245.54	14172.7	2318.89	82.8202
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	3.49949E+08	1.16650E+08	1.44877 NS
Temperature	1	7.40720E+07	7.40720E+07	.919965 NS
+or- granules	1	1.39119E+08	1.39119E+08	1.72785 NS
Error	8	6.44129E+08	8.05161E+07	-
Total	11	9.94078E+08	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	21979.5	50684.6	7027.18	16894.9
+or- granules	38069.5	87788.4	8340.24	14631.4

RIA-MTG : 03

Table 3.89 : R.I.A. standard curve values for measured T.

% Bound = ((STND - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
1200.	-79.3282	-43.5061	-89.5689	-70.8011
600.	-42.6493	-28.4918	-55.9092	-42.3501
300.	-18.6999	-28.451	-9.84635	-18.9991
150.	12.3487	-29.1854	-6.37838	-7.73836
75.0001	-2.8696	-12.0087	20.223	1.78156
37.5	-14.0895	62.2875	9.8191	19.339
18.75	21.243	44.2948	75.833	47.1236
9.375	76.0778	90.4801	52.006	72.8546
4.6875	63.1443	93.0504	68.1626	74.7858
2.34375	80.0353	72.2426	96.4776	82.9185
1.17187	65.9594	147.967	93.2952	102.407
.585938	75.1802	62.0835	62.5731	66.6122
.292969	55.3515	102.394	88.4809	82.0753

Total mean : 167587. counts

BLK mean : 16187.3 counts

BKG mean : 13736.3 counts

Curve formulae :

Curve I : Y = -17.1309 X + 72.6529 R = .895871

Curve II : Y = -20.424 X + 100.879 R = .882993

Curve III : Y = -19.9751 X + 89.7175 R = .911202

Mean : Y = -19.1767 X + 87.7496 R = .940801

Curve with greatest correlation coefficient used to calculate sample protein with concentrations : Mean

One way Anovar using orthogonal linear contrasts.

Table 3.90 : Supernatant 1. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	5.18157	20680.7	3.65519E+09	.24281
Replicates	3	3	3	3
Means	1.72719	6893.57	1.21840E+09	8.09377E+02

LINEAR CONTRAST COEFFICIENTS :

Temperature	1	-1	0	0
+of- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	3.34010E+18	1.11337E+18	.999999 NS
Temperature	1	7.12463E+07	7.12463E+07	6.39917E-11 NS
+or- granules	1	2.96897E+18	2.96897E+18	2.66666
Error	8	8.90694E+18	1.11337E+18	-
Total	11	1.22470E+19	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	2.58461E+09	5.96011E+09	6891.84	1.98670E+09
+or- granules	4.47667E+09	1.03232E+10	1.21839E+09	1.72054E+09

Table 3.91 : Supernatant 2. results and analysis, all values derived from measured total T. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	24.5594	5822.28	2.82846E+11	3.29901E-03
Replicates	3	3	3	3
Means	8.18647	1940.76	9.42820E+10	1.09967E-03
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	2.00006E+22	6.66682E+21	1 NS
Temperature	1	5.60226E+06	5.60226E+06	8.40320E-16 NS
+or- granules	1	1.77782E+22	1.77782E+22	2.66667 NS
Error	8	5.33346E+22	6.66682E+21	-
Total	11	7.33350E+22	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	2.00002E+11	4.61205E+11	1932.57	1.53735E+11
+or- granules	3.46414E+11	7.98831E+11	9.42820E+10	1.33138E+11

Table 3.92 : Supernatant 3. results and analysis, all values derived from measured total T. in µg/ml

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	65.86	1417.62	5.14167E+10	2.58428
Replicates	3	3	3	3
Means	21.9533	472.54	1.71389E+10	.861426
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	6.60920E+20	2.20307E+20	1.24764 NS
Temperature	1	304542.	304542.	1.72467E-15 NS
+or- granules	1	5.87485E+20	5.87285E+20	3.32703 NS
Error	8	1.41263E+21	1.76579E+20	-
Total	11	2.07355E+21	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	3.25496E+10	7.50594E+10	450.586	2.50198E+10
+or- granules	5.63776E+10	1.30007E+11	1.71389E+10	2.16678E+10

RIA-MTG : 04

Table 3.93 : R.I.A. standard curve values for measured M.A.P.

% Bound = ((STND - BKG) x 100) / (BLK - BKG)

Protein conce. NG/100 microl.	Curve I . Bound	Curve II . Bound	Curve III % Bound	Mean curve % Bound
4800.01	-104.204	-111.986	-83.0053	-99.7316
2400.	-101.252	-84.0787	-50.	-78.4436
1200.	-60.7334	-39.2665	-71.1985	-57.0662
600.	-25.313	-49.7316	-29.8747	-34.9731
300.	54.6512	3.13061	-3.5778	18.068
150.	35.331	60.8229	-9.74953	28.8014
75.0001	39.6243	-184.705	113.417	-10.5545
37.5	81.2165	-37.3882	87.3882	43.7388
18.75	92.4866	19.4991	31.3059	47.7639
9.375	133.542	45.5277	65.1163	81.3954
4.6875	35.8676	95.7066	116.369	82.6476
2.34375	54.6512	78.2648	79.6064	70.8408
1.17187	65.3846	66.9946	65.1163	65.8319

Total mean : 6241.67 counts

BLK mean : 1598 counts

BKG mean : 1225.33 counts

Curve formulae :

Curve I	: Y = -22.0493	X + 118.371	R = .803816
Curve II	: Y = -20.9729	X + 79.9959	R = .678187
Curve III	: X = -21.711	X + 117.654	R = .843999
Mean	: Y = -21.5778	X + 105.34	R = .931499

Curve with greatest correlation coefficient used to calculate sample protein concentrations : Mean

One way Anovar using orthogonal linear contrasts.

Table 3.94 : Supernatant 1. results and analysis, all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	187.991	28.171	127.115	6.44204
Replicates	3	3	3	3
Means	62.6638	9.39035	42.3683	2.14735
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	7252.45	2417.48	2.81364 NS
Temperature	1	4257.09	4257.09	4.95471 NS
+or- granules	1	80.4234	80.4234	9.36026E-02 NS
Error	8	6873.6	859.2	-
Total	11	14126.1	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	71.7997	165.57	53.2734	55.1901
+or- granules	124.361	286.776	6.34127	47.796

Table 3.95 : Supernatant 2. results and analysis, all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	1.57752	21.5168	5.32491E-02	.108716
Replicates	3	3	3	3
Means	.525839	7.17226	1.77497E-02	3.62388E-02
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	110.087	36.6957	32.1456 ***
Temperature	1	66.2624	66.2624	58.0461 ***
+or- granules	1	29.3577	29.3577	25.7175 ***
Error	8	9.13239	1.14155	-
Total	11	119.22	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	2.61711	6.03507	6.64642	2.01169
+or- granules	4.53298	10.453	3.8313	1.74217

Table 3.96 : Supernatant 3. results and analysis, all values derived from measured M.A.P. in µg/ml.

	MT+G 4°	MT+G 37°	MT ONLY 37°	G ONLY 37°
Totals	147.089	1.70481	.104197	532.872
Replicates	3	3	3	3
Means	49.0297	.568272	3.47322E-02	177.624
LINEAR CONTRAST COEFFICIENTS :				
Temperature	1	-1	0	0
+or- granules	1	1	-2	0

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F
Treatment	3	63129.4	21043.1	.874961 NS
Temperature	1	3522.77	3522.77	.146475 NS
+or- granules	1	1226.54	1226.54	5.09987E-02 NS
Error	8	192403.	24050.4	-
Total	11	255532.	-	-

	- TOTAL CONTRASTS -		- MEAN CONTRASTS -	
	Standard error	Confidence interval	Mean effect	Confidence interval
Temperature	379.871	875.983	48.4615	291.994
+or- granules	657.956	1517.25	24.7643	252.875

Only two significant differences between the treatment types were shown in the results of these four experiments. In experiment R-01 the overall effect of treatment and the effect of temperature was significant ($P < 0.05$) in the third supernatant results. The other significant results was obtained in the second supernatant of the fourth experiment, R-04. Overall treatment, temperature and the presence or

absence of granules was very highly significant ($P < 0.001$) when the levels of M.A.P. were assayed.

Radioimmunoassay for a pancreas time course experiment.

The time course experiment described in 'PANC : 22' had the second supernatant (polymerised T.) re-assayed using the R.I.A. for T. The results were analysed by a one way analysis of variance using orthogonal polynomials to partition the treatment effect, all calculations being done using a computer programme.

RIA-PANC : 01

Table 3.97 : R.I.A. standard curve values for measured T.

% Bound = ((STND - BKG) x 100) / (BLK-BKG)

Protein conc. NG/100 microl.	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
4000.01	-67.1297	50.9259	189.815	57.8703
2000.	-43.5186	-21.2964	55.0925	-3.2408
1000.	57.8703	49.537	-14.3519	31.0185
500.	-18.5186	134.259	30.0925	48.6111
250.	23.1481	32.8703	-7.40746	16.2036
125.	-31.0186	130.093	49.537	49.537
62.5	-25.463	128.704	120.37	74.537
31.25	-7.40746	38.4259	12.037	14.3518
15.625	49.537	96.7592	99.537	81.9444
7.8125	98.1481	157.87	327.315	194.444
3.90625	113.426	93.9814	198.148	135.185
1.95313	10.6481	5.09254	17.5925	11.1111
.976563	118.981	167.593	95.3703	127.315

Total mean : 2855.33 counts

BLK mean : 800.333 counts

BKG mean : 728.333 counts

Curve formulae :

Curve I : Y = -16.14 X + 88.1805 R = .707239

Curve II : Y = -8.85169 X + 118.512 R = .396475

Curve III : Y = -9.22602 X + 128.393 R = .252048

Mean : Y = - 11.4059 X + 111.695 R = .532221

Curve with greatest correlation coefficient used to calculate sample protein concentrations : Curve I

One way Anovar using orthogonal polynomial coefficients.

Table 3.98 : Orthogonal polynomial coefficients used to partition treatment effect over the time intervals.

Treatment Levels	Levels	-Orthogonal Polynomial Coefficients- Linear	Quadratic	Cubic
10	0	-2.3	3.15	-2.71
20	1	-1.3	-0.89	3.95
30	2	-0.3	-2.94	1.76
45	3.5	1.2	-2.25	-5.02
60	5	2.7	2.93	2.02

Table 3.99 : Supernatant 2. results and analysis, all values derived from measured polymerised T. in µg/ml.

Time interval	- Replicates - 1	2	3	Mean
10	2.88	0.97	0.59	1.48
20	0.10	0.25	18.13	6.16
30	0.56	0.67	0.06	0.43
45	3.28	0.85	90.06	37.40
60	2.15	2.24	1.22	1.87

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio - F	
Total	14	7447.30	-	-	
Treatment	4	2063.47	515.87	0.96	NS
Linear	1	184.63	184.63	0.34	NS
Quadratic	1	411.02	411.02	0.76	NS
Cubic	1	955.65	955.65	1.78	NS
Residual	1	512.17	512.17	0.95	NS
Error	10	5383.83	538.38	-	

Here no significant effect of treatment could be demonstrated at any level partitioned or otherwise.

Measurement of MT. proteins using the radioimmunoassay technique - summary of results and analyses.

The MT. granule binding experiments in this group are summarised in the table below. Note that 'RIA-MTG : 01' and 'RIA-MTG : 03' measured T. by radioimmunoassay whereas the other two experiments measured M.A.P. The final experiment in the group 'RIA-PANC : 01' used the R.I.A. for T. to measure the levels of polymerised T. in the pancreas supernatants produced in experiment 'PANC : 22'.

Table 3.100 : Summary of mean effect, confidence intervals and significances for experiments RIA-MTG : 01 to RIA-MTG : 04.

Expt. No.	SN No.	Mean temp effect	± C.I.	Sig^{n} P.<	Mean granule effect	±C.I.	Sig^{n} P.<
01	1	-10.3	24.2	NS	-11.1	20.9	NS
(T)	2	+ 2.5	12.9	NS	- 2.5	11.2	NS
	3	+ 6.4	5.0	.05	- 3.6	4.4	NS
02	1	-89983.5	153701	NS	-45905.8	133109	NS
(MAP)	2	+294480	533182	NS	-192906	461749	NS
	3	+7027.2	16894.9	NS	-8340.2	14631.4	NS
03	1	+6891.84	2.0×10^{9}	NS	$+1.2\times10^{9}$	1.7×10^{9}	NS
(T)	2	+1932.6	1.5×10^{11}	NS	$+9.4\times10^{10}$	1.3×10^{11}	NS
	3	+450.6	2.5×10^{10}	NS	$+1.7\times10^{10}$	2.2×10^{10}	NS
04	1	-53.3	55.2	NS	+6.3	47.8	NS
(MAP)	2	+6.6	2.0	.001	-3.8	1.7	.001
	3	=48.5	292.0	NS	-24.8	252.9	NS

The only granule binding experiments which produced significant results were 'RIA-MTG : 01' and 'RIA-MTG : 04', the other two not only showed no significant treatment effects but also had T. and M.A.P.

measurements which were far too high to be considered realistic. Obvi ously in the case of these two experiments the antibody binding was rather non-specific producing inaccurate results. In the case of experiment 'RIA-MTG : 01' the significant treatment effect was shown in the third supernatant alone and then only as an effect of temperature, no effect of granule addition being detected. Similarly 'RIA-MTG : 04' showed a significant treatment effect in only one supernatant, the second, only in this case both temperature and granule effects were significant.

The final experiment in this group using the R.I.A. looked at the levels of polymerised T. in the pancreas supernatants of mice stimulated with Pilocarpine. The supernatants assayed were the same on es previously assayed by ^{3}H-colchicine binding for T. in experiment 'PANC : 22'. As was found using this assay no significant effects of treatment were detectable in the R.I.A. results.

DISCUSSION

Methods and Experimental Procedures.

The object of the experiments outlined previously was to test the hypothesis that MT. are in some way involved in exocrine pancreas secretion. This was approached in two ways, both from the point of view of the necessity for secretory granule binding to MT. and from the need for alterations in the cellular MT. and free T. pools. Although experimentally the isolation procedures used were conventional the combination of the isolated cellular portions and the assay procedures used to quantify their effects on one another were novel or at least relatively recent innovations. Of necessity considerable numbers of trial experiments and modifications were made to obtain the results presented. Thus the references given for individual procedures were used only to provide a backbone for the actual methods required. In this first section of the discussions the points of particular note in the methods and experimental procedures will be dealt with before discussing the actual experimental results in detail.

So far as the isolation procedures required for the experimental materials are concerned the methods used were well established. Conventional differential centrifugation to isolate the exocrine pancreatic zymogen granules was used throughout. The choice of material was made on the basis of availability and purity; mouse pancreas provided a readilyavailable source of uncontaminated granules. Porcine slaughter house material and rat pancreas were both found to be too high in collagen to enable rapid homogenisation and the production of uncontaminated preparations. Although rat pancreas was used in some of the granule binding experiments it was never used for E.M. studies since collagen fibre contaminants made rapid detection of MT-granule interactions difficult. Indeed for E.M. study the isolated mouse granules

were usually further purified after differential centrifugation by centrifuging through a Urografin-dense medium. The production of granule 'ghosts' by leaving the granules in sucrose-free buffer also facilitated rapid E.M. isolation of granule-MT. interactions.

Although the isolation of MT. proteins from brain material by assembly/disassembly cycles is well known, the actual conditions chosen distinctly alter the yields and nature of the isolated proteins. Initially glycerol was always included in the assembly cycles, however, later exclusion of glycerol in the first assembly cycle produced higher yields of MT. proteins, particularly the M.A.P's. A second modification was the reduction in GTP concentration, it was found that reducing the concentration of GTP in the MT. isolation buffer from 1mM to 0.1mM had no significant effect on the yields of MT. proteins. The yields of MT. protein, quoted as typically being about 3ml at 30mg protein per ml. after isolation, were extremely sensitive to minor modifications of the isolation procedure and also to storage, experimental material was aften at a concentration less than 10mg/ml when used after storage for a prolonged period. Even so, the activity of the proteins always proved to be consistent, assembled MT. being produced upon incubation under the correct conditions.

Experiments which required the MT. proteins to be separated used phosphocellulose column chromatography to separate T. from the M.A.P's; the use of a large column (16 x 600 mm) produced high yields of pure M.A.P's with low concentration T. solutions while using small (80 x 15 mm) disposable columns (Pharmacia) produced T. solutions with a much higher concentration. Where M.A.P's alone were required the heat denaturation method was employed since it was much more rapid and provided high yields of M.A.P's.

The use of an MT. stabilising buffer to separate cellular assembled

MT. from depolymerised T. proved to be more effective when carried out at 4°C. Surprisingly the yields of assembled MT. from pancreas homogenates in MTS. buffer were greater when kept at 4°C than if kept at room temperature prior to centrifugation. In common with the findings of Ostlund, Leung and Hajek (1979) the DMSO and glycerol were capable of maintaining assembled MT. even at a temperature at which they would normally depolyermise, this low temperature prevented degradation of the MT. by homogenate enzymes as was the case when the homogenates were kept at room temperature. Higher ^{3}H-colchicine binding assay results were also produced when the pancreas supernatants were treated to remove excess insoluble fat globules by ultrafiltration. Passing the supernatants through 0.8µm pore filters (Millipore) produced clear samples which proved to have lost little of their ^{3}H-colchinine binding activity. It is important to note that all pancreas homogenates were diluted one to ten (weight to volume) with buffer and then diluted to one to twenty with the other necessary buffer to produce a 50 : 50 mixture of MTS. and TS. buffers. Thus the samples were all corrected for pancreas weight. In addition the fact that all three supernatants were in the same buffer meant that the third supernatant ^{3}H-colchicine binding assay values could be used as blanks for the other two supernatants. It was found that blanks in TS. buffer produced different values to those in MTS. buffer, and often that these blank values were higher than those of the third supernatants; consequently the mean third supernatant value was used.

The values of T. determined by ^{3}H-colchicine binding assay were calculated from the formula estimate of bound colchicine in nmoles per 110µl of sample by assuming that 1 mole of T. binds 1 mole of colchicine and that the molecular weight of T. is 110,000 daltons; the final concentration of the samples was always given in µg/ml. Individual samples were always determined in triplicate and the calculations for

conversion of β-counter output counts to T. concentrations and consequent analysis of these concentrations were always carried out by computer with no elimination of spurious results. This meant that often the determination variances were higher than they needed to be, but it also ensured that no bias was allowed by eliminating individual results.

The use of the ^{3}H-colchicine binding assay to determine the levels of assembled MT. and depolymerised T. in pancreas secreting and non-secreting mice encountered a number of logistic problems. In the first place no more than ten pancreas homogenates could be centrifuged at a time in the 10 well rotor. Also very large numbers of mice produced so many supernatants for T. assay that the time taken to process them made the experimental determination variances too high. To overcome these problems it was necessary to carry out the injection of the mice, separation of the MT. and depolymerised T. and the T. determination entirely at random. Despite sample randomisation the processing of large numbers of mouse pancreas supernatants, such as was necessary when examining the effect of Pilocarpine over the time after injection (experiments 'PANC : 19 to 22'), meant sacrificing low assay determination variation in favour of larger numbers of mice and consequently lower mouse variation. This step was necessary since the earlier pancreas experiments showed that the variation in T. levels between mice was very high, often so high as to exclude any possibility of detecting an effect of differential treatment. Obviously the mice were grouped at random into treatment groups, but even selection of mice which were as close as possible to one another in age, size, weight and litter relationship failed to reduce the enormous variation in pancreas T. levels. The levels of T. detected did not seem to be dependent on any of these factors, nor on the weight of the pancreas before homogenisation.

Problems encountered in carrying out the RIA for T. and M.A.P's were not so much related to mouse variation as to that of the determinations. Initially the main problem encountered was one of non-specific binding of ^{125}I activity to the walls of the assay tubes. This was overcome by adding Triton X-100 detergent to the first incubation mixture, however this led to the final second antibody precipitated pellets breaking up and being lost. To counter this effect potato starch was incorporated in the second incubation medium. Although this prevented the tendency of the pellets to fall apart, they still tended to slide down the outside of the tube whilst pouring out the supernatants. This made rapid treatment of a large number of samples and standards difficult and produced a high determination variance.

Electron microscope preparative methods were fairly conventional, the main problems encountered were in maintaining the granule pellets after fixation, producing an embedding medium suitable for the material and staining the sections for both MT. and granule membranes. To overcome the break-up of the pellets during handling for dehydration and embedding pieces of pellet were embedded in agarose after fixation. Subsequently the embedded pieces were cut into small pieces for conventional E.M. preparation. Agarose embedding had little effect on the views of MT-granule interactions and also did little to alter the texture of the pellets seen under the S.E.M. Standard Spurr resin mixture was used to embed the dehydrated pellets, however the tendency for alcohol to be left in the pellets and produce brittle blocks was countered by prolonged soaking in resin and the incorporation of extra flexibiliser in the Spurr resin mixtures. Finally the optimum section staining regime was determined by comparing <u>in vitro</u> zymogen granule and MT. sections after dual staining with uranyl acetate and lead citrate

in a variety of solvents and at a number of temperatures. The best all round method used 1% magnesium uranyl acetate in 50% aqueous ethanol and aqueous lead citrate, both being used at 60°C.

The iodination of large protein molecules such as M.A.P's necessitated a longer period of incubation with chloramine-T than would normally be used, to prevent excess protein degradation the concentration of this compound was reduced from 5mg/ml to 0.1mg/ml. The large molecular weight of M.A.P's and T. enabled straightforward separation of iodinated protein from free iodine using a small bed volume column, this was more rapid than with a larger column and readily separated the protein into the void volume fractions with little or no iodine contamination.

The modifications discussed above enabled the roles of MT. during secretion to be examined both in vitro and in vivo, the mainstay of the resultant experimental observations lay with the statistical analyses outlined in the appendix. These analyses were of necessity rather complex and were therefore carried out by computer programme. This enabled the experimental results to be analysed both rapidly and repeatedly using various statistical procedures. Since most of the tests used were ones not in common usage the computer programmes had to be written and were not part of the available system software. These programmes are also listed in the appendix.

Macroscopic examination of the interaction between isolated zymogen granules and MT. proteins.

The photographs P1 to P4 demonstrate definite morphological differences between the experimental regimes used in the in vitro study. Incubation of MT. proteins with zymogen granules at two different temperatures resulted in suspensions of markedly different properties. Photograph P1 shows that the mixture incubated at 37°C (β) was much more turbid than that kept at 4°C (α). This increase in turbidity was much greater than could be assigned simply to MT. assembly alone (as seen in the tube labelled δ). We can see by careful observation of photograph P1 that the zymogen granules in tube α were beginning to settle out of suspension as was the case with tube Δ containing only granules; although the increased MT. protein concentration in tube α reduced the rate of settling. However there was no sign of any settling out of the zymogen granules in tube β despite a similar MT. protein concentration to tube α. This difference can be explained in one of two ways, a complex MT. network formed by the in vitro assembly of MT. proteins could have produced both the increase in turbidity and viscosity and prevented the settling out of the zymogen granules. Alternatively a specific interaction between the assembled MT. and the zymogen granules in association with the formation of an MT. network could have brought about the alterations in viscosity associated with the differing incubation temperatures. It is true to say that increased viscosity was one of the key effects of combining actin and MT. protein in similar in vitro experiments conducted by Griffin and Pollard (1978 a,b.).

That some factor included in the MT. protein/granule mixture was removed after incubation at 37°C and subsequent centrifugation is

apparent from photograph P2. The mixture incubated at 37°C (β) had a resultant supernatant of much reduced volume by comparison with the mixture kept at 4°C (α). This volume reduction can only have been the result of MT. protein loss during centrifugation. This was reflected in the turbidity of the different supernatants, the tubes labelled αs_1 and γs_1 showed the presence of MT. protein by their turbidity while Δs_1 and particularly βs_1 were clear indicating the absence of MT. proteins. Photographs P3 and P4 show that the lost MT. proteins did in fact cause an increased pellet volume, the B sample being two or three times the volume of the other pellets. This is hardly surprising when one considers that a combination of MT. meshwork and trapped granules would have been present after incubation, a complex likely to sediment very readily. At the low centrifugation rates used, MT. fail to sediment out of solution on their own (no pellet was found in the γ tube even though the increase in viscosity and cloudiness after incubation at 37°C indicated the presence of assembled MT.), however, such low speed spins easily brought the MT. out of solution when granules were present. Even so, the pellet was not compressed to the same extent as if granules alone (Δ) or granules and disassembled MT. protein (α) had been present. This may well be the result of the rigidity of assembled MT. and the interaction between these and the zymogen granules.

The increase in size of the pellet from MT.protein/granule mixtures incubated at 37°C as against those incubated at 4°C was apparent if fixation took place before or after centrifugation. This indicates that the interaction between MT. and granules occurred spontaneously in solution and was not the result of either centrifugation nor fixation. There was a slight increase in the size of the pellet from the cold incubated mixture if fixed before centrifugation, this could have been

the result of either glutaraldehyde induced swelling or some other non-specific combination of MT. protein with the granules under the influence of glutaraldehyde.

The macroscopic observations of combined MT. proteins and zymogen granules gives us the first indications that there may be a specific interaction between assembled MT. and zymogen granules in vitro.

Scanning electron microscope study of the interaction between isolated zymogen granules and MT. proteins.

The scanning electron micrographs of experimental pellets were examined to compare the effect of assembled MT. as against unpolymerised MT. proteins on the ultrastructural texture and morphology of the zymogen granule pellets already contrasted macroscopically. The photographs P5 to P12 show various views of the pellets after conventional fixation, dehydration and critical point drying. It is worth noting that they show broken, inner surfaces of the pellets splutter coated with gold. This will have caused the dimensions of the individual components of the pellets to be increased. The photographs selected are representative of the S.E.M. view of the pellets as a whole. In each case the areas selected for close study were on the broken inner surface of the pellet.

Comparison of photographs P5 and P7, detailed views of the pellets derived from MT. proteins and granules incubated at $4^{\circ}C$ and granules alone incubated at $37^{\circ}C$ respectively, shows that the inclusion of MT. protein kept at $4^{\circ}C$ does not alter the appearance of the granule pellet. However, photograph P8 shows a morphologically, totally different view of the pellet derived from MT. proteins and granules warmed to $37^{\circ}C$. The former pair of photographs, P5 and P7, show a 'granular' pellet resulting from closely packed zymogen granules, individual granules were difficult to identify probably because centrifugation could have caused the normally spherical granules to distort in shape. This 'granular' appearance of granules alone or granules in the presence of cold, disassembled MT. proteins was unaffected by fixation prior to centrifugation. As photograph P6 shows such a pellet of granule and cold MT. proteins (α_f) still has a similar 'granular' morphology although

the texture is slightly less angular probably as a result of more frequent or longer gluteraldehyde bonding.

By contrast with these 'granular' pellets the granule pellets which had assembled MT. included (β and β_F) had a totally distinct texture. Photograph P8 shows this as a complex meshwork of gold-coated MT. completely obscuring the granule outlines, although hints of granule masses can sometimes be seen below this mesh. In photograph P10 it is possible to discern individual coated MT. and MT. groups. Once again pre-centrifugation, fixation had little effect on the morphological texture of these pellets. In both photographs P9 and P10, the complex MT. web is still apparent although in the former photograph it appears much more closely packed, this could be the result of shorter gluteraldehyde bonding or simply variations in the local MT. protein concentrations. In some instances individual granules were visible firmly attached to the MT. web surface, P11, or lying near the surface of the web but still coated by the meshwork, P12. Even in such cases the resolution of the S.E.M. was insufficiently high to enable delineation of the nature of the MT.-granule interaction although it has provided evidence for the existence of such an interaction.

The complex meshwork resulting from the polymerisation of MT. proteins *in vitro* could still have been providing a 'trap' for the zymogen granules. The exact nature of any specific interaction between this web and the granule membranes, if present, was not discernable in the S.E.M. Nonetheless, the MT. mesh visualised here *in vitro* is certainly reminiscent of the MT. cell web seen in fluorescent antibody stained cells by Osborn and Weber (1976). Therefore we might expect similar interactions between the granules and the MT. to take place in this model system as may occur *in vivo*. Certainly the granule membranes appear (photograph P12) to be specifically coated by the MT.

web in a way not likely to occur if they were simply trapped in the meshwork fortuitously. Indeed the granules may well have been acting as nucleation sites for MT. assembly. The MT. web seems to be capable of maintaining the spherical outline of the granule membranes against the centrifugal sedimentation forces which produced the peculiar 'granular' texture apparent in the pellets formed in the absence of assembled MT.

Transmission electron micrographs of in vitro assembled MT. and isolated zymogen granules.

The views of MT. assembled *in vitro* show that they were of a structure comparable to the structures apparent *in vivo*. About 13 subunits could be seen in the t.s. and the photofilament arrangements visible in the l.s. were also typical. Side arms, probably less apparent *in vivo*, could be seen clearly in these *in vitro* sections. Regular decoration of the MT. walls indicated the morphological presence of side arm proteins.

Fixed and sectioned exocrine pancreatic granules readily showed the presence of their zymogen contents when isolated in isotonic buffer. However transference to sucrose-free MT. isolation buffer produced 'granule ghosts' which occasionally had some contents remaining. Those ghosts shown in photographs P15 and P16, which appear to be in the process of losing their contents, could well have been isolated in the process of secretion *in vivo*. This would account for the localisation of the zymogen at one pole of the granule.

The loss of granule contents enabled examination of the membrane structure much more easily. This was particularly important when looking for an interaction between MT. and the granule membrane. Internal zymogen removal not only made the membrane more distinctive but removed the possibility of chance interference of free zymogen in the MT. attachment to granule membranes. Such close association of MT. fragments with the 'granule ghosts' is visible in both photographs P15 and P16, a t.s. in the former and an l.s. in the latter was associated with the granule membrane.

Transmission electron microscope study of the interaction between isolated zymogen granules and MT. proteins.

As might be expected from the S.E.M. study of the zymogen granule pellets formed in the presence and absence of assembled MT. there were noticeable differences in sections of these same pellets when examined under the T.E.M. Pellets produced in the complete absence of MT. protein or from granules incubated with MT. proteins at 4°C were always more tightly packed than similar pellets produced with MT. proteins incubated at 37°C. The former pellets rarely exhibited the presence of assembled MT. in association with the granules. Occasionally the odd fragment of MT. could be found. This probably resulted from the presence of either cold stable MT. proteins or proteins which had lost their ability for assembly/disassembly. Alternatively some fragements, particularly those seen in t.s., could have been portions of T. rings of short helices often found under certain conditions as stages in MT. assembly (Penningroth, Cleveland and Kirschner, 1976). However, the presence of such fragments in pellets kept under conditions of MT. disassembly was rare; not once did such fragments appear in close association with the granules. Similarly, granules which had previously been associated with assembled MT., when placed under MT. disassembly conditions and repelleted, rarely demonstrated MT. fragments. These observations indicate that the alterations in granule pellet texture and morphology associated with the inclusion of MT. proteins was the result of their association with the granules, a feature only present when the proteins were assembled into MT.

This association between MT. and zymogen granules could be accounted for in a number of ways. Firstly, it could simply have been an artefactual consequence of sedimentation by centrifugation of the two

organelles or their gluteraldehyde fixation. Alternatively it could have been the result of their close contact, the web formed on assembly of the MT. proteins simply trapping the granules. Finally, there may have been a real physical interaction between the two organelles producing some form of structural bond. The explanation for the association between zymogen gramules and MT. as being artefactual can be negated on a number of grounds. As was shown by the previously mentioned S.E.M. study, the interaction occurred similarly if fixation was carried out before or after centrifugation, this shows us that a relationship between the two types of organelle was formed prior to centrifugation and also prior to fixation. Indeed this was confirmed by the earlier macroscopic study, since the samples containing granules together with assembled MT. were of a totally different consistency to those containing either granules alone or granules with disassembled MT. proteins. Further confirmation comes from the fact that when collagen fibre contamination was present, in early rat pancreas extracted zymogen granule suspensions, no interaction occurred on addition of assembled MT. proteins between collagen and granules.

Thus it can be concluded that either the granules are trapped in a complex MT. meshwork altering their sedimentation characteristics or that there is a true structural interaction between the two organelles. It seems unlikely that even a web of MT. would be capable of holding back large zymogen granules against the effect of sedimentation forces of about 1,000g for 10 minutes without the formation of some form of structural or chemical linkage between the granules and the MT. Evidence for the presence of such an interaction was sought in the T.E.M. photographs P17 to P33.

The first group of photographs, P17 to P23, showed 'granule ghosts' in close association with MT. in various planes of section. Photographs

P17, P18 and P20 in particular showed MT. of considerable length aligned against the granule. Together with these large pieces of MT. in the plane of section, all the photographs in this group contained numerous fragments of MT. surrounding the granule membrane, both in and out of contact. This confirms the findings of the S.E.M. study that the granules were enmeshed within a complex web of assembled MT. Photograph P18 showed such a situation where MT. going in all directions surrounded the 'granule ghost'; a similar view was presented in photograph P23 only this time most of the MT. were in t.s. In all these photographs it was noticeable that the sectioned MT. had various characteristic morphologies. This depended upon the way in which the plane of the section cut the MT. tube. In some cases, P18, the section went through the centre of the tube in l.s. bringing out the side walls and clear central core; in other cases sections through the side wall, P19, presented a clearer view of the protofilaments which go to make up the MT. Also apparent on the MT. side walls were some indications of decoration presumed to represent the points of attachment of side arms. The regular periodicity of this decoration would seem to indicate the presence of MT. associated side arms demonstrated by Sloboda et al (1976) to be derived from the M.A.P.'s

Despite the apparent indication of the presence of side arms none of these lower magnification pictures, P17 to P23, really showed any structural linkage between the MT. and the granule membranes. Indeed no true side arms of any real length were seen at all, only indications of their presence. However, they did provide evidence that the granules and MT. were somehow associated closely with one another. To observe the structural nature of the granule-MT. interaction we must look to the higher magnification T.E.M. view of MT. in close proximity to the granule membrane shown in photographs P24 to P33.

The types of interaction between the assembled MT. and granule membranes fell into three main categories. The first category was represented by photograph P24 which showed long strands of wispy material connecting the MT. to the membrane. Such strands were also visible at the top of photograph P19 and a similar single strand connected the MT. t.s. at the top of P23 to the granule membrane. These strands did not seem to be reminiscent of MT. side arm decoration and are believed to be fixed contaminant protein strands. Alternatively they could have been non-assembled MT. protein precipitated out of solution by gluteraldehyde fixation. Indeed such strand like material was commonly encountered in gluteraldehyde fixed MT. protein solutions even when zymogen granules were not present.

The more typical MT. granule interactions were shown in the other high magnification T.E.M. photographs. These were of two kinds, close contact between the granule membrane and the MT. wall with no structural connection and actual side arm connection between the two. The close juxtaposition of MT. to the granule wall was shown in l.s. in photograph P25, here the granule membrane almost overlapped the MT. wall. The MT. may actually have been intruding into the granule membrane although it is more likely that the plane of section cut through the point above where the organelles were in contact and what we could see was the curve of the granule membrane overlapping our view of the MT. That we could see the outer wall of the MT. is certain since the individual protofilaments were visible. The close contact between MT. and granule membranes were seen more clearly when the MT. were cut in t.s. Photograph P31 showed this particularly clearly, the MT. being in intimate association with the granule membrane. Less intimate association was shown in photographs P30 and P32, an outer coating around the MT. separating it from the granule membrane. This

coating could have been our view of the rest of the MT. out of focus behind the t.s. or more likely was a coating derived from the tannic acid/glutaraldehyde fixation technique used.

True structu ral connections between granules and MT. were sometimes clearly discernable, P28 and P29, although often the side arms arising from the MT. were only partially present, P26 and P27. The side arm connection from the MT. to the granule membrane shown in photograph P28 coincided well with the periodic decoration on the MT. wall. In photograph P29 the complete connection was one of four other links hinted at by outgrowths in the granule membrane and coincident decoration of the MT. wall. Such hints of MT.-granule interconnections were more common than complete links; photograph P26 had three or four of such links and P27 about three. Linkage between the two organelles was also shown in t.s. in photograph P33.

What conclusions can be drawn from these different types of association seen between the isolated zymogen granules and assembled MT. in vitro ? Certainly the strand like connections typified by photograph P24 is more likely to have been the result of fortuitous precipitation of MT. protein than any other cause. This, then leaves two other types of association, close contact and side arm connections, between the organelles. Should any interaction be present, consider the problems involved in the alignment of a straight rigid structure such as an MT. with the curved surface of a zymogen granule. The MT. would truly come into contact with the membrane at only one point, as a tangent does to a sphere. Such sparse contact would not be strong enough to resist sedimentation forces in the region of 1,000g. It seems more likely that such close contact and related close juxtapositioning of the MT. and granule membrane, seen particularly in t.s., was simply the consequence of the alignment of a curved surface

against a straight surface.

True attachment of MT. to the granule membrane was achieved by side arms of varying length, such variation being visible in photograph P27. Further, these side arms appear to have arisen from the MT. side walls since they coincide well with the decoration previously correlated with side arm attachment. Certainly some portion of the linkage could have been provided by the granule membrane, bulges in the area of attachment were sometimes present. Side arms of varying length were necessary to accommodate the curvature of the granule membrane, these differences in length were noticeable in photograph P27. However, in some cases, especially photograph P19, it appeared that the granule membrane itself was somehow flattened to make attachment to the MT. more straightforward. This though,may have been a consequence of the effect of the sedimentation forces during centrifugation. It is worth noting that, although devoid of their supportive contents, most of the 'granule ghosts' were not compressed by centrifugation; this may have been prevented by their spherical nature or could have been the result of support provided by the surrounding MT. network.

Although a well preserved side arm was displayed connecting the MT. to the granule membrane in photograph P28 the more frequent arrangement was that typified by photographs P27 and P26. Here side arms were only present in part, this was possibly caused by poor fixation of the proteins constituting the side arms or possibly because more than one type of granule membrane-MT. interaction existed. Perhaps in some parts of the membrane true structural linkage was reinforced by electrostatic or chemical interactions between the coincident bulges shown both on the membrane and the MT. side walls. Such reinforcing links would be necessary since, even with close contact between the MT. and the granules, the supposed helical arrangement of MT. side arms would only

contact the curved membrane infrequently to bond the two together. That the two do contact is certain though whether this is simply an artefact of fixation is uncertain. The occurrence of these bonds, both before and after centrifugation, and the fact that other fibrous proteins do not bind to the 'granule ghosts' negates this possibility.

This in vitro model system appears to consist of a complex web of assembled MT. which are physically supporting the zymogen granules either by structural side arm attachment, electrostatic or chemical bonding or both. These interactions are not present in control mixtures containing either granules alone or MT. alone, nor are they present in conditions unfavourable to MT. assembly.

^{3}H-colchicine binding assay studies of the interaction between isolated zymogen granules and MT. proteins.

Once we had shown the presence of a morphological link between pelleted zymogen granules and assembled MT. the next step was to look for a biochemical alteration in the levels of MT. protein in the supernatants. One would expect that if MT. bound to granules were being taken out of solution during centrifugation, there would be a corresponding reduction in the levels of MT. protein in the supernatant left behind. Similarly where zymogen granules were combined with unpolymerised MT. protein, one would expect that since the granules appeared to pellet alone there would be no corresponding alteration in the MT. proteins left in the supernatant.

The ways in which the zymogen granules and MT. proteins were combined experimentally were such that two main effects were investigated. These were, firstly the effect of differences in incubation temperature (either 4^{o}C of 37^{o}C) and secondly, the effect of the presence or absence of zymogen granules. The effects of these two experimental conditions were compared by measuring the ^{3}H-colchicine bound T. levels in the supernatants. Not only were the T. levels in the first supernatants analysed but also the corresponding levels in further supernatants produced by resuspending the initially pelleted granules in fresh buffer under MT. disassembly conditions. The effect of incubation temperature on the T. levels in the supernatants was found by statistical comparison of the two parts of the experimental regime containing both MT. proteins and zymogen granules. The only difference between this pair was their initial incubation temperature; one set being incubated under MT. assembly conditions at 37^{o}C while the other was maintained under disassembly conditions at 4^{o}C. To assess the effect of addition

of zymogen granules to the MT. proteins the mean T. levels of the sets of samples containing both granules and MT. proteins already compared were statistically compared with the set of samples containing only MT. proteins and no granules. The final control sample sets, containing only zymogen granules, were used to check that no T. was present in the initially isolated granule suspension.

That zymogen granules did actually remove MT. protein from the first supernatants was shown by the fact that in the majority of the experiments these first supernatants from samples which did not contain granules had higher T. levels than those containing granules. This 'mean granule effect' brought about an increase in the amount of T. left in the first supernatant of samples without granules as compared with those with granules, with a magnitude of between about 17 and 232µg/ml. The only experiments where no increase in the first supernatant was apparent were 'MT-G : 06 and 08'; the latter having an insignificant mean granule effect and the former a significant ($P < 0.05$) decrease in T. levels of granule-free samples as opposed to those with granules. In the case of experiment 'MT-G : 06' the lack of a granule effect seemed to be due to the low level of T. in the granule-free sample most probably brought about by pipetting error when apportioning MT. protein to these samples. The particularly high removal of T. from the first supernatant of the sample containing both granules and MT. protein and incubated at 37°C produced the unsignificant granule effect in experiment 'MT-G : 08'.

Not only were zymogen granules shown to remove MT. protein from the first supernatants after centrifugation but they also only did this at a particular temperature. Comparison of samples containing both MT. proteins and zymogen granules incubated at the two different temperatures (4°C or 37°C) showed that those incubated at the higher temperature

had T. levels well below that of those kept at the lower temperature. The higher incubation temperature supplied the energy necessary for MT. assembly and the presence of assembled MT. as opposed to depolymerised MT. proteins produced an increased loss of T. from the first supernatants of between 35 and 271µg/ml. This 'mean temperature effect' was apparent in all the experiments apart from 'MT-G : 03' where M.A.P. removal from the MT. proteins used in one part of the experiment produced an insignificant effect.

The removal of T. from the first supernatants incubated at 37°C was mirrored by an increase in the amount of T. released after cold depolymerisation in the second supernatants. In nearly all the experiments the mean temperature effect on the second supernatants was to produce an increase in the levels of T. when samples originally incubated at 37°C were compared with those incubated at 4°C. This increase, of between 11 and 442µg/ml of T. in the second supernatant, was produced by the release of granule-bound MT. proteins following cold disassembly. That the resuspended granules released this bound T. is reflected in the fact that the mean granule effect on the second supernatants was to produce an overall reduction in T. levels in the granule free samples as against those containing granules; this reduction varied between 2 and 384µg/ml. In only one experiment did this second supernatant pattern alter, in this case ('MT-G : 04') the use of a low concentration of MT. proteins meant that the levels of T. in the second supernatant were too low to measure.

In general granule supernatants collected after further MT. depolymerisation washes in buffer produced zero levels of T. This indicates that all the T. bound to the granules was released during the first period of depolymerisation. In only one experiment, 'MT-G : 08', was there any detectable T. in the third supernatant, however, no

incubation temperature effect could be shown on the T. levels in this supernatant although there was a significant ($P < 0.001$) granule effect. The curious observation in this experiment was that the levels of T. measured in the third supernatant were the same in both the presence of assembled MT. and of depolymerised MT. proteins. More peculiar was the fact that similar levels of T. were detected in the third supernatant of samples which contained only zymogen granules and buffer with no added MT. proteins. Low levels of T. were sometimes detected in other experiments in these samples in the first and occasionally the second supernatants but these were probably derived from exocrine pancreas, cellular T. co-isolated with the zymogen granules. In the case of experiment 'MT-G : 08' no T. was detectable in the second supernatant of samples containing only granules; in fact the low binding of depolymerised MT. proteins to the granules produced similar low T. levels in the second supernatant derived from these samples also. Yet much higher levels of T. were detectable in all three sets of third supernatants derived from samples containing granules. The reason for this observation was that the third supernatants were collected after the granule had been kept in buffer overnight at $4^{\circ}C$. This led to the release of native zymogen granule, membrane-bound T. not normally released during cold MT. disassembly conditions but released here as a result of membrane breakdown during prolonged cold incubation. Indeed T.E.M. photographs of such granules after experimental treatment showed the membranes to be rather fuzzy in apperance, no more typical bilayer being detectable.

In a couple of experiments, 'MT-G : 03 and 05', in addition to using whole MT. proteins, zymogen granules were combined with MT. proteins with the M.A.P's removed by cellulose phosphate cation exchange chromatography. In the case of experiment 'MT-G : 05', the levels of T.

measured in supernatants derived from such samples were too low to be detectable by ^{3}H-colchicine binding assay. Detectable T. levels were present in the supernatants derived from M.A.P-free MT. proteins in experiment 'MT-G : 03' however. Despite this no incubation temperature effect was demonstrable in either the first or the second supernatants. Nontheless there was certainly a significant ($P < 0.001$) reduction in the mean levels of first supernatant T. in the samples containing granules as against those without granules, regardless of incubation temperatures. This indicates the presence of some non-specific MT. protein binding by the granules which was not influenced by the incubation temperature. In the absence of M.A.P's the granule effect constituted 42% of the total T. added (that present in the samples containing only MT. proteins) as against a value of 17% for samples containing M.A.P's.

This non-specific binding of T. to the granule membranes was not simply a facet of the M.A.P-free MT. proteins but occurred to some extent in all the experiments. Generally the levels of T. in the first supernatants were higher in the samples containing only MT. proteins than in those containing both MT. proteins and granules regardless of incubation temperatures. In particular, though, the MT. protein-granule mixtures kept at 4°C nearly always had lower T. levels in the first supernatants than the samples containing only MT. proteins. This is surprising when we consider the fact that granule-T. binding seemed to be dependent on the presence of MT. proteins as assembled MT. This non-specific T. binding by the granules may well have been caused by the presence of MT. fragments which failed to disassemble but still bound to the granules, however such fragments were not seen in T.E.M. views of cold incubated granule membranes. It may also have been due to electrostatic or biochemical interactions between T. and the granule

membrane, certainly it was not dependent on the presence of M.A.P's. That such non-specific binding did occur is certainly reflected in the T. levels of the second supernatants, the levels of T. released from the granule pellets incubated at $4^{o}C$ usually being higher than those detected from T. binding to the test tube used for the MT. protein-only samples after removal of the supernatants.

The analysis of the T. levels in the supernatants discussed above has shown us that indeed the levels of T. did fall when assembled MT. were pelleted in association with zymogen granules. The associations we have seen in the electron micrographs of the granule pellets were the result of the loss of MT. proteins, in particular T, from the supernatants to the pellet as bound assembled MT. This T. bound to the zymogen granules was readily released again once the MT. attached to the granules were disassembled. In addition to this specific association of granule membranes with the MT. we were able to detect some non-specific binding of T. to the membranes. This may represent a particular part of the mechanism necessary for binding assembled MT. to the granules but is more likely to have been the result of simple electrostatic or biochemical adsorption of T. onto the membranes. Probably the volume of the granule pellets would have taken down with it a small portion of the contents of the supernatant during centrifugation. Some T. was certainly left adhering to the reaction tubes and could be similarly released in the second supernatants. What is certain is that the second, cold attachment of T. to the granules was certainly independent of the presence of M.A.P. This was not the case with the MT-associated attachment, the removal of M.A.P's from the MT. proteins produced no difference in the binding of the granules to T. whether polymerised into MT. or otherwise, whereas whole MT. proteins bound preferentially to the granules when assembled into MT.

Finally T. bound to the zymogen granules was detected which was not readily released upon cold depolymerisation, this T. is believed to represent MT. proteins native to the granule membranes only released as they broke down over a considerable period (about 48 hours). This native T. may have a role in the binding of MT. to the granule membrane possibly via its affinity for the M.A.P. side arms. The amounts of T. bound both as assembled MT. and non-specifically was very variable and depended on the original concentration of MT. proteins in the depolymerised solution from brain added to the zymogen granules as well as on the number of granules in the original suspension isolated from the pancreas material. All that could be done was to ensure that the MT. protein solution and granule suspension used in each individual experiment were completely uniform.

The effects of exocrine pancreas stimulation on the MT-free T. equilibrium.

The establishment of an *in vitro* interaction between exocrine pancreatic zymogen granules and assembled MT. in the previous section provided evidence for a role for MT. in pancreatic secretion. In order to obtain further evidence for an *in vivo* role for MT. in secretion the levels of both assembled MT. and depolymerised T. were investigated in the exocrine pancreas. These two pools of T. were separated and measured by ^{3}H-colchicine binding assay. Assays were carried out for the concentrations of T. in weight to volume pancreas supernatants derived from mice kept at basal pancreas secretion levels and at actively secreting levels generally under the influence of Pilocarpine.

We might expect that if MT. are indeed important to one or more of the processes of exocrine pancreatic secretion alterations in the relevant T. pools would occur. As secretion begins more T. may enter the MT. pool to provide more MT. for binding and possibly transport. Alternatively T, could move from the MT. pool to the free-T. pool as granule release, associated with MT. breakdown, occurs. The *in vitro* granule binding study provided evidence that MT. attachment to the zymogen granules was both possible and readily reversible, giving no clue as to which is more important *in vivo*.

The results presented in this section are somewhat inconclusive, although two experiments provided evidence for a significant effect of treatment for pancreas secretion. The experiments which showed that Pilocarpine treatment (to induce active exocrine pancreatic secretion) did in fact alter the MT-free T. equilibrium in the pancreas were experiments 'PANC : 09' and 'PANC : 18'. In both cases the Pilocarpine-treated mice had pancreas with an overall lower depolymerised T. content

than did mice given straightforward saline injections. These differences were both statistically significant at the 5% level ($P < 0.05$), indicating that such findings are possible by chance once in twenty times. In the case of experiment 'PANC : 09' the overall level of depolymerised T. in the actively secreting (Pilocarpine-treated) pancreas supernatants was 2.44µg/ml lower than in the basal secreting pancreas supernatants; with a confidence interval of 2.42µg/ml. The corresponding difference in experiment 'PANC : 18' was 3.50µg/ml with a confidence interval of 3.04µg/ml. This indicates that the differences observed in these two experiments were in fact not only of the same order but also in the same direction. Despite the presence of a treatment effect on the depolymerised T. pancreas pool in these two experiments, no corresponding effect was demonstable on the polymerised pool; indeed no such effect was present in any of the experiments.

Why were no significant treatment effects demonstrated in any of the other sixteen experiments? The analysis of the experiments pooled as groups and into blocks shows us that there was no reason to believe that there was any significant difference between the individual experiments. The effect of treatments in the individual experiments must be looked at in the light of both the variations in the mice and the variations in the T. determination values. Three T. determinations were made for each mouse pancreas supernatant, both first and second corresponding to depolymerised T. and polymerised T. respectively. So far as the first supernatants were concerned, there were considerable differences between the 'within mice' determination variances for the individual experiments. It is worth noting that the variances for experiments 'PANC : 09' and 'PANC : 18', where a significant effect of treatment was found, were both very low, 0.9 and 0.3 respectively. The 'within treatments' mice variances for the first supernatants also

showed a considerable range but once again the experiments with significant treatment effects had low variances, although in both cases they were significant.

The occurrence of a significant effect of treatment at the $P<0.05$ level in two out of seventeen experiments does not provide concrete evidence for an overall difference in the depolymerised T. levels of the exocrine pancreas, despite the fact that it is statistically significant. However, the presence of considerable variations in the T. levels of individual mice and the problems with high determination variances, may indicate that a real treatment effect does exist. This difference in the depolymerised T. levels of secreting and resting pancreas must, if present, be very small. The experiments described in this section certainly do not seem to be capable of measuring it satisfactorily. The effect of treatment only showed up when the 'within mice' determination and 'within treatment' mice variances were small. The small determination variances in turn made the small mice variance significant. The peculiarity was also present in the second supernatant results, significant mice variation was brought about by the low determination variance. Despite this fact, there were still experiments where both the first and second supernatant results had low mice and determination variances and yet no significant effect of treatment was demonstrated.

In the overall analysis, when the experiments were examined as a group the experiments themselves showed no significant variation, but both the mice and determinations had fairly high, significant variances. Although the second supernatants had insignificant mice variation in the first group no treatment effect was demonstrated. As was mentioned at the outset the results of these experiments were inconclusive, nonetheless they provided some hints of a difference in secreting pancreas T. levels

and a spur to design experiments better suited to showing this difference.

Examination of the microtubule, free tubulin equilibrium during the stimulated exocrine pancreas time course.

The attempts made at showing differences in the two T. pools of the exocrine pancreas during secretion and rest discussed in the previous section proved to be inconclusive. The immense variation between individual mice and the problems of producing low assay determination variability made it practically impossible to measure the small differences in T. levels likely to be present. For this reason a second series of experiments, discussed in this section, were designed to attempt to show the presence of a real difference in T. levels between the actively secreting and basal secreting exocrine pancreas. Here the levels of T, both polymerised and depolymerised, were measured over a prolonged period after injection. The periods over which the levels of T. were studied were from 10 to 60 minutes after injection (experiments 'PANC : 19, 21 and 22') and between 0 and 20 minutes after injection (experiments 'PANC : 20'). Experiments 'PANC : 19, 20 and 22' studied the effect of Pilocarpine alone on the pancreas T. levels whereas experiment 'PANC : 21' compared the effects of Pilocarpine and saline injections.

Significant alterations in the levels of pancreas T. as a result of treatment by Pilocarpine injection were shown only in experiment 'PANC : 19' in the period 10 to 60 minutes after injection. Comparison of the saline and Pilocarpine injected mice over the time period after injection (experiment 'PANC : 21') showed a significant interaction between the two treatments over this same time period. There was no significant difference in the Pilocarpine-induced T. levels over the period 0 to 20 minutes after injection (experiment 'PANC : 20'), nor in the period 10 to 60 minutes after injection when only three replicate mice were compared for each time interval (experiment 'PANC : 22').

Looking first at the results of experiment 'PANC : 19', the analysis of variance using orthogonal polynomials indicate that there was a significant ($P < 0.05$) quadratic effect of treatment upon the pancreas depolymerised T. levels and a significant ($P < 0.01$) linear effect on the polymerised T. levels. These effects occurred over the period 10 to 60 minutes after injection. Thus assuming that there is a polynomial relationship between the levels of T. and the time after Pilocarpine injection; if there is not truly a quadratic term, in the case of depolymerised T, and a linear term, in the case of polymerised T, in the relationships then we have witnessed two very unlikely events. The response curve for the levels of depolymerised T. had the formula $Y = 6.5 + 0.4X + 1.0X^2$, where Y was the level of depolymerised T. and X was the time after Pilocarpine injection. This curve, when plotted, shows its significant quadratic nature by rising from a basal depolymerised T. level of 2.5µg/ml at 10 minutes after injection to a peak of about 10µg/ml at about 38 minutes, only to fall to 4.6µg/ml at 60 minutes. Thus the levels of depolymerised T. rose, peaked and fell over the period after Pilocarpine injection.

The concurrent alteration in pancreas polymerised T, after Pilocarpine injection, had a significant linear trend. The response curve for this trend had the formula $Y = 1.0 - 0.2X$, producing a line with negative slope falling from 1.6µg/ml at 10 minutes to 0.3µg/ml after 60 minutes. When we consider that depolymerised T. and assembled MT. are in equilibrium or at least interdependent; it is suprising, given the negative linear trend for polymerised T, that no concomitant positive linear trend is evident in the depolymerised T. levels. What we do have in the depolymerised T. levels is an initial positive linear trend from 10 to about 38 minutes after injection followed by a steady reduction in the levels from 38 to 60 minutes. It appears, therefore, that the

effect of Pilocarpine injection on the exocrine pancreas T. pools was to produce an overall reduction of polymerised T. which was accompanied by an initial rise in the depolymerised pool followed by a fall at about 38 minutes after injection. Thus it seems that exocrine pancreatic secretion was paralleled by a decrease in assembled MT. and that the disassembly of these MT. produced a rise in the levels of depolymerised T. However, this rise did not continue throughout the MT. disassembly period, depolymerised T. began to be removed from the cellular pool after about 38 minutes of MT. disassembly. The fate of this removed T. is uncertain, it may simply have been broken down by lysosomal action, it may have been sequestered in a cellular site inaccessible to ^{3}H-colchicine binding or it may even have been inactivated so that colchicine would not bind to it.

Extrapolation of the response curves discussed above would be very dangerous and likely to lead to spurious conclusions. The second experiment 'PANC : 20' was designed to examine the effect of Pilocarpine on the T. levels immediately after injection, ie., in the period 0 to 20 minutes. However, analysis of the results showed that there was no significant polynomial relationship between the time after injection and both the levels of polymerised and depolymerised pancreas T. The response curve for these effects would therefore correspond to a straight line with no slope at the mean values for the T. levels. These lines, when plotted on the graphs of the response curves analysed in the previous experiment ('PANC : 19'),do in fact overlap with the curves previously plotted in the case of the depolymerised T. However the polymerised T. curves do not overlap, even when account is taken of the confidence bands for the two lines. It cannot therefore, necessarily be concluded that the mice treated with Pilocarpine in the first experiment in this group were from the same population as those in the

second experiment. Despite this the second experiment does indicate that there were no Pilocarpine treatment effects on the T. levels within the first 10 minutes after injection. It seems likely that the first experiment did in fact cover the time range during which alterations in the T. pools took place. The first ten minutes after injection was the period taken for the sub-cutaneous Pilocarpine injection to reach the pancreas and build up to a level capable of stimulating pancreatic secretion. This time lag was present even though the dosage of Pilocarpine was very high.

Having shown that exocrine pancreatic stimulation with Pilocarpine affected the assembled MT.-free pool T. equilibrium, the next step was to compare this effect with the effect of control saline injection over a similar time period after injection. Hence a similar methodology was applied to two groups of mice, one injected with Pilocarpine in saline and the other with saline alone. The effect of the two types of treatment were then not only examined alone but also the interactions between the treatments over the time after injection were compared. Initial analysis of the results of this experiment ('PANC : 21') showed no significant effects of treatment upon either the polymerised or depolymerised pancreas T. levels. Nor were there any significant interactions between the two types of treatment in either case. However, the presence of a zero T. value in the second supernatant results warranted the transformation of the data by adding one to the determined T. values and converting to logarithms. Although this showed up no significant effects in the depolymerised T. data, the polymerised T. data had a significant ($P < 0.05$) treatment interaction over the time period which was shown to be at the quadratic level.

When the average effects of the two treatments (Pilocarpine and saline injection) together over the time period after injection was

analysed no significant deviations were present. However when the two treatments were isolated and compared with one another over the same period a significant ($P < 0.05$) quadratic relationship was demonstrated. Looking at the response curves for the two treatments this is not surprising since the two curves are almost mirror images of one another. Combining the two would cancel any polynomial relationship leaving a straight, non-sloping line at the mean of the two treatment effects. The relationship lies in the difference between the two curves, this has a significant quadratic nature.

After injection the two treatment curves behaved almost in opposition, the levels of polymerised T. in the saline treated mice rose, peaked and then fell; whereas the levels in the Pilocarpine treated mice fell, troughed and then rose again. The saline treatment curve peaked at about 43 minutes after injection while the Pilocarpine curve troughed at about 36 minutes. The shape of the Pilocarpine-treated curve compares to a certain extent with the polymerised T. curve produced in experiment 'PANC : 19', certainly from 10 to 30 minutes after injection both curves show a decline in the levels of polymerised T. However, 30 minutes after injection the decline in polymerised T. exhibited in experiment 'PANC : 19' was not present in 'PANC : 21', instead the levels of polymerised T. levelled off and eventually rose again. The levels of polymerised T. encompassed by the two experimental curves are also similar between 10 and 30 minutes after injection, they coincide when the confidence bands are compared although the curve for experiment 'PANC : 21' has the larger range. It is noticeable that this range also covers the levels of polymerised T. measured in experiment 'PANC : 20'. When the curves are compared later than 30 minutes after injection they are obviously less coincident since the curve for experiment 'PANC : 21' begins to rise while the 'PANC : 19' curve continues to fall. It appears

that the regime followed in experiment 'PANC : 21' produced a more rapid alteration in the polymerised T. levels and an even more rapid return to the non-secreting levels. This may have been due to variations in the Pilocarpine dosage but was probably more likely to have been due to the variations between the individual groups of experimental mice.

The most surprising result of experiment 'PANC : 21' was that, not only did Pilocarpine elicit alterations in the levels of polymerised T. over the period after injection, but such alterations were also produced by straightforward saline injection, only in this case in the opposite direction. Saline injection produced a rise followed by a fall in polymerised pancreas T. It appears, then, that either the saline itself or possibly the stress associated with injection induced an effect on the pancreas assembled MT. This makes the opposite effects of Pilocarpine, injected borne in saline, even more remarkable. Despite the significance of the differences in effect of the two types of treatment, Pilocarpine and saline injection, on the levels of polymerised pancreas T, no concomitant significant effects were detected in the levels of depolymerised T. This may have been due to the rapidity with which excess free T. was sequestered to another site as discussed previously or possibly to the enormous variation in the levels of depolymerised T in the experimental mice.

Coming finally to consider the last experiment in this group, experiment 'PANC : 22', the T. levels, after Pilocarpine injection, were compared again over the period 10 to 60 minutes after injection, this time using only three replicate mice per time point group. This low level of replication failed to produce a significant treatment effect in either the first or second supernatant results. Even transformation to a logarithmic scale after the addition of 1µg/ml to each mouse mean failed to show any significant effect of treatment. This lack of a

treatment effect was the result of excess mouse variation being difficult to eliminate in the smaller sample.

Despite the presence of this final insignificant effect of treatment, the other experiments show, particularly experiment 'PANC : 19', that Pilocarpine injection had a definite effect on the levels of exocrine pancreas T. This effect was most marked in the levels of polymerised T. where at about 10 minutes after Pilocarpine injection, the levels of T. began to fall. This fall continue for 60 minutes after injection ('PANC : 19') or levelled off and began to rise after 36 minutes ('PANC : 21'). The effect of this falling presence of exocrine pancreas MT. on the levels of depolymerised T. were less marked. It seems that the depolymerising MT. released free T. which was detected as a rise in the level of pancreas depolymerised T. However this rise did not continue for the full 60 minutes after injection. Some of the free T. was removed either by breakdown or cellular storage, this removal could have been so rapid as to prevent detection as a depolymerised pancreas T. rise. Thus the level of pancreatic free-pool T. could be maintained at a constant level despite alterations in the number of assembled MT. during secretion. These Pilocarpine injection effects on the mouse exocrine pancreas could have been the direct result of drug-induced effects on cellular MT. More probable, though, is that exocrine secretion induced by Pilocarpine, produced a physiological reduction in the numbers of assembled MT. The effect of saline injection, as a control, in inducing an opposite increase in pancreatic MT. indicates that any stress or osmotic pressure related effect would not induce this fall in exocrine pancreas MT. numbers but would be more likely to have the opposite effect. Thus we can conclude that during exocrine pancreas secretion, certainly in the initial phase from 10 to 60 minutes after stimulation, the numbers of pancreatic MT. are reduced.

This reduction may be accompanied by an increase in free MT. proteins, although the level of these may be kept constant by their rapid active removal from the detectable pool. This removal could encourage the further shift of the free T, MT. equilibrium towards the free T. side inducing further MT. disassembly.

Measurement of MT. proteins using the radioimmunoassay technique

The ^{3}H-colchicine binding assay for T. used in the previous sections to assay both in vitro zymogen granule bound T. and pancreas supernatant T. produced considerable determination variations in some experiments and was incapable of detecting very low levels of T. In an attempt to overcome these problems and also to measure M.A.P's associated with T., a radioimmunoassay technique was adopted. The results of these RIA's for both granule-bound and pancreas MT. proteins proved to be as variable as those of the ^{3}H-colchicine assays.

Two of the granule binding experiments when assayed by RIA produced results which were ridiculously high. The cause of this can be credited to the non-specific binding of the antisera used and also the problems encountered in keeping the bound pellets together discussed earlier. Apparent low percentage binding obviously caused the assay results to err on the high side. Often excellent correlation between T. standard concentrations and percentage binding was countered by the high variations in the individual experimental determinations. For these reasons the results of experiments 'RIA-MTG : 02' and 'RIA-MTG : 03' are probably best ignored, not least because the treatment effects investigated were certainly not significant.

Significant treatment effects encountered using the RIA for T. and M.A.P's were in experiments 'RIA-MTG : 01' and 'RIA-MTG : 04'. The former experiment exhibited a significant effect of treatment, attributable to alteration in the incubation temperature, in only the third supernatant. Although the first and second supernatants had effects consistent with the findings of the ^{3}H-colchicine binding assays for T, in the light of the high error values produced by a high determination variance they are insignificant. However a low value for error mean square in

the third supernatant produced a significant ($P<0.05$) temperature effect. In common with the experiments assayed by ^{3}H-colchicine binding the levels of T. released from granule binding were higher in the warm incubated samples than in the cold incubated ones. Unusually, though this difference was detectable in the third supernatant showing that even after one hour under T. depolymerisation conditions, more T. was released from the warm incubated granules after a further overnight depolymerisation period. This may represent T. more tightly bound to the granules or possibly T. derived from the granule membranes themselves.

When the RIA technique was used to measure the M.A.P. content of zymogen granule bound supernatants no significant treatment effect was detected in the first supernatant. However, the levels of M.A.P. measured in these samples paralleled those of the T. detected by ^{3}H-colchicine binding (see experiment 'MT-G : 08'), a high error mean square value prevented them from being significant. Significant ($P<0.001$) treatment effects were exhibited by the second supernatant samples both for incubation temperature and addition or exclusion of granules. The effect due to alteration of the incubation temperature was + 6.6µg/ml in favour of the warm incubated samples, while the granule addition effect was - 3.8µg/ml against the samples without granules. These values compare favourably with those obtained for the effects on the ^{3}H-colchicine binding T. levels, the M.A.P. values being 13 to 15 % of the T. ones; especially when we consider that the M.A.P's constitute on average 13% of total isolated MT. protein fraction (Sloboda et al, 1976). Once again the third supernatant M.A.P. values had a high error mean square, due to high determination variation, resulting in insignificant treatment effects.

It appears that, where significant differences in M.A.P. levels were detected, they were consistent with the differences in T. levels.

This indicates that M.A.P's remained in a stoichiometric relationship to T. even after disassembly of the MT. after binding to zymogen granule membranes.

The final experiment in this section, 'RIA-PANC : 01' used the RIA for T. to remeasure the levels of polymerised T. in supernatants assayed by ^{3}H-colchicine binding in experiment 'PANC : 22'. No significant treatment effect was shown by either method of assay. The levels of T. in both cases showed excessive variation due both to the differences in mouse T. levels and determination consistency. Also the actual assembled T. values for both methods were inconsistent, particularly the measurements made at 30 and 45 minutes after Pilocarpine injection. This is believed to be due to the inconsistency of the RIA producing a number of erroneously high values on the one hand and the failure of the ^{3}H-colchicine binding assay to measure some of the lower values on the other.

Generally the use of an RIA to measure MT. proteins, rather than producing more consistent and sensitive results, has introduced more problems. In particular the consistency of determinations on individual experimental samples has been very low. This may of course be eliminated after further trials, however without the improvement there seems little advantage in the RIA since it takes much longer to perform than the ^{3}H-colchicine binding assay. Its main advantage lies in its use to measure M.A.P's since they are difficult to quantify by any other method. Indeed the results obtained using M.A.P. antisera proved to be more consistent, producing values comparable with the corresponding T. levels measured by ^{3}H-colchicine binding assay.

Conclusions and Prospects.

The available literature discussed in the introduction has examined the cellular secretory process from two main viewpoints. Firstly several studies have been done on the effect of known microtubule poisons, such as colchicine and vinblastine, on secretion and other cellular processes thought to involve MT. Secondly, a number of groups have examined secretory cells in the electron microscope to try to produce evidence for a structural relationship between MT. and the secretory pathway. Consequently a number of models have been proposed to provide an explanation of the wide variety of cellular mechanisms thought to involve MT.

From the point of view of the secretory process two main mechanisms seem to explain MT. action. MT, possibly in conjunction with other components of the cytoskeleton notably microfilaments, could provide the motive force or directionality necessary for the transport of secretory granules to the cell membrane. Alternatively they could act to prevent granule movement towards the membrane, secretion being the result of a temporary breakdown of the MT. cell cytoskeleton. The electron microscope study of MT. and isolated zymogen granules combined in vitro does not favour either secretory model. However it does show that the interaction between MT. and granule membranes is not simply a passive trapping process. Rather, specific structural connections were seen apparently produced from the M.A.P. side-arms and possibly parts of the granule membrane. This MT-granule bonding proved capable of resisting powerful centrifugation forces to produce pellets which in S.E.M. view were reminiscent of the freeze-dried cytoskeleton replicas produced by by Heuser and Kirschner (1980). Zymogen granules were capable of effecting between 14 and 100% removal of MT. proteins from solution,

this removal being dependent on the presence of assembled MT. proteins and M.A.P's, although some non-specific removal of disassembled T. was detected.

The secretory role of MT. in vivo proved much more difficult to elucidate since the changes in the MT-free T. equilibrium during exocrine pancreas secretion were very small in comparison with the degree of variability in total tubulin between experimental animals. However measurement of the MT-T.pools over the period after artificially-induced pancreatic secretion with Pilocarpine did produce changes in both pools. These changes seemed to confirm the 'trapping model' for the role of MT. in secretion since the levels of polymerised T. fell as secretion was induced. Concomitantly free pool T. rose but later began to decline. Indeed free T. seemed to be removed rapidly from the detectable pool, possibly by breakdown or sequestration. The pancreas MT. system also seemed to be sensitive to alterations induced by saline injection, although changes were in the opposite direction to those induced by Pilocarpine and saline together.

It appears that exocrine pancreatic secretion is accompanied by alterations in the MT-free pool T. equilibrium. The binding of zymogen granules to assembled MT. suggests that MT. might be directly involved in the secretory process. Since MT. depolymerisation seems to occur during secretion it seems likely that the MT. act to release trapped granules so that other cytoskeletal elements can carry them towards the acinar cell lumen.

Despite this final conclusion a number of aspects of this process are still unclear. The fate of the M.A.P. side-arms during this process is not certain, they may be left attached to the MT. or remain attached to the zymogen granule membrane for removal during membrane protein recycling. The mechanism for granule movement from the Golgi

complex to the apex of the acinar cells is also unknown. MT. are involved in holding the granules in position, what provides the motive force for this movement ?, microfilaments seem likely candidates. These questions are yet to be answered, the results presented here provide only clues to the role of MT. in secretion. The in vitro model needs to be better studied, especially in the light of other in vitro systems and to elucidate the role of nucleotides and M.A.P's in the binding process. A more sensitive assay for MT. proteins is necessary to better examine the in vivo role of MT. in secretion, the RIA needs to be modified to provide more consistent determinations.

The procedures and results discussed in this study are well suited to the examination of the role of MT. in secretion. To produce an overall picture other tissues need to be examined as do other cellular organelles. MT. certainly are involved in a wide variety of cellular processes, protein secretion being one which is well suited to further study.

Statistical methods.

In this section of the appendix are outlined the statistical procedures used to analyse the experimental results. These were mainly modifications of the analysis of variance procedure although regression analysis, the correlation coefficient and analysis of covariance were also used. In each case the experimental results were transferred by computer programme to another programme capable of carrying out the statistical tests required. The tables of results and analyses were then printed out by the computer and checked by hand for significant values by comparison with statistical tables. The levels of significance used were always the same being denoted by an asterix system outlined in the first part of this section. The main references used for statistical procedures were Ridgman (1975), Snedecor and Cochran (1967) and Dawkins (1975) although Cochran and Cox (1957) and Pollard (1977) were also consulted; the statistical tables used were White, Yeats and Shipworth (1974).

Analysis of variance using orthogonal linear contrasts.

This statistical test was used to assess the variations present in the MT-zymogen granule binding experiments. The effect of treatment was partitioned into two contrasts, the in vitro effect of temperature on the mixture of MT. proteins and zymogen granule and the effect of zymogen granule addition on the level of T. in the supernatants left after centrifugation. The null hypotheses tested were : (1) There was no difference in the supernatant T. content of samples initially incubated at 37°C and 4°C before the first centrifugation; and (2) The addition or exclusion of zymogen granules made no difference to the post-centrifugation supernatant T. levels. These null hypotheses were tested for each of the supernatants collected regardless of the incubation temperatures of the later supernatants. All calculations were made using a custom-written computer programme.

Initially the components of the analysis of variance table were calculated in the usual manner. The first column, headed 'Source of variation', shows the sources of variation which could be isolated in the experiment; this includes the total variation, treatment variation and residual or error variation. The second column shows the degrees of freedom (D.F.) for each source of variation; for total variation this was the total number of observations less one, for treatment variation this was the number of treatments less one and for error variation this was the total variation D.F. less the treatment variation D.F. The third column in the analysis of variance table shows the sum of squares (or sum of squares of the deviations from the mean) for each source of variation. This value for total variation, assuming the observations form the array $x_1, x_2, x_3 \ldots\ldots x_n$, with mean $\bar{x}$ was

calculated as :

$$\Sigma(x_i-\bar{x})^2 = \Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}$$

The sum of squares (S.S.) for treatment variation, where T_1, T_2, T_3, ... are the treatment totals and r_1, r_2, r_3, ... are the number of the respective replicates, was calculated as :

$$\frac{T_1^2}{r_1} + \frac{T_2^2}{r_2} + \frac{T_3^2}{r_3} \quad ..... \quad - \frac{(\Sigma x)^2}{n} \quad .$$

Error S.S. was calculated from the difference between total S.S. and treatment S.S. These values for S.S. were used to calculate the unbiased estimate of variance or average spread for each source of variation; this value, known as the 'mean square' (M.S.), was calculated as the S.S. divided by the D.F. for each source of variation. The M.S. for total variation was not calculated since it represents the mixture of variation separated into treatment and error variations.

So far we have followed a conventional analysis of variance methodology, the difference now comes in partitioning the treatment variation into its component parts. This involved the comparison of orthogonal contrasts using a table of linear coefficients to partition the S.S. The number of contrasts permissible is the same as the number of D.F. for treatment variation. In this case 3 D.F. for treatments permitted three contrasts, however, only two were necessary to analyse the experiments, i.e., comparing the temperature effect and the granule effect. The effect of temperature was found by comparing the T. content of the supernatants from the samples containing both MT. protein and granules while the granule effect was found by comparing these two groups combined with the samples containing no granules only MT.protein.

In practice this involved the calculation of linear coefficients for each contrast. These coefficiants were found by determining the total number of individual values on each side of the contrast and then finding the lowest common multiple of these numbers. The required coefficient for either side was then this value divided by the number of individual values on that side. The signs on each side of the contrast were made opposite so that the sum of the coefficients was zero. Before calculations were made of the partitioned treatment S.S. the coefficients were checked for orthogonality. Assuming the matrix of coefficients for two contrasts to be :

1st contrast	$k_{1,1}$	$k_{1,2}$	$k_{1,3}$ .....
2nd contrast	$k_{2,1}$	$k_{2,2}$	$k_{2,3}$ .....

and the number of replicates for each column to be r_1, r_2, r_3 ..... then the tests of orthogonality were :

$$k_{1,1}\cdot r_1 + k_{1,2}\cdot r_2 + k_{1,3}\cdot r_3 + \ldots\ldots = 0 ,$$

$$k_{2,1}\cdot r_1 + k_{2,2}\cdot r_2 + k_{2,3}\cdot r_3 + \ldots\ldots = 0 \text{ and}$$

$$k_{1,1}\cdot k_{2,1}\cdot r_1 + k_{1,2}\cdot k_{2,2}\cdot r_2 + k_{1,3}\cdot k_{2,3}\cdot r_3 + \ldots\ldots = 0.$$

If the contrasts selected were confirmed as orthogonal the S.S. for each contrast was calculated. In the case shown above where the treatment totals were T_1, T_2, T_3, ... then the 1st contrast S.S. would be :

$$\frac{(T_1\cdot k_{1,1} + T_2 k_{1,2} + T_3\cdot k_{1,3}\ldots)^2}{(r_1\cdot k_{1,1}^2 + r_2\cdot k_{1,2}^2 + r_3\cdot k_{1,3}^2 \ldots)} \text{ , the}$$

second contrast S.S. being calculated similarly. These partitioned S.S. were used to calculate the respective M.S. by dividing by the contrast D.F. - always one. Once the M.S. column of the analysis of variance column was completed with these values, the variance ratio or F- value for each contrast was calculated in the normal manner as :

$$F = \frac{\text{M.S. for contrast variation}}{\text{M.S. for the error variation}} \text{ and}$$

also added to the table. The level of significance of these F values

were then checked in tables showing the percentage points of the F distribution for normally distributed random variates. Assuming the data to consist of normally distributed random variates with the same variance for each treatment, if the experiment gave an F value larger than the table value at the chosen level, then if there was not a true effect of treatment on the difference between the means then we had witnessed an event which can occur less frequently than the chosen level. In this case the levels chosen were indicated by a star notation:

		$P > 0.05$	NS
5% level	:	$P < 0.05$	*
1% level	:	$P < 0.01$	**
0.1% level	:	$P < 0.001$	***

Finally to summarise the data the total effect of treatment, standard error of this effect, mean effect of treatment and 95% confidence limits were calculated for each contrast. The total effect was calculated as $\leq kT$ with a standard error of $S.E. = \sqrt{\text{error M.S. } (r_1 . k_{1,1}^2 + r_2 . k_{1,2}^2 + \ldots)}$. These total contrasts were converted to mean contrasts by dividing by $\frac{1}{2} \leq |rk|$ for the relevant contrast.

Analysis of variance using a hierarchical design.

This test was used to analyse the results of the experiments which compared the mouse pancreas T. pools during the active and basal secretory states. The same method was used for the individual experiments and also on the combined experimental data. The problem associated with these experiments was that the variation due to treatment (for pancreas secretion or otherwise) also contained variation due to the mice within each treatment group together with variation due to the assay determinations for each mouse. Consequently the determination of the treatment effect required the isolation of both these other effects.

The experiments were further complicated by the fact that not all the variations were random-effect factors. The substitution of one treatment for another would have altered the experiment completely making it a different experiment altogether. Hence the treatment for secretion was a 'fixed-effect factor', while the mice used within the treatment groups and determinations within the mice could both be readily substituted without alteration to the experiment making them 'random-effect factors'. The difference between these two types of factor is that random-effect factors have true population variances which can be estimated by their M.S. while fixed-effect factors have M.S. which only estimate variation between the levels of the factor. For example two models are possible for a single factor experimental design with p treatments each with r replications :

Source of variation	D.F.	Expected M.S. Fixed-effect factor	Expected M.S. Random-effect factor
Treatments	p-1	$\sigma^2 + r A^2$	$\sigma^2 + r \sigma_A^2$
Error	p(r-1)	σ^2	σ^2

In the case of the individual pancreas secretion experiments the only fixed effect factor was treatment for secretion so the parameters

estimated were :

Treatment for secretion : $\sigma_D^2 + 3\sigma_M^2 + 9T^2$,

Mice within treatment groups : $\sigma_D^2 + 3\sigma_M^2$,

Determinations within individual mice: σ_D^2,

and for the ten experiments analysed as a group they were :

Experiments : $\sigma_D^2 + 3\sigma_M^2 + 9T^2 + 18\sigma_E^2$

Treatments within experiments : $\sigma_D^2 + 3\sigma_M^2 + 9T^2$

Mice within treatment groups : $\sigma_D^2 + 3\sigma_M^2$

Determinations within mice : σ_D^2

The practical estimate of the parameters above is the M.S. for each individual variable. However, the determination of the S.S.and hence the M.S. for each variable did not follow the normal methodology since some of the M.S. contained a portion attributable to the M.S. from other variables. In practice the first step in the analysis was to determine the D.F. for each variable. For individual experiments there were two treatments for secretion groups so the D.F. for treatments was (2-1) or 1. Each treatment group contained three mice so had (3-1) or 2 D.F. and since there were two groups the D.F. for mice within treatment groups was 4. Each mouse had three determinations carried out on it so had (3-1) or 2 D.F. and there were six mice in the experiment making a total of 12 D.F. for determinations within mice. When summed together the total number of D.F. came to 17 which is as was expected for 18 individual values.

In calculating the variable S.S. the next step was to calculate the total of the three determinations for each mouse (M), then the total of these for each treatment group (G) and finally the grand total ($\sum x$) of all the determinations (x) together. The S.S. for each variable were then calculated using the following formulae :

Treatment for secretion S.S. $= \frac{\Sigma G^2}{9} - \frac{(\Sigma x)^2}{18}$

Mice within treatment S.S. $= \frac{\Sigma M^2}{3} - \frac{\Sigma G^2}{9}$

Determinations within mice S.S. $= \Sigma x^2 - \frac{\Sigma M^2}{3}$

Note that each S.S. contains a component from the S.S. above it apart from treatment S.S. which has the correction factor.

Using these S.S. values the M.S. were determined by dividing by the relevant D.F. The F values were then found in a slightly different manner to the usual. The test for evidence that σ_M^2 was other than zero was done using an F value for the mice within treatment variable found from :

$$\frac{\text{M.S. for mice within treatments}}{\text{M.S. for determinations within mice}} .$$

Similarly the evidence for a significant T^2 value was found from the F value :

$$\frac{\text{M.S. for treatment}}{\text{M.S. for mice within treatments}} .$$

Where these F values were found to be significant from F tables the individual estimates of variances and effects were then determined. The estimate of σ_D^2 was the M.S. due to determinations within mice. The estimate of σ_M^2 was found from :

$$\frac{\text{M.S. for mice within treatments} - \text{M.S. for determinations}}{3} ,$$

and of T^2 from :

$$\frac{\text{M.S. for treatments} - \text{M.S. for mice within treatments}}{9} .$$

These formulae f ollow from the model of the parameters estimated, outlined earlier. The confidence limits of these estimates do not follow a true normal distribution but rather approximate to the χ^2 distribution and were estimate from χ^2 tables. The formula for

calculating these confidence limits is :

$$\frac{(n-1)s^2}{\chi^2_{\frac{1}{2}(1-\alpha)}} \qquad \sigma^2 \qquad \frac{(n-1)s^2}{\chi^2_{\frac{1}{2}(1+\alpha)}}$$

where s^2 is the estimate of the true variance σ^2 with (n-1) D.F. and probability α.

The analysis of the pancreas experiments combined followed a similar hierarchical methodology but was combined with a conventional two way analysis of variance treating each experimental treatment group mean as an individual observation. Analysis was then carried out for variation between treatments and experiments tested conventionally against the error M.S.

Analysis of variance using orthogonal polynomials.

In the first part of the statistics section the use of orthogonal linear contrasts to partition the treatment variation into its component parts was described. In this section the use of orthogonal polynomials to consider the relationship between two parameters will be discussed. This method of analysis was used to investigate the relationships between the levels of T. in the two pancreas pools and time after Pilocarpine injection to induce exocrine pancreatic secretion. Two types of experiment were analysed, one involving only the relationship mentioned above and a second to investigate the interactions between two treatments to induce secretion or otherwise over the time period after injection.

In general the model used to analyse data of this type, where the relationship of some measure to the level of a particular factor is considered, would be a polynomial relationship of the form $y = a+bx+cx^2+dx^3+ \ldots\ldots\ldots\ldots qx^p$. Here y is the variate measured, x the level of treatment and a, b, c, d, etc. constants. In practice, however, we are seldom interested in any relationship beyond cubic, powers greater than this are difficult to account for biologically and are usually combined to give a value for the 'residual' relationship. So far as the experiments analysed here were concerned four main null hypotheses were made : (1) there is no linear relationship between measured T. and time after injection; (2) there is no quadratic effect; (3) there is no cubic effect; and (4) there is no deviation from the linear, quadratic and cubic effects. In other types of experiments the level to which we can stretch this argument depends upon the number of D.F. available, one D.F. being necessary for each level. This follows from the fact that a linear effect only needs two points to plot its

straight line while a quadratic effect requires at least three for its curve and a cubic effect four.

The formula above cannot be solved directly for a, b, c, d, etc. but requires three orthogonal contrasts to partition the treatment S.S. and test each separately. These contrasts are found by solving the equivalent formula $Y = A + BK_1 + CK_2 + DK_3$ where K_1 is a linear function of x, K_2 a quadratic function and K_3 a cubic function; and the K's are orthogonal to each other. The values of the orthogonal polynomials for evenly spaced levels of x can be found from tables (Fisher and Yates, 1963) but unevenly spaced levels have to be found by solving the equation above. The method used is illustrated below using the levels 10, 20, 30 and 40, the units being immaterial (in this case they would be minutes after injection). Orthogonal polynomials for linear contrast are found initially :

Levels	Reduced Levels		Linear effect	Polynomials	
		x	$K_1 = a + x$	K_1	$K_1' = K_1 . \lambda$
10	1	0	a	$-\frac{3}{2}$	-3
20	2	1	$a + 1$	$-\frac{1}{2}$	-1
30	3	2	$a + 2$	$+\frac{1}{2}$	$+1$
40	4	3	$a + 3$	$+\frac{3}{2}$	$+3$
Totals :	-	-	$4a + 6$	0	0

These values follow from the orthogonality of the contrasts which must sum to zero, thus $4a + 6 = 0$ and $a = -\frac{3}{2}$. The polynomials are simplified by the common multiplier λ to give the K_1' values used in the analysis. The next stage is to find the polynomials for quadratic contrast :

Levels	Quadratic Effect	Multiplication	Polynomials
	$K_2=a+bx+x^2$	$K_1'(a+bx+x^2)$	K_2'
0	a	- 3a	+ 1
1	a + b + 1	- a - b - 1	- 1
2	a + 2b + 4	+ a + 2b + 4	- 1
3	a + 3b + 9	+ 3a + 9b + 27	+ 1
Totals :	4a + 6b + 14	10b + 30	0

Once again the orthogonality of the contrasts means that the product of any two must sum to zero producing $10b + 30 = 0$ and $b = -3$. Substituting in $4a + 6b + 14 = 0$ gives $a = 1$; using these values for a and b in the second column gives the polynomials with no simplification being necessary, $\lambda = 1$. Finally the polynomials for cubic contrast are found :

Levels	Cubic effect	Multiplication and substitution	
	$K_3=a+bx+cx^2+x^3$	$K_1'(a+bx+cx^2+x^3)$	$K_2'(a+bx+cx^2+x^3)$
0	a	- 3a	+ a
1	a + b + c + 1	- a - b - c - 1	- a - b - c - 1
2	a + 2b + 4c + 8	+ a + 2b + 4c + 8	- a - 2b - 4c - 8
3	a + 3b + 9c + 27	+ 3a + 9b +27c + 81	+ a + 3b + 9c + 27
Totals :	4a + 6b +14c + 36	10b +30c + 88	4c + 18

Polynomials

K_3	K_3'
$-\frac{3}{10}$	- 1
$+\frac{9}{10}$	+ 3
$-\frac{9}{10}$	- 3
$+\frac{3}{10}$	+ 1

Both a, b and c are solved using the orthogonality of the contrasts, the substituted K_3 values then being simplified by multiplying by $\lambda = \frac{10}{3}$.

This procedure provides simplified polynomials for the three contrasts required, however in practice the procedure outlined above was carried out by a computer programme with no simplification being necessary.

The polynomials were used to partition the treatment S.S. into linear, quadratic and cubic contrasts. The treatment totals for each level were set out at the head of the matrix of polynomials, in the case of our example :

Levels :	10	20	30	40
Totals (y') :	T_{10}	T_{20}	T_{30}	T_{40}
K_1' :	- 3	- 1	+ 1	+ 3
K_2' :	+ 1	- 1	- 1	+ 1
K_3' :	- 1	+ 3	- 3	+ 1

From this matrix was calculated the total effect, $(\leqslant K'.y')$, for each contrast. This was used to find the S.S. for each contrast - $(\leqslant K'.y')^2/(r \leqslant K'^2)$, where r was the number of replicates making up each treatment total. The residual S.S. was found by subtracting the sum of the linear, quadratic and cubic contrast S.S. from the overall treatment S.S. A conventional analysis of variance table was then constructed incorporating the contrast S.S. each with 1 D.F. apart from residual variation which contained what was left over from the overall treatment D.F. From this table were calculated the M.S. for each contrast and for error, each contrast being F-tested against error for its significance.

Where a significant contrast was shown the points of the response curve were calculated. This involved calculating the mean polynomial effects up to and including the highest order of contrast considered significant. The mean effect is the total effect divided by the sum of squares of the coefficients or $(\leqslant K'y') / (r \leqslant K'^2)$. These mean

effects were then set at the head of the polynomial matrix, in our example :

	Mean	Linear	Quadratic	Cubic
Mean effects	A	B'	C '	D'
Y-values				
10	+ 1	- 3	+ 1	- 1
20	+ 1	- 1	- 1	+ 3
30	+ 1	+ 1	- 1	- 3
40	+ 1	+ 3	+ 1	+ 1

Here the cubic effect would be the highest order significant contrast so both the quadratic and linear effects must be included together with the general mean (A). This follows from the curve model $Y = A + B'K_1' + C'K_2' + D'K_3'$. By multiplying each mean effect by its appropriate polynomial and finding the sum for each value of Y the points of the response curve were found. Where significant contrasts were found this curve was plotted together with its confidence band. This was calculated using the standard error for each point of

$$\sqrt{\text{error M.S.}\left(\frac{1}{n} + \frac{K_1'}{r\Sigma K_1'} + \frac{K_2'}{r\Sigma K_2'} + \frac{K_3'}{r\Sigma K_3'} + \ldots\ldots\right)}$$

up to the highest significant order. The curve was then plotted only within the limits of treatment since extrapolation outside these limits was liable to lead to erroneous conclusions.

The comparison of two treatments for pancreatic secretion over the same time period after injection required more complex analysis. Here the contrasts used not only involved linear, quadratic and cubic effects but also comparison of the interaction of these effects for the two types of treatment. The null hypotheses would then become : (1) there is no linear, quadratic or cubic effect nor a deviation from these in the relationship between measured T. and time after injection; and (2) the linear, quadratic and cubic effects and deviations from these

are the same for both treatments. Orthogonal polynomials for the time intervals were calculated as before but each treatment was given a coefficient of either +1 or -1. This enabled the building-up of a matrix of orthogonal polynomials producing interaction coefficients obtained by multiplying the treatment coefficient (-1 or +1) by the time interval coefficients. Using the polynomials calculated previously this would be :

	Treatment 1				Treatment 2			
Levels	10	20	30	40	10	20	30	40
Treatments	-1	-1	-1	-1	+1	+1	+1	+1
K_1'	-3	-1	+1	+3	-3	-1	+1	+3
K_2'	+1	-1	-1	+1	+1	-1	-1	+1
K_3'	-1	+3	-3	+1	-1	+3	-3	+1
Trt x K_1'	+3	+1	-1	-3	-3	-1	+1	+3
Trt x K_2'	-1	+1	+1	-1	+1	-1	-1	+1
Trt x K_3'	+1	-3	+3	-1	-1	+3	-3	+1

This type of matrix was then used to calculate the total effects and S.S. for the contrasts and interactions by placing the relevant treatment total at the head of each column. Where significant contrasts were demonstrated the same matrix was used to calculate the response curve points. Mean effects used in determining the curve points included all those up to and including the highest significant order of main effect, all those up to and including the highest significant order of interaction and any main effect whose interaction was significant even though it may not have been significant itself. The same level of order also being used to calculate the standard errors for each point and hence the confidence bands.

Regression analysis and the correlation coefficient.

The R.I.A. used in this project produced standard curves of percentage antibody bound iodinated protein (T. or M.A.P.) against total protein present. In order to decide which standard curve was the best for estimating unknown sample protein contents from their percentage antibody binding the correlation coefficient for each was determined and then the formula of the curve with the highest correlation coefficient used to determine the sample protein contents. The main problem with this methodology was that in the standard curve the independent variable (x) had to be the amount of protein in the standards while the dependent variable (y) was the percentage antibody binding. This meant that prediction from the regression curve of y on x of protein content from the percentage binding meant a prediction of x from measured y values. This makes the calculation of the confidence limits for the predicted value of x from y more complicated than usual.

The model used in linear regression analysis is $y = \bar{y} + b(x - \bar{x})$ where $\bar{y}$ and $\bar{x}$ are the means of the variables y and x. In this case the roughly plotted standard curve points produced a curve rather than a perfect straight line. In order to convert this curve to a line the protein standards ($_x$) were converted to a logarithmic scale. The next step was to find the S.S. due to the y variable ($S.S._y$), the S.S. due to the x variable ($S.S._x$) and the sum of products of the x andy variable ($S.P._{xy}$). These were found using the following formulae :

$$S.S._y = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$$

$$S.S._x = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

$$S.P._{xy} = \Sigma xy - \frac{\Sigma x \Sigma y}{n}$$

where x and y represent the individual variables and n the total number of variables or points on the line. From these values were found the slope of the line or regression coefficient (b), the intercept or regression constant (a) and regression S.S. :

$$b = S.P._{xy} / S.S._{x}$$

$$a = \bar{y} - b\bar{x}$$

$$\text{Regression S.S.} = (S.P._{xy})^2 / S.S._{x}$$

To check the significance of the line or linear effect an analysis of variance of y was carried out. The total S.S. for this test were $S.S._{y}$ while the error or residual S.S. was found by subtracting the regression S.S. from this total S.S. The analysis of variance was carried out in the usual manner with 1 D.F. for regression and (n - 2) for residual variations. Assuming that a significant value was found for the F-test of the linear effect then the next stage was to find the correlation coefficient for the line. The correlation coefficient (r) was found by first finding the coefficient of determination (r^2) :

$$r^2 = \frac{\text{Regression S.S.}}{\text{Total S.S.}} \quad \text{or} \quad \frac{(S.P._{xy})^2}{S.S._{x}\ S.S._{y}} ,$$

r was then the square root of this value.

Once the correlation coefficients had been determined for the standard curves and the mean standard curve these were compared to see which was the largest. The curve with the greatest value of r was then used to calculate the protein concentrations (x') from the unknown sample percentage binding values (y'). These concentrations were found by transforming the model curve formula :

$$y = \bar{y} + b(x-\bar{x})$$

so that :

$$x' = \bar{x} + \frac{(y'-\bar{y})}{b}$$

where $\bar{y}$ and $\bar{x}$ were the mean standard curve values and x' and y' were the sample values. The confidence limits for a value of x found from an individual y value are found from the formula :

$$(x-\bar{x}) = \frac{y'-\bar{y}}{b} \pm \frac{t}{b}\sqrt{M.S_e\left[\frac{(n+1)(1-c^2)}{n} + \frac{(x'-\bar{x})^2}{S.S._x}\right]} \Big/ 1-c^2$$

where $$c^2 = \frac{1}{S.S._x}\left[\frac{t\sqrt{M.S._e}}{b}\right]^2$$

This formula is rather complex but fortunately if b is significant (which we checked by analysis of variance earlier) c is 1 and c^2 becomes negligible. The simplified formula used for the confidence limits therefore was :

$$(x-\bar{x}) = \frac{y'-\bar{y}}{b} \pm \frac{t}{b}\sqrt{M.S._e\left[1 + \frac{1}{n} + \frac{(x'-\bar{x})^2}{S.S._x}\right]}$$

where y' and x' were the sample curve values, $M.S._e$ the residual M.S. and t the table value of t at the 5% level.

Computer programmes.

All the programmes listed are in the Hewlitt Packard 2000 A^{C}_{A}cess System language of BASIC.

1. INTRODUCTION

1.1 Using a Terminal

(a) Teletypes

Press START button.
Select DUPLEX position on three-way switch.
Press RETURN.
Press LINE FEED.
After message "PLEASE LOG-IN" appears, go to section 1.2.

(b) V.D.U.s.

Switch POWER-ON.
Press LINE key.
Press RETURN key.
Press LF key.
After message "PRESS LOG-IN" appears, go to section 1.2.

1.2 Logging in

In response to PLEASE LOG-IN the user types

HEL–idcode,password — the–(dash) and,(comma) must be included.

e.g. HEL–A901,MINE

1.3 Special Charecters

Note

For terminals without a specific RETURN key the keys CTRL and M should be pressed *simultaneously* instead of RETURN at the end of a line of data etc.

X^{C} means press CTRL key and X simultaneously.

Character	Effect
RETURN or M^{C}	Terminates line of input.
BREAK	Stops program running. Can be used whether or not input is expected.
X^{C} (CTRL and X together)	Deletes line of input.
Backspace or H^{C} (CTRL and H together)	Backspace, deletes last character typed.

When Access responds with ERROR

(i) If error description required, type any character, e.g. : followed by RETURN or M^{C}.

(ii) If full description not required, type RETURN or M^{C} only.

2. SYSTEM COMMANDS

Assume that PROG1 is the name of the program in the users workspace.

APPEND	
APP–PROG1A	appends PROG1A to end of PROG1.
BYE	
BYE	user is logged off.
CATALOG	
CAT	lists user directory.
CAT–MA	lists from filename beginning MA or later.
CSAVE	
CSA	save PROG1 in partly compiled form.
DELETE	
DEL–50	delete PROG1 from line 50 onwards.
DEL–50,99	delete PROG1 from line 50 to 99 inclusive.
DEL–50,50	delete line 50 only.
EXECUTE	
EXE–PROG2	PROG2 is loaded into workspace, deleting PROG1, and is run from line 1.
EXE–$PRG1	Program PRG1 from the system library is executed.
EXE–*PRG2	Program PRG2 from the group library is executed.
GET	
GET–PROG2	PROG2 is loaded into workspace deleting PROG1.
GET–$PRG1	Program PRG1 is loaded from the system library.
GET–*PRG2	Program PRG2 is loaded from the group library.
GROUP	
GRO	lists group directory.
GRO–LIB	lists from filename beginning LIB or later.
HELLO	
HEL–A901,MINE	logs on user with idcode A901, password MINE.
KEY	
KEY	returns control to keyboard after TAPE input.
LENGTH	
LEN	gives length of PROG1 in words.
LIBRARY	
LIB	lists system directory.
LIB–SOR	lists from filename beginning SOR.
LIST	
LIS	lists PROG1.
LIS–200,250	lists PROG1 from line 200 to 250.
LIS–200,200	lists line 200 only.
MESSAGE	
MES–HELLO OPERATORS	send message HELLO OPERATORS to operator console.
NAME	
NAM–PROG2	renames PROG1 as PROG2.
PURGE	
PUR–FILEA	deletes FILEA from user library.
RENUMBER	
REN	renumber whole of PROG1 at intervals of 10, i.e. 10, 20, 30, etc.
REN–20,10,50,100	renumber PROG1 from 20, at intervals of 10, starting with old line 50, ending with old line 100.
RUN	
RUN	runs PROG1 from first statement.
RUN–100	runs PROG1 from statement 100.
SAVE	
SAV	saves PROG1 in user library.
SCRATCH	
SCR	deletes PROG1 from workspace.
TAPE	
TAP	starts input from tape reader.
TIME	
TIM	gives terminal time used.

3. BASIC STATEMENTS

CHAIN	
260 CHAIN "PROG2"	terminates current program, loads and executes PROG2.
260 CHAIN V1,"PROG2",100	as above, but PROG2 executes from statement 100, V1 becomes 0 if OK.
COMMON	
10 COM A(10),B$(100)	A & B$ are placed in area common to current and chained programs.
DATA	
100 DATA 457,"STRING DATA"	provides data for READ.
DEF FN	
40 DEF FNA(B)=B*B–X1*Y1	defines function.
DIM	
20 DIM A(10),B(5,5),A$(80)	defines (max) length of arrays and strings.

END
999 END — terminates program.

ENTER
100 ENTER #V — port number is returned in V.
120 ENTER A,B,Cs — A seconds to input Cs. B is set to actual time taken.
300 ENTER #V,A,B,Cs — combination of above examples.

FOR
100 FOR I=1 TO 10 — executes statements between 100 and "NEXT I" until I, which starts at 1, becomes greater than 10.
50 FOR I=1 TO 10 STEP 3 — as above but I is incremented by 3 instead of 1.
160 FOR I = 1 TO -A/2 STEP-1 — I will be decremented by 1, statements executed until I passes -A/2.

GOSUB
100 GOSUB 590 — branch to subroutine at 590 (see RETURN)
150 GOSUB N OF 510,590,620 — branch to subroutine at Nth number listed. N can be an expression.

GOTO
100 GO TO 105 — branch to 105.
90 GO TO X-Y OF 50,52,54 — branch to (X-Y)th number listed.

IF...THEN
60 IF A<B THEN 50 — if A<B branch to 50, otherwise continue with next statement. See section 5.
30 IF As = "YES" THEN 70 — see section 7.2 for string relations.
90 IF X=9 AND Y=20 THEN 20 — see section 5.

IMAGE
40 IMAGE 2(4DX),"STRING",X4A — see PRINT USING (below) and section 8.

INPUT
20 INPUT A — input value for A from terminal.
30 INPUT A,Y(3),As(N,N+20) — input values for A,Y(3) and As(N) to As(N+20) from terminal.

LINPUT
20 LINPUT As — see section 7.4.

LET
130 LET A=B=C=0
100 LET As="ABC" — the word LET is optional.

NEXT
30 NEXT I — marks end of FOR loop which begins FOR I=...

PRINT
20 PRINT A,B;Cs — prints values specified. semicolon separators give 3 character fields. Comma separators pack output into field widths of multiples of 15.
50 PRINT — leaves blank line.
60 PRINT A; — next print list will be on same line as A except under special conditions.

PRINT USING
30 PRINT USING 40;A,B,Cs — prints A,B,Cs according to image defined in line 40. see section 8.

READ
30 READ A,B,Cs — read A,B,Cs from DATA statements.

REM
10 REMARK... — any comments following REM do not cause errors.

RESTORE
50 RESTORE — data pointer is reset to first DATA statement.
70 RESTORE 30 — pointer reset to first DATA statement on or after line 30.

STOP
999 STOP — terminates execution. May be anywhere.

4. BASIC FUNCTIONS

ABS(X) gives absolute value of expression X.
ATN(X) gives angle in radians whose tangent is X.
COS(X) gives cosine of X (X in radians).
EXP(X) gives value of e↑X.
INT(X) gives integer part of X, e.g. INT(3.6) gives 3 but INT(-3.6) gives -4
LOG(X) gives log to base e of X.
RND(X) gives random number in range $0 \leqslant R \leqslant 1$
X<0, random number based on X
X≥0, random number based on last number or time.
SGN(X) gives 1 if X>0, 0 if X=0, -1 if X<0.
SIN(X) gives sine of X (X in radians).
SQR(X) gives positive square root of X.
TAN(X) gives tangent of X (X in radians).
TIM(X) gives time, depending on X
X=0 TIM(X) gives current minute (0-59)
X=1 TIM(X) gives current hour (0-23)
X=2 TIM(X) gives current day (1-366)
X=3 TIM(X) gives current year (1-99)
X=4 TIM(X) gives current seconds (0-59)

5. OPERATORS

5.1 Arithmetic Operators

+	addition
-	subtraction
*	multiplication
/	division
↑ **	exponentiation

5.2 Relational Operators

<	less than
<= or =<	less than or equal to
>	greater than
>= or =>	greater than or equal to
=	equal to
<> or #	not equal to

5.3 Logical Operators

AND ∧	A AND B only true if A*B ≠ 0
OR ∨	A OR B only true if A or B ≠ 0
NOT ~	NOT A only true if A=0

5.4 MIN/MAX Operators

MIN	A MIN B =	A if A<B B if A>B
MAX	A MAX B =	A if A>B B if A<B

5.5 Order of Evaluation

first	** ↑
	unary - unary + NOT
	* /
	+ -
	MIN MAX
	< <= = => > <> #
	AND
last	OR

6. MAT STATEMENTS

Notes
Array names are one letter only.
Maximum array size is 5000
Maximum number of dimensions is 2, e.g. A(10,10)
Array subscripts range from 1 to array size.
A(5,7) means row 5, column 7.

Statements

DIM
10 DIM A(10,20) — allocates 200 elements to A, 10 rows, 20 columns. Not needed if number rows and columns is less than or equal to 10.

MAT...+
30 MAT A=A+B — elemental addition A(I,J)=A(I,J)+B(I,J)

MAT...-
40 MAT B=A-B — elemental subtraction B(I,J)=A(I,J)-B(I,J)

```
MAT...=
 50 MAT A=B                    elemental assignment
                               A(I,J)=B(I,J)

MAT...*
 20 MAT X=(N)*Y                Scalar multiplication; X,Y are
                               arrays.

MAT...*
 30 MAT A=B*C                  Matrix multiplication; A,B,C, all
                               arrays.

MAT...CON
 60 MAT A=CON                  all elements of A become 1.
 70 MAT A=CON(5,20)            as above, but A is redimensioned.

MAT...IDN
 80 MAT B=IDN                  B becomes identity matrix.
 90 MAT B=IDN(10,10)           as above but is redimensioned.

MAT INPUT
 20 MAT INPUT A,B              input arrays A,B row by row from
                               terminal.
 30 MAT INPUT A(100)           redimensions A.

MAT...INV
 50 MAT B=INV(A)               B becomes the inverse matrix of
                               A.
 60 MAT A=INV(A)               A is inverted.

MAT PRINT
 90 MAT PRINT A;B              print A&B row by row.
                               1-dimensional arrays are listed
                               down page.

MAT PRINT USING
 100 MAT PRINT USING 200;A     see sections 3 and 8.

MAT READ
 20 MAT READ A                 input A row by row from DATA
                               statements.

MAT...TRN
 40 MATA=TRN(B)                A becomes transpose of B.
                               MAT A=TRN(A) is not allowed.

MAT...ZER
 20 MAT A=ZER                  all elements of A become 0.
 30 MAT A=ZER(5,20)            redimensions A.
```

7. STRINGS

7.1 String Manipulation

String variables must be:

(i) named as letter A–Z followed by $
(ii) less than 256 characters (elements) long.
(iii) dimensioned in DIM statement to maximum expected length if more than 1 character long.
(iv) in the following examples, where A$= "ABCDEF"

A$ references entire string i.e. ABCDEF
A$(3) references from 3rd character CDEF
A$(2,2) references 2nd character B
A$(1,4) references 1st to fourth characters ABCD

7.2 Relations on strings

=	"AB"="AB"	is the same string as.
<> or ><	"AB"<>"BA"	is not the same string as.
<	"ABC"<"ACC"	is earlier in alphabetical order than
>	"XYZ">"WXYZ"	is later in alphabetical order than
=<, <=	means either = or <	
=>, >=	means either = or>	

7.3 The order of characters for comparison is as follows:

0 1 2 3 4 5 6 7 8 9 : ; < = > ? space ! " # £ % & ' ( ) * + , – . /
@ A B C D E F G H I J K L M N O P Q R S T U V W X Y Z [\ ] ↑
new line

7.4 BASIC Statements Relating to Strings

```
CONVERT
 200 CONVERT A+B TO A$         numeric value of A+B is con-
                               verted to string of characters
                               which represent that value.
 220 CONVERT L$(1,5) TO X,900
                               convert characters in L$(1,5) to
                               number and place in X. Go to 900
                               if L$(1,5) does not represent a
                               number.

LINPUT
 30 LINPUT A$                  entire line of data is put into A$
                               (no prompt sent to terminal).
```

7.5 String Functions

```
CHR$
 200 A$(1)=CHR$(72)            A$(1) contains character whose
                               code is 72.

LEN
 50 A=LEN(B$)                  A contains current (logical) length
                               of B$.

NUM
 70 N=NUM(A$)                  N contains numeric equivalent of
                               first character in A$.

POS
 45 P=POS(A$,B$)               P contains position in A$ where
                               B$ first occurs.
                               P contains 0 if A$ does not con-
                               tain B$.
                               P contains 1 if B$ is a null string.

UPS$
 100 A$=UPS$(B$)               A$ becomes B$ but all chars
                               upper case.
```

8. IMAGE CODES FOR PRINT USING

8.1 Print Functions

LIN(N)	skip N lines
LIN(–N)	skip N lines without carriage-return
SPA(X)	skip X spaces
TAB(N)	skip to column N

8.2 Format Characters

A reserves a character position for string.
D reserves a digit position for number.
S defines position of sign for number.
. defines position of decimal point.
E defines position of E in floating point output.
X reserves blank character in string or number.
repetition factor 1–255 may precede A,X or D.

8.3 Examples

Format Spec.	*Value*	*Output*
6D	–1234	▽–1234
DX3D.3D	1234.5	1▽234.500
S8D	1234	▽▽+1234
2D.4D	–.456	–0.4560
4D.DDXE	4.82716×10^{21}	4827.16 E+18
8A	ABCDEF	ABCDEF▽▽
4A	ABCDEF	ABCD
"TOTALS"/2(DDDD)	12,13	TOTALS ▽▽12▽▽13

9. FILE HANDLING

FIL1	exists on user library
*FIL1	exists on group library
$FIL1	exists on system library
FIL1.Z999	exists on user library Z999.

9.1 Commands relating to file handling

```
CREATE
  CRE–FILEA,48                 creates BASIC disc file FILEA, 48
                               blocks long.
  CRE–FILEA,48,64              As above, but records are 64
                               words long.

PURGE
  PUR–FILEA                    deletes FILEA from library.
CATALOG }
GROUP   }                      see section 2.
LIB     }
```

9.2 BASIC Statements relating to file handling

V1 is a return variable.

The FILES statement is mandatory.

FILES

```
30 FILES FILEA,*,FILED.M350
```
file 1 will mean FILEA.
file 2 will be reserved until assigned.
file 3 will mean FILED from library of user id M350.

READ

```
30 READ #1;A,B,Cs
```
A,B,Cs are read from file 1.

```
40 READ #N,2;A
```
A is read from 2nd record of file N.

```
50 MAT READ #1;A
```
Matrix A is read in from file 1.

```
60 READ #1,1
```
reset file pointer to start of file.

PRINT

```
50 PRINT #1;A,B,Cs
```
A,B,Cs are written onto one record of file 1.

```
30 PRINT #2,5;A
```
A is written to 5th record of file 2.

```
60 PRINT #1;END
```
End-of-file marker written to file 1.

```
70 MAT PRINT #1;A
```
Matrix A is written to file 1.

IF END

```
90 IF END #2 THEN 120
```
branch to 120 when end of file detected on file 2. The effect is global.

UPDATE

```
40 UPDATE #2;A
```
update next item in file 2 to A.

ADVANCE

```
20 ADVANCE #2;X,V1
```
skip X items in file 2, V1 is 0 if skip OK.

CREATE

```
20 CREATE V1,"FILEA",100,72
```
create file FILEA 100 blocks long, 72 characters per record. Record size optional.

PURGE

```
20 PURGE V1,"FILEA"
```
FILEA is deleted from library, V1 is 0 if purge OK.

ASSIGN

```
100 ASSIGN "FILEA",3,V1
```
assign file name FILEA to position reserved for file no. 3 in FILES statement.
V1 is return variable 0–8.

```
110 ASSIGN *,3,V1
```
close file number 3.

9.3 Functions relating to file handling

ITM

```
20 X=ITM(3)
```
X contains number of data items between beginning of currently accessed record and position of file pointer, for file 3.

REC

```
50 R=REC(N)
```
R becomes record number currently being accessed in file N.

TYP

```
60 T=TYP(N)
```
T indicates type of next item in file N.
T=1 for number
T=2 for string
T=3 for end of file
T=4 for end of record

COMPUTER CENTRE

Quick Guide for HP Access Basic

USER NUMBER

MTGINP

```
10  REM ****************************************
20  REM * INPUT OF DATA FOR MTGTRT             *
30  REM * DATA AS DPM FROM B-COUNTER PRINTOUT *
40  REM ****************************************
50  FILES *
60  DIM A$[6],B$[1],C$[1],X$[9]
70  DIM A[20,4]
80  DIM C[10],D[10],E[10],F[10]
90  DIM G[500]
100  PRINT "ENTER NAME OF FILE FOR STORAGE OF DATA"
110  INPUT A$
120  ASSIGN A$,1,V1
130  PRINT "WITH GLYCEROL :"
140  PRINT TAB(20)"ENTER NO. OF BLANKS -comma- NO. OF STANDARDS"
150  INPUT B1,S1
160  PRINT "WITHOUT GLYCEROL :"
170  PRINT TAB(20)"ENTER NO. OF BLANKS -comma- NO: OF STANDARDS"
180  INPUT B2,S2
190  PRINT "ENTER NO. OF SUPERNATANTS"
200  INPUT S3
210  MAT A=ZER[S3*4,4]
220  X=-3
230  FOR I=1 TO S3
240  X=X+4
250  PRINT "FOR SN.-";I;":"
260  PRINT "ENTER :NO. TRT.'s -comma- NO. DILN.'s -comma- NO. REP.
270  INPUT A[X,1],A[X,2],A[X,3],A[X,4]
280  NEXT I
290  FOR I=1 TO S3*4 STEP 4
300  FOR X=I+1 TO I+3
310  FOR J=1 TO 4
320  A[X,J]=A[I,J]
330  NEXT J
340  NEXT X
350  NEXT I
360  PRINT "ARE THERE ANY EXCEPTIONS ? ENTER y OR n"
370  INPUT B$
380  IF B$="n" THEN 610
390  PRINT "ENTER SN. OF EXCEPTION"
400  INPUT X1
410  PRINT "ENTER TYPE : TRT.(1),DILN.(2),REP.(3),MAX DILN.(4)"
420  INPUT X2
430  PRINT "ENTER SN. TYPE : ALPHA (0),BETA (1),GAMMA (2),DELTA (3
440  INPUT X3
450  IF X2=1 THEN 500
460  IF X2=2 THEN 520
470  IF X2=3 THEN 540
480  X$="MAX DILN."
490  GOTO 550
500  X$="TRT.      "
510  GOTO 550
520  X$="DILN.     "
```

```
530  GOTO 550
540  X$="REP.      "
550  PRINT "FOR SN -";X1;": ENTER EXCEPTION VALUE FOR ";X$
560  INPUT X4
570  A[((X1*4)-3+X3),X2]=X4
580  PRINT "ARE THERE OTHER EXCEPTIONS ? ENTER y OR n"
590  INPUT C$
600  IF C$="y" THEN 390
610  PRINT "WITH GLYCEROL :"
620  PRINT TAB(20)"ENTER BLANKS"
630  MAT  INPUT C[B1]
640  PRINT TAB(20)"ENTER STANDARDS"
650  MAT  INPUT D[S1]
660  PRINT "WITHOUT GLYCEROL :"
670  PRINT TAB(20)"ENTER BLANKS"
680  MAT  INPUT E[B2]
690  PRINT TAB(20)"ENTER STANDARDS"
700  MAT  INPUT F[S2]
710  A=0
720  FOR I=1 TO S3*4
730  A=A+(A[I,1]*A[I,2]*A[I,3])
740  NEXT I
750  PRINT "ENTER SAMPLE DPM.'s IN SN.  THEN ALPHABETICAL ORDER"
760  MAT  INPUT G[A]
770  READ #1,1
780  PRINT #1;B1,S1,B2,S2,S3,A
790  MAT  PRINT #1;A,C,D,E,F,G
800  PRINT "DATA STORED IN ";A$;" FOR TREATMENT"
810  END
```

MTGTRT

```
10  REM ***********************************************
20  REM * TREATMENT OF MT.-GRANULE EXPT. RESULTS *
30  REM * CONVERTS DPM TO MICROG./ML.  T.          *
40  REM ***********************************************
50  FILES *,*
60  DIM A$[6],B$[6]
70  DIM A[20,4]
80  DIM C[10],D[10],E[10],F[10],H[10]
90  DIM G[500],I[500],J[500]
100  PRINT "ENTER NAME OF FILE FOR TREATMENT"
110  INPUT A$
120  ASSIGN A$,1,V1
130  PRINT "ENTER NAME OF FILE FOR TREATED DATA"
140  INPUT B$
150  ASSIGN B$,2,V2
160  READ #1,1
170  READ #1;B1,S1,B2,S2,S3,A
180  MAT A=ZER[S3*4,4]
190  MAT C=ZER[B1]
200  MAT D=ZER[S1]
210  MAT E=ZER[B2]
220  MAT F=ZER[S2]
230  MAT G=ZER[A]
240  MAT  READ #1;A,C,D,E,F,G
250  REM BLK.& STND.  MEANS
260  X=B1
270  MAT H=ZER[B1]
280  MAT H=C
290  GOSUB 470
300  M1=T1/B1
310  X=S1
320  MAT H=ZER[S1]
330  MAT H=D
340  GOSUB 470
350  M2=T1/S1
360  X=B2
370  MAT H=ZER[B2]
380  MAT H=E
390  GOSUB 470
400  M3=T1/B2
410  X=S2
420  MAT H=ZER[S2]
430  MAT H=F
440  GOSUB 470
450  M4=T1/S2
460  GOTO 520
470  T1=0
480  FOR I=1 TO X
490  T1=T1+H[I]
500  NEXT I
510  RETURN
520  REM SAMPLE PROTEIN CONC.'s
```

```
530  A1=0
540  FOR I=1 TO 4
550  A1=A1+(A[I,1]*A[I,2]*A[I,3])
560  NEXT I
570  MAT I=ZER[A]
580  FOR I=1 TO A1
590  IF G[I] <= M1 THEN 620
600  I[I]=((G[I]-M1)/(M2-M1))*1.1*1000
610  GOTO 630
620  I[I]=0
630  NEXT I
640  FOR I=A1+1 TO A
650  IF G[I] <= M3 THEN 680
660  I[I]=((G[I]-M3)/(M4-M3))*1.1*1000
670  GOTO 690
680  I[I]=0
690  NEXT I
700  REM SAMPLE PROTEIN CONC.'s BEFORE DILN.
710  MAT J=ZER[A]
720  A2=1
730  A4=0
740  FOR X=1 TO S3*4
750  A3=A[X,1]*A[X,2]*A[X,3]
760  A4=A4+A3
770  D=A[X,4]-A[X,2]
780  FOR I=A2 TO A4 STEP A[X,3]
790  D=D+1
800  FOR Y=I TO I+A[X,3]-1
810  J[Y]=I[Y]*D
820  NEXT Y
830  NEXT I
840  A2=A2+A3
850  NEXT X
860  REM STORAGE OF TREATED DATA
870  READ #2,1
880  PRINT #2;A
890  MAT  PRINT #2;A,G,I,J
900  REM PRINTOUT
910  PRINT LIN(5)
920  PRINT "WITH GLYCEROL :"
930  PRINT
940  PRINT TAB(20)"   BLANK MEAN -";M1
950  PRINT TAB(20)"STANDARD MEAN -";M2
960  PRINT
970  PRINT "WITHOUT GLYCEROL :"
980  PRINT
990  PRINT TAB(20)"   BLANK MEAN -";M3
1000  PRINT TAB(20)"STANDARD MEAN -";M4
1010  PRINT LIN(3)
1020  PRINT "SAMPLE VALUES :"
1030  PRINT
1040  PRINT " ","DILUTED","SAMPLE"
1050  PRINT " ","PROTEIN CONC.","PROTEIN CONC."
1060  PRINT "DPM","MICROG./ML.","MICROG./ML."

1070  PRINT
1080  FOR I=1 TO A
1090  PRINT G[I],I[I],J[I]
1100  NEXT I
1110  PRINT
1120  PRINT "TREATED DATA STORED IN ";B$;" FOR ANALY:
1130  END
```

AVMGIN

```
10  REM ****************************
20  REM * DATA INPUT FOR AOVMTG   *
30  REM * INPUT AS MICOG./ML.  T. *
40  REM ****************************
50  FILES SAMPLE,*,*,*
60  DIM M[100],A[25,4]
70  READ #1,1
80  READ #1;A,R
90  MAT M=ZER[A]
100  MAT  READ #1;M
110  PURGE V4,"SAMPLE"
120  A=A/(3*R)
130  DIM B[25,4],C[25,4]
140  MAT A=ZER[R,A]
150  MAT B=ZER[R,A]
160  MAT C=ZER[R,A]
170  Q=0
180  FOR I=1 TO A*R STEP R
190  Q=Q+1
200  V=0
210  FOR X=I TO I+R-1
220  V=V+1
230  A[V,Q]=M[X]
240  B[V,Q]=M[X+(A*R)]
250  C[V,Q]=M[X+(2*A*R)]
260  NEXT X
270  NEXT I
280  CREATE R1,"SNI",1
290  ASSIGN "SNI",2,V2
300  READ #2,1
310  PRINT #2;A,R
320  MAT  PRINT #2;A
330  CREATE R2,"SNII",1
340  ASSIGN "SNII",3,V3
350  READ #3,1
360  PRINT #3;A,R
370  MAT  PRINT #3;B
380  CREATE R3,"SNIII",1
390  ASSIGN "SNIII",4,V4
400  READ #4,1
410  PRINT #4;A,R
420  MAT  PRINT #4;C
430  CHAIN "AVMTGR"
440  END
```

AOVMTG

```
10  REM ****************************************
20  REM * ONE WAY ANALYSIS OF VARIANCE         *
30  REM * USING ORTHOGONAL LINEAR CONTRASTS *
40  REM * FOR MT.-G.  EXPTS.                   *
50  REM ****************************************
60  FILES *,*
70  DIM A$[6],B$[2]
80  DIM C$[6]
90  DIM A[50,4],B[2,4],C[2,4]
100  PRINT "ENTER NAME OF FILE FOR ANALYSIS"
110  INPUT C$
120  ASSIGN C$,1,V1
130  PRINT "ENTER NAME OF PRINTOUT FILE"
140  INPUT A$
150 'ASSIGN A$,2,V2
160  GOTO 210
170  PRINT "ENTER NO.  OF TREATMENTS"
180  INPUT A
190  PRINT "ENTER HIGHEST NO.  OF REPLICATES"
200  INPUT R
210  PRINT "WHICH SN.  IS TO BE TREATED. ENTER 1,2 OR 3"
220  INPUT Z0
230  READ #1,Z0;A,R
240  PRINT A,R
250  MAT A=CON[R,A]
260  MAT A=(-1)*A
270  MAT  READ #1,Z0;A
280  GOTO 380
290  PRINT "ENTER REPLICATES FOLLOWED BY -1 :"
300  PRINT
310  FOR J=1 TO A
320  PRINT "FOR TREATMENT ";J
330  FOR I=1 TO R
340  INPUT A[I,J]
350  IF A[I,J]=-1 THEN 370
360  NEXT I
370  NEXT J
380  REM Trt.  totals, squares and no.
390  S1=0
400  MAT B=ZER[2,A]
410  FOR J=1 TO A
420  T1=N=0
430  FOR I=1 TO R
440  IF A[I,J]=-1 THEN 490
450  T1=T1+A[I,J]
460  S1=S1+(A[I,J] ** 2)
470  N=N+1
480  NEXT I
490  B[1,J]=T1
500  B[2,J]=N
510  NEXT J
520  REM Total ss and trt. ss
```

```
530  N=T3=T2=0
540  FOR J=1 TO A
550  T2=T2+B[1,J]
560  T3=T3+((B[1,J] ** 2)/B[2,J])
570  N=N+B[2,J]
580  NEXT J
590  S1=S1-((T2 ** 2)/N)
600  S2=T3-((T2 ** 2)/N)
610  S3=S1-S2
620  REM Degrees of freedom
630  F1=N-1
640  F2=A-1
650  F3=F1-F2
660  F4=F5=1
670  REM Linear contrast coefficients
680  PRINT "ENTER TRT. NO.'S FOR DIRECT COMPARISON"
690  INPUT X,Y
700  PRINT "ENTER TRT. NO. FOR COMPARISON WITH THESE COMBINED"
710  INPUT Z
720  A1=B[2,X]
730  B1=B[2,Y]
740  GOSUB 1370
750  L1=C1
760  A1=B[2,X]+B[2,Y]
770  B1=B[2,Z]
780  GOSUB 1370
790  L2=C1
800  MAT C=ZER[2,A]
810  C[1,X]=L1/B[2,X]
820  C[1,Y]=-L1/B[2,Y]
830  C[2,X]=C[2,Y]=L2/(B[2,X]+B[2,Y])
840  C[2,Z]=-L2/B[2,Z]
850  REM Check of orthogonality
860  B$="OT"
870  Z1=Z2=Z3=0
880  FOR J=1 TO A
890  Z1=Z1+(B[2,J]*C[1,J])
900  Z2=Z2+(B[2,J]*C[2,J])
910  Z3=Z3+(B[2,J]*C[1,J]*C[2,J])
920  NEXT J
930  IF Z1 <> 0 THEN 970
940  IF Z2 <> 0 THEN 970
950  IF Z3 <> 0 THEN 970
960  GOTO 1000
970  PRINT "COMPARISONS NOT ORTHOGONAL"
980  B$="NO"
990  GOTO 1100
1000  REM Linear contrast ss
1010  T4=T5=R1=R2=0
1020  FOR J=1 TO A
1030  T4=T4+(B[1,J]*C[1,J])
1040  R1=R1+(B[2,J]*(C[1,J] ** 2))
1050  T5=T5+(B[1,J]*C[2,J])
1060  R2=R2+(B[2,J]*(C[2,J] ** 2))
```

```
1070  NEXT J
1080  S4=(T4 ** 2)/R1
1090  S5=(T5 ** 2)/R2
1100  REM Ms + vr
1110  M1=S1/F1
1120  M2=S2/F2
1130  M3=S3/F3
1140  V2=M2/M3
1150  IF B$="NO" THEN 1530
1160  M4=S4/F4
1170  M5=S5/F5
1180  V4=M4/M3
1190  V5=M5/M3
1200  REM Se, cl + mean contrasts
1210  E4=SQR(M3*R1)
1220  E5=SQR(M3*R2)
1230  PRINT "ENTER VALUE OF t FOR ";F3;" DEGREES OF FREEDOM,P=0.05.
1240  INPUT T9
1250  C4=T9*E4
1260  C5=T9*E5
1270  K1=K2=0
1280  FOR J=1 TO A
1290  K1=K1+ABS(B[2,J]*C[1,J])
1300  K2=K2+ABS(B[2,J]*C[2,J])
1310  NEXT J
1320  K4=(ABS(T4))/(K1/2)
1330  K5=(ABS(T5))/(K2/2)
1340  L4=C4/(K1/2)
1350  L5=C5/(K2/2)
1360  GOTO 1530
1370  C1=A1*B1
1380  IF A1<B1 THEN 1400
1390  GOTO 1430
1400  E1=B1
1410  B1=A1
1420  A1=E1
1430  D1=A1
1440  D1=D1-B1
1450  IF D1>B1 THEN 1440
1460  IF D1=B1 THEN 1510
1470  IF D1=0 THEN 1510
1480  A1=B1
1490  B1=D1
1500  GOTO 1430
1510  C1=C1/B1
1520  RETURN
1530  REM Printout
1540  PRINT LIN(5)
1550  PRINT "ONE WAY ANOVAR :"
1560  PRINT #2;"ONE WAY ANOVAR :"
1570  PRINT LIN(2)
1580  PRINT #2;LIN(2)
1590  PRINT " ","T. + G.","T. + G.","T. ONLY","G. ONLY"
1600  PRINT #2;" ","T. + G.","T. + G.","T. ONLY","G. ONLY"
```

```
1610  PRINT " ","4 DEG.","37 DEG.","37 DEG.","37 DEG."
1620  PRINT #2," ","4 DEG.","37 DEG.","37 DEG.","37 DEG."
1630  PRINT
1640  PRINT #2," "
1650  PRINT "TOTALS :",B[1,1],B[1,2],B[1,3],B[1,4]
1660  PRINT #2;"TOTALS :",B[1,1],B[1,2],B[1,3],B[1,4]
1670  PRINT "REPLICATES :",B[2,1],B[2,2],B[2,3],B[2,4]
1680  PRINT #2;"REPLICATES :",B[2,1],B[2,2],B[2,3],B[2,4]
1690  PRINT "MEANS :",B[1,1]/B[2,1],B[1,2]/B[2,2],B[1,3]/B[2,3],B[1
1700  PRINT #2;"MEANS :",B[1,1]/B[2,1],B[1,2]/B[2,2],B[1,3]/B[2,3],
1710  IF B$="NO" THEN 1780
1720  PRINT "LINEAR CONTRAST COEFFICIENTS :"
1730  PRINT #2;"LINEAR CONTRAST COEFFICIENTS :"
1740  PRINT "TEMPERATURE :",C[1,1],C[1,2],C[1,3],C[1,4]
1750  PRINT #2;"TEMPERATURE :",C[1,1],C[1,2],C[1,3],C[1,4]
1760  PRINT "+or- GRANULES :",C[2,1],C[2,2],C[2,3],C[2,4]
1770  PRINT #2;"+or- GRANULES :",C[2,1],C[2,2],C[2,3],C[2,4]
1780  PRINT LIN(3)
1790  PRINT #2;LIN(3)
1800  PRINT "SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
1810  PRINT #2;"SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
1820  PRINT "VARIATION","FREEDOM","SQUARES","SQUARE","RATIO - F"
1830  PRINT #2;"VARIATION","FREEDOM","SQUARES","SQUARE","RATIO - F"
1840  PRINT
1850  PRINT #2," "
1860  PRINT "TREATMENT :",F2,S2,M2,V2
1870  PRINT #2;"TREATMENT :",F2,S2,M2,V2
1880  IF B$="NO" THEN 1950
1890  PRINT
1900  PRINT #2," "
1910  PRINT "TEMPERATURE :",F4,S4,M4,V4
1920  PRINT #2;"TEMPERATURE :",F4,S4,M4,V4
1930  PRINT "+or- GRANULES :",F5,S5,M5,V5
1940  PRINT #2;"+or- GRANULES :",F4,S5,M5,V5
1950  PRINT
1960  PRINT #2," "
1970  PRINT "ERROR :",F3,S3,M3," - "
1980  PRINT #2;"ERROR :",F3,S3,M3," - "
1990  PRINT "TOTAL :",F1,S1," - "," - "
2000  PRINT #2;"TOTAL :",F1,S1," - "," - "
2010  IF B$="NO" THEN 2160
2020  PRINT LIN(3)
2030  PRINT #2;LIN(3)
2040  PRINT " "," - TOTAL CONTRASTS -"," - MEAN CONTRASTS -"
2050  PRINT #2;" "," - TOTAL CONTRASTS -"," - MEAN CONTRASTS -"
2060  PRINT " ","STANDARD","CONFIDENCE","MEAN","CONFIDENCE"
2070  PRINT #2;" ","STANDARD","CONFIDENCE","MEAN","CONFIDENCE"
2080  PRINT " ","ERROR","INTERVAL","EFFECT","INTERVAL"
2090  PRINT #2;" ","ERROR","INTERVAL","EFFECT","INTERVAL"
2100  PRINT
2110  PRINT #2," "
2120  PRINT "TEMPERATURE :",E4,C4,K4,L4
2130  PRINT #2;"TEMPERATURE :",E4,C4,K4,L4
2140  PRINT "+or- GRANULES :",E5,C5,K5,L5

2150  PRINT #2;"+or- GRANULES :",E5,C5,K5,L5
2160  END
```

PANINP

```
10  REM ******************************************
20  REM * DATA INPUT FOR PANTRT                  *
30  REM * INPUT AS DPM FROM B-COUNTER PRINTOUT *
40  REM ******************************************
50  PRINT "ENTER NAME OF FILE FOR STORAGE OF DATA"
60  INPUT A$
70  DIM A$[6]
80  FILES *
90  ASSIGN A$,1,V1
100  IF V1>0 THEN 710
110  B=A=S=0
120  PRINT "ENTER NO. OF BLANKS"
130  INPUT B
140  PRINT "ENTER NO. OF STANDARDS"
150  INPUT S
160  PRINT "ENTER NO. OF SAMPLES"
170  INPUT A
180  MAT C=CON[A,5]
190  MAT C=(-1)*C
200  MAT A=ZER
210  MAT B=ZER
220  MAT  PRINT #1;A,B,C
230  DIM A[35],B[10],C[500,5]
240  PRINT "ENTER BLANKS"
250  MAT  INPUT A[B]
260  PRINT "ENTER STANDARDS"
270  MAT  INPUT B[S]
280  PRINT "FOR FIRST TREATMENT :"
290  PRINT "ENTER : NO. OF FIRST SAMPLE-comma-NO. OF SAMPLES"
300  INPUT S1,N1
310  PRINT "FOR SECOND TREATMENT :"
320  PRINT "ENTER : NO. OF FIRST SAMPLE-comma-NO. OF SAMPLES"
330  INPUT S2,N2
340  PRINT "ENTER NO. OF REPLICATES"
350  INPUT R1
360  V=0
370  FOR I=S1 TO S1+N1-1
380  FOR J=1 TO R1
390  V=V+1
400  C[V,1]=11000+(I*10)+J
410  NEXT J
420  NEXT I
430  FOR I=S2 TO S2+N2-1
440  FOR J=1 TO R1
450  V=V+1
460  C[V,1]=12000+(I*10)+J
470  NEXT J
480  NEXT I
490  FOR I=S1 TO S1+N1-1
500  FOR J=1 TO R1
510  V=V+1
520  C[V,1]=21000+(I*10)+J
```

```
530  NEXT J
540  NEXT I
550  FOR I=S2 TO S2+N2-1
560  FOR J=1 TO R1
570  V=V+1
580  C[V,1]=22000+(I*10)+J
590  NEXT J
600  NEXT I
610  PRINT "ENTER SAMPLE DPM'S"
620  FOR J=1 TO A
630  INPUT C[J,2]
640  NEXT J
650  READ #1,1
660  PRINT #1;B,S,A
670  MAT  PRINT #1;A,B,C
680  MAT  PRINT C
690  READ #1,1
700  PRINT "STORED IN ";A$;" FILE FOR TREATMENT"
710  END
```

PANTRT

```
10  REM ************************************
20  REM * TREATMENT OF PANC. EXPT. DATA  *
30  REM * CONVERTS DPM TO MICROG./ML. T. *
40  REM ************************************
50  PRINT "ENTER NAME OF DATA FILE"
60  INPUT A$
70  GOTO 120
80  PRINT "ENTER NAME OF PRINTOUT FILE"
90  INPUT B$
100  PRINT "ENTER NAME OF ANALYSIS FILE"
110  INPUT C$
120  DIM A$[6],B$[6]
130  DIM C$[6]
140  FILES *,*,*
150  ASSIGN A$,1,V1
160  ASSIGN B$,2,V1
170  GOTO 190
180  ASSIGN C$,3,V1
190  B=S=A=T=U=V=W=Z=Y=B1=B2=B3=S1=S2=S3=A1=A2=A3=A4=0
200  P=H=0
210  READ #1;B,S,A
220  GOTO 290
230  PRINT "NO. OF BLANKS";B
240  PRINT #2;"NO. OF BLANKS",B
250  PRINT "NO. OF STANDARDS";S
260  PRINT #2;"NO. OF STANDARDS",S
270  PRINT "NO. OF SAMPLES";A
280  PRINT #2;"NO. OF SAMPLES",A
290  DIM A[40],B[20],C[500,5]
300  MAT A=ZER[B]
310  MAT B=ZER[S]
320  MAT C=ZER[A,5]
330  MAT  READ #1;A,B,C
340  FOR U=1 TO B
350  LET B1=A[U]
360  B2=B2+B1
370  NEXT U
380  B3=B2/B
390  FOR T=1 TO S
400  LET S1=B[T]
410  S2=S2+S1
420  NEXT T
430  S3=S2/S
440  GOTO 490
450  PRINT "BLANK MEAN:";B3
460  PRINT #2;"BLANK MEAN :",B3
470  PRINT "STANDARD MEAN:";S3
480  PRINT #2;"STANDARD MEAN :",S3
490  FOR W=1 TO A
500  LET A1=C[W,2]
510  IF A1<B3 THEN 540
520  A2=((A1-B3)/(S3-B3))*1.1
```

```
530  GOTO 550
540  LET A2=0
550  Z=Z+1
560  GOSUB 590
570  NEXT W
580  GOTO 620
590  LET C[Z,3]=A2
600  A2=0
610  RETURN
620  FOR V=1 TO A
630  LET A3=C[V,3]
640  LET A4=A3*1000
650  LET Y=Y+1
660  GOSUB 690
670  NEXT V
680  GOTO 720
690  LET C[Y,4]=A4
700  LET A4=0
710  RETURN
720  FOR H=1 TO A
730  A5=C[H,4]
740  A6=A5*20
750  P=P+1
760  GOSUB 790
770  NEXT H
780  GOTO 820
790  C[P,5]=A6
800  A6=0
810  RETURN
820  PRINT
830  GOTO 900
840  PRINT " "," ","COLCH. BOUND","TUBULIN CONC.","TUBULIN"
850  PRINT #2;" "," ","COLCH. BOUND","TUBULIN CONC.","TUBULIN"
860  PRINT "SAMPLE NO.","DPM","NM./110MICROL.","MICROG./ML.","MICRO
870  PRINT #2;"SAMPLE NO.","DPM","NM./110MICROL.","MICROG./ML.","MI
880  MAT  PRINT C
890  MAT  PRINT #2;C
900  DIM D[170,4]
910  MAT D=ZER[A/3,4]
920  X=0
930  FOR D1=1 TO A/2 STEP 3
940  X=X+1
950  D[X,2]=C[D1,4]
960  NEXT D1
970  X=0
980  FOR D2=2 TO A/2 STEP 3
990  X=X+1
1000  D[X,3]=C[D2,4]
1010  NEXT D2
1020  X=0
1030  FOR D3=3 TO A/2 STEP 3
1040  X=X+1
1050  D[X,4]=C[D3,4]
1060  NEXT D3
```

```
1070  X=A/6
1080  FOR D4=((A/2)+1) TO A STEP 3
1090  X=X+1
1100  D[X,2]=C[D4,4]
1110  NEXT D4
1120  X=A/6
1130  FOR D5=((A/2)+2) TO A STEP 3
1140  X=X+1
1150  D[X,3]=C[D5,4]
1160  NEXT D5
1170  X=A/6
1180  FOR D6=((A/2)+3) TO A STEP 3
1190  X=X+1
1200  D[X,4]=C[D6,4]
1210  NEXT D6
1220  X=0
1230  FOR D7=1 TO A STEP 3
1240  X=X+1
1250  D[X,1]=INT((C[D7,1])/10)
1260  NEXT D7
1270  CREATE V1,"TREAT",5
1280  ASSIGN "TREAT",3,V2
1290  PRINT #3;A
1300  MAT  PRINT #3;D
1310  PRINT
1320  GOTO 1360
1330  PRINT " ","1","REPLICATES","3"
1340  PRINT "SAMPLE NO.","MICROG./ML.","MICROG./ML.","MICROG./ML."
1350  MAT  PRINT D
1360  PRINT "DATA STORED IN ";B$;" FOR PRINTOUT,AND ";C$;" FOR ANAL
1370  CHAIN "AOVALL"
1380  END
```

AOVPVS

```
10  REM ****************************************
20  REM * HIERARCHICAL ANALYSIS OF VARIANCE *
30  REM * FOR INDIVIDUAL PANC. EXPTS.       *
40  REM ****************************************
50  REM Data Entry
60  DIM A$[15],B$[15]
70  DIM C$[3]
80  DIM Q[125,4],A[65,4],B[65,4]
90  DIM Z[4,5]
100  DIM D[1,60],E[1,20],F[1,10]
110  DIM G[1,60],H[1,20],I[1,10]
120  GOTO 150
130  PRINT "ENTER NAME OF FILE FOR ANOVAR"
140  INPUT A$
150  ASSIGN "TREAT",1,V1
160  PRINT "ENTER NAME OF FILE FOR PRINTOUT"
170  INPUT B$
180  PRINT "IS ANOVAR FOR ONE EXPT. OR ALL EXPT.'S? : ENTER ONE OR
190  INPUT C$
200  FILES *,*,*
210  GOTO 230
220  ASSIGN A$,1,Z1
230  ASSIGN B$,2,Z2
240  READ #1,1
250  READ #1;A
260  G=A/3
270  PRINT "TOTAL NO. OF ROWS =";G
280  PRINT "ENTER NO. OF ROW WHERE DATA STARTS"
290  INPUT Q1
300  PRINT "ENTER NO. OF ROW WHERE DATA ENDS"
310  INPUT Q2
320  PRINT "ENTER NO. OF REPLICATES"
330  INPUT C
340  PRINT "ENTER NO. OF TREATMENTS"
350  INPUT T
360  PRINT "ENTER NO. OF MICE IN EACH EXPERIMENTAL TREATMENT"
370  PRINT "EACH EXPERIMENTAL TREATMENT MUST HAVE THE SAME NO. OF M
380  INPUT M
390  MAT A=ZER[(Q2-(Q1-1)),C+1]
400  MAT Q=ZER[G,C+1]
410  R=Q2-(Q1-1)
420  READ #1,1
430  READ #1;A
440  MAT  READ #1;Q
450  ASSIGN *,1,V2
460  PURGE X1,"TREAT"
470  V=0
480  FOR Q3=Q1 TO Q2
490  V=V+1
500  FOR Q4=1 TO C+1
510  A[V,Q4]=Q[Q3,Q4]
520  NEXT Q4
```

```
530  NEXT Q3
540  IF C$="one" THEN 710
550  R=R-1
560  REM SORT OF DATA
570  MAT B=ZER[R,C+1]
580  X=-5
590  FOR I=1 TO R/2 STEP 3
600  X=X+6
610  FOR J=1 TO C+1
620  B[X,J]=A[I,J]
630  B[X+1,J]=A[I+1,J]
640  B[X+2,J]=A[I+2,J]
650  B[X+3,J]=A[I+30,J]
660  B[X+4,J]=A[I+31,J]
670  B[X+5,J]=A[I+32,J]
680  NEXT J
690  NEXT I
700  GOTO 730
710  MAT B=ZER[R,C+1]
720  MAT B=A
730  REM CORRECTION FACTOR
740  C1=C2=0
750  FOR I=1 TO R
760  FOR J=2 TO C+1
770  C1=C1+B[I,J]
780  NEXT J
790  NEXT I
800  C2=(C1 ** 2)/(R*C)
810  REM SUM( EACH REPLICATE )**2
820  R1=0
830  FOR I=1 TO R
840  FOR J=2 TO C+1
850  R1=R1+(B[I,J] ** 2)
860  NEXT J
870  NEXT I
880  REM SUM( TOTAL FOR EACH MOUSE )**2
890  MAT D=ZER[1,R]
900  V=M1=M2=0
910  FOR I=1 TO R
920  V=V+1
930  FOR J=2 TO C+1
940  M1=M1+B[I,J]
950  NEXT J
960  GOSUB 990
970  NEXT I
980  GOTO 1030
990  D[1,V]=M1
1000  M2=M2+(M1 ** 2)
1010  M1=0
1020  RETURN
1030  REM SUM( TOTAL FOR EACH TREATMENT WITHIN AN EXPERIMENT )**2
1040  MAT E=ZER[1,R/M]
1050  T1=T2=V=0
1060  FOR J=1 TO R STEP M
```

```
1070  V=V+1
1080  FOR J1=J TO J+(M-1)
1090  T1=T1+D[1,J1]
1100  NEXT J1
1110  GOSUB 1140
1120  NEXT J
1130  GOTO 1180
1140  E[1,V]=T1
1150  T2=T2+(T1 ** 2)
1160  T1=0
1170  RETURN
1180  REM SUM( TOTAL FOR EACH EXPERIMENT )**2
1190  MAT F=ZER[1,R/(T*M)]
1200  V=E1=E2=0
1210  FOR J=1 TO R/M STEP T
1220  V=V+1
1230  FOR J1=J TO J+(T-1)
1240  E1=E1+E[1,J1]
1250  NEXT J1
1260  GOSUB 1290
1270  NEXT J
1280  GOTO 1330
1290  F[1,V]=E1
1300  E2=E2+(E1 ** 2)
1310  E1=0
1320  RETURN
1330  REM SUM OF SQUARES (DEVIANCE)
1340  IF C$="one" THEN 1400
1350  Z[1,3]=(E2/(T*M*C))-C2
1360  Z[2,3]=(T2/(M*C))-(E2/(T*M*C))
1370  Z[3,3]=(M2/C)-(T2/(M*C))
1380  Z[4,3]=R1-(M2/C)
1390  GOTO 1440
1400  MAT Z=ZER[3,5]
1410  Z[1,3]=(T2/(M*C))-C2
1420  Z[2,3]=(M2/C)-(T2/(M*C))
1430  Z[3,3]=R1-(M2/C)
1440  REM DEGREES OF FREEDOM
1450  IF C$="one" THEN 1510
1460  Z[1,2]=(R/(T*M))-1
1470  Z[2,2]=(T-1)*(R/(T*M))
1480  Z[3,2]=(M-1)*(R/(T*M))*T
1490  Z[4,2]=R*(C-1)
1500  GOTO 1540
1510  Z[1,2]=T-1
1520  Z[2,2]=(M-1)*T
1530  Z[3,2]=(C-1)*(T*M)
1540  REM MEAN SQUARES
1550  Z[1,4]=Z[1,3]/Z[1,2]
1560  Z[2,4]=Z[2,3]/Z[2,2]
1570  Z[3,4]=Z[3,3]/Z[3,2]
1580  IF C$="one" THEN 1600
1590  Z[4,4]=Z[4,3]/Z[4,2]
1600  REM VARIANCE RATIOS
```

```
1610  Z[1,5]=Z[1,4]/Z[2,4]
1620  Z[2,5]=Z[2,4]/Z[3,4]
1630  IF C$="one" THEN 1650
1640  Z[3,5]=Z[3,4]/Z[4,4]
1650  REM MEANS
1660  MAT G=ZER[1,R]
1670  MAT G=(1/C)*D
1680  MAT H=ZER[1,R/M]
1690  MAT H=(1/(C*M))*E
1700  MAT I=ZER[1,R/(M*T)]
1710  MAT I=(1/(C*M*T))*F
1720  REM STANDARD DEVN.,COEFF. OF VARIATION AND %DIFF. DETECTABLE
1730  D1=D2=D5=0
1740  FOR J=1 TO R
1750  D1=D1+(G[1,J] ** 2)
1760  D2=D2+G[1,J]
1770  NEXT J
1780  D3=D1-((D2 ** 2)/R)
1790  D4=SQR(D3/(R-1))
1800  FOR J=1 TO R/6
1810  D5=D5+I[1,J]
1820  NEXT J
1830  D6=D5/(R/6)
1840  D7=(D4/D6)*100
1850  D8=(2*D7*SQR(2))/(SQR(C))
1860  REM PRINT OUT
1870  PRINT
1880  PRINT "RAW DATA:"
1890  PRINT #2;"RAW DATA:"
1900  PRINT
1910  PRINT #2;" "
1920  PRINT " "," 1 ","REPLICATES"," 3 "
1930  PRINT #2;" "," 1 ","REPLICATES"," 3 "
1940  PRINT "SAMPLE NO.","MICROG./ML.","MICROG./ML.","MICROG./ML."
1950  PRINT #2;"SAMPLE NO.","MICROG./ML.","MICROG./ML.","MICROG./ML
1960  MAT  PRINT B
1970  MAT  PRINT #2;B
1980  PRINT
1990  PRINT #2;" "
2000  PRINT "SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
2010  PRINT #2;"SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
2020  PRINT "VARIATION","FREEDOM","SQUARES","SQUARE","RATIO"
2030  PRINT #2;"VARIATION","FREEDOM","SQUARES","SQUARE","RATIO"
2040  PRINT
2050  PRINT #2;" "
2060  IF C$="one" THEN 2160
2070  PRINT "EXPERIMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2080  PRINT #2;"EXPERIMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2090  PRINT "TREATMENTS",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2100  PRINT #2;"TREATMENTS",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2110  PRINT "MICE",Z[3,2],Z[3,3],Z[3,4],Z[3,5]
2120  PRINT #2;"MICE",Z[3,2],Z[3,3],Z[3,4],Z[3,5]
2130  PRINT "REPLICATES",Z[4,2],Z[4,3],Z[4,4]," - "
2140  PRINT #2;"REPLICATES",Z[4,2],Z[4,3],Z[4,4]," - "
```

```
2150  GOTO 2220
2160  PRINT "TREATMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2170  PRINT #2;"TREATMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2180  PRINT "MICE",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2190  PRINT #2;"MICE",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2200  PRINT "REPLICATES",Z[3,2],Z[3,3],Z[3,4]," - "
2210  PRINT #2;"REPLICATES",Z[3,2],Z[3,3],Z[3,4]," - "
2220  PRINT
2230  PRINT #2;" "
2240  PRINT " ","MICE","TREATMENT","EXPERIMENTAL"
2250  PRINT #2;" ","MICE","TREATMENT","EXPERIMENTAL"
2260  PRINT "SAMPLE NO.","MEANS","MEANS","MEANS"
2270  PRINT #2;"SAMPLE NO.","MEANS","MEANS","MEANS"
2280  V=-1
2290  X=0
2300  FOR J=1 TO R STEP 6
2310  V=V+2
2320  X=X+1
2330  PRINT B[J,1],G[1,J]
2340  PRINT #2;B[J,1],G[1,J]
2350  PRINT B[J+1,1],G[1,J+1],H[1,V]
2360  PRINT #2;B[J+1,1],G[1,J+1],H[1,V]
2370  PRINT B[J+2,1],G[1,J+2]
2380  PRINT #2;B[J+2,1],G[1,J+2]
2390  PRINT " "," "," ",I[1,X]
2400  PRINT #2;" "," "," ",I[1,X]
2410  PRINT B[J+3,1],G[1,J+3]
2420  PRINT #2;B[J+3,1],G[1,J+3]
2430  PRINT B[J+4,1],G[1,J+4],H[1,V+1]
2440  PRINT #2;B[J+4,1],G[1,J+4],H[1,V+1]
2450  PRINT B[J+5,1],G[1,J+5]
2460  PRINT #2;B[J+5,1],G[1,J+5]
2470  IF C$="one" THEN 2510
2480  PRINT "*************************************************************
2490  PRINT #2;"*************************************************************
2500  NEXT J
2510  PRINT
2520  PRINT "COEFFICIENT OF VARIATION FOR MICE :";D7
2530  PRINT #2;"COEFFICIENT OF VARIATION FOR MICE :";D7
2540  PRINT "% DIFFERENCE DETECTABLE BETWEEN MICE :";D8
2550  PRINT #2;"% DIFFERENCE DETECTABLE BETWEEN MICE :";D8
2560  PRINT
2570  PRINT "ANOVAR STORED IN FILE ";B$;" FOR PRINTOUT"
2580  DIM M[10,2]
2590  PRINT "IS ANOVAR OF TREATMENTS AS BLOCKS REQUIRED? Y OR N?"
2600  INPUT D$
2610  IF D$="n" THEN 2770
2620  MAT M=ZER[R/(T*M),T]
2630  V=0
2640  FOR J=1 TO R/M STEP T
2650  V=V+1
2660  X=0
2670  FOR J1=J TO J+(T-1)
2680  X=X+1
2690  M[V,X]=H[1,J1]
2700  NEXT J1
2710  NEXT J
2720  CREATE V1,"XXXXXX",1
2730  ASSIGN "XXXXXX",3,V2
2740  PRINT #3;R,T,M
2750  MAT  PRINT #3;M
2760  CHAIN "AOVMNS"
2770  END
```

```
GATH10

10  REM ************************************
20  REM * PROGRAMME COLLECTING TOGETHER *
30  REM * PANC. EXPTS.                  *
40  REM ************************************
50  FILES t1,t2,t3,t4,t5,t6,t7,t8,t9,t10,pvs110
60  GOTO 230
70  DIM A$[6],B$[6],C$[6],D$[6],E$[6],F$[6],G$[6],H$[6],I$[6],J$[6
80  PRINT "ENTER NAMES OF TREATED EXPERIMENTAL FILES"
90  INPUT A$,B$,C$,D$,E$,F$,G$,H$,I$,J$
100  ASSIGN A$,1,V1
110  ASSIGN B$,2,V2
120  ASSIGN C$,3,V3
130  ASSIGN D$,4,V4
140  ASSIGN E$,5,V5
150  ASSIGN F$,6,V6
160  ASSIGN G$,7,V7
170  ASSIGN H$,8,V8
180  ASSIGN I$,9,V9
190  ASSIGN J$,10,V0
200  PRINT "ENTER NAME OF FILE FOR STORAGE"
210  INPUT K$
220  ASSIGN K$,11,V
230  DIM D[14,4]
240  A2=0
250  FOR F=1 TO 10
260  READ #F;A1
270  A2=A2+A1/3
280  NEXT F
290  FOR F=1 TO 10
300  READ #F,1
310  READ #F;A
320  MAT D=ZER[A/3,4]
330  MAT  READ #F;D
340  MAT  PRINT #11,F;D
350  NEXT F
360  DIM E[122,4]
370  MAT E=ZER[A2,4]
380  READ #11,1
390  MAT  READ #11;E
400  DIM F[122,4]
410  MAT F=ZER[A2,4]
420  V=-2
430  X=1
440  Y=109
450  GOSUB 570
460  X=X+3
470  Y=Y+3
480  GOSUB 570
490  V=V+1
500  X=X+3
510  Y=Y+4
520  GOSUB 570
```

```
530  X=X+3
540  Y=Y+3
550  GOSUB 570
560  GOTO 690
570  FOR I=X TO Y STEP 12
580  IF I>114 THEN 600
590  GOTO 610
600  I=I+1
610  V=V+3
620  FOR J=1 TO 4
630  F[V,J]=E[I,J]
640  F[(V+1),J]=E[(I+1),J]
650  F[(V+2),J]=E[(I+2),J]
660  NEXT J
670  NEXT I
680  RETURN
690  FOR J=1 TO 4
700  F[61,J]=E[115,J]
710  F[122,J]=E[122,J]
720  NEXT J
730  READ #11,1
740  PRINT #11;A2
750  MAT  PRINT #11;F
760  READ #11,1
770  READ #11;A2
780  PRINT "A2 =";A2
790  MAT  READ #11;F
800  MAT  PRINT F
810  PRINT "FILES : ";A$;B$;C$;D$;E$;F$;G$;H$;I$;J$
820  PRINT "STORED IN ";K$;" FOR TREATMENT"
830  END
```

AVPALL

```
10  REM ****************************************
20  REM * HIERARCHICAL ANALYSIS OF VARIANCE *
30  REM * FOR COMBINED PANC. EXPTS.          *
40  REM ****************************************
50  REM Data Entry
60  DIM A$[15],B$[15]
70  DIM C$[3]
80  DIM Q[125,4],A[65,4],B[65,4]
90  DIM Z[4,5]
100  DIM D[1,60],E[1,20],F[1,10]
110  DIM G[1,60],H[1,20],I[1,10]
120  PRINT "ENTER NAME OF FILE FOR ANOVAR"
130  INPUT A$
140  PRINT "ENTER NAME OF FILE FOR PRINTOUT"
150  INPUT B$
160  PRINT "IS ANOVAR FOR ONE EXPT. OR ALL EXPT.'S? : ENTER ONE OR
170  INPUT C$
180  FILES *,*,*
190  ASSIGN A$,1,Z1
200  ASSIGN B$,2,Z2
210  READ #1,1
220  READ #1;A
230  G=A/3
240  PRINT "TOTAL NO. OF ROWS =";G
250  PRINT "ENTER NO. OF ROW WHERE DATA STARTS"
260  INPUT Q1
270  PRINT "ENTER NO. OF ROW WHERE DATA ENDS"
280  INPUT Q2
290  PRINT "ENTER NO. OF REPLICATES"
300  INPUT C
310  PRINT "ENTER NO. OF TREATMENTS"
320  INPUT T
330  PRINT "ENTER NO. OF MICE IN EACH EXPERIMENTAL TREATMENT"
340  PRINT "EACH EXPERIMENTAL TREATMENT MUST HAVE THE SAME NO. OF M
350  INPUT M
360  MAT A=ZER[(Q2-(Q1-1)),C+1]
370  MAT Q=ZER[G,C+1]
380  R=Q2-(Q1-1)
390  READ #1,1
400  READ #1;A
410  MAT  READ #1;Q
420  ASSIGN *,1,V2
430  PURGE X1,"TREAT"
440  V=0
450  FOR Q3=Q1 TO Q2
460  V=V+1
470  FOR Q4=1 TO C+1
480  A[V,Q4]=Q[Q3,Q4]
490  NEXT Q4
500  NEXT Q3
510  IF C$="one" THEN 670
520  REM SORT OF DATA
```

```
530  MAT B=ZER[R,C+1]
540  X=-5
550  FOR I=1 TO R/2 STEP 3
560  X=X+6
570  FOR J=1 TO C+1
580  B[X,J]=A[I,J]
590  B[X+1,J]=A[I+1,J]
600  B[X+2,J]=A[I+2,J]
610  B[X+3,J]=A[I+R/2,J]
620  B[X+4,J]=A[I+(R/2)+1,J]
630  B[X+5,J]=A[I+(R/2)+2,J]
640  NEXT J
650  NEXT I
660  GOTO 690
670  MAT B=ZER[R,C+1]
680  MAT B=A
690  REM CORRECTION FACTOR
700  C1=C2=0
710  FOR I=1 TO R
720  FOR J=2 TO C+1
730  C1=C1+B[I,J]
740  NEXT J
750  NEXT I
760  C2=(C1 ** 2)/(R*C)
770  REM SUM( EACH REPLICATE )**2
780  R1=0
790  FOR I=1 TO R
800  FOR J=2 TO C+1
810  R1=R1+(B[I,J] ** 2)
820  NEXT J
830  NEXT I
840  REM SUM( TOTAL FOR EACH MOUSE )**2
850  MAT D=ZER[1,R]
860  V=M1=M2=0
870  FOR I=1 TO R
880  V=V+1
890  FOR J=2 TO C+1
900  M1=M1+B[I,J]
910  NEXT J
920  GOSUB 950
930  NEXT I
940  GOTO 990
950  D[1,V]=M1
960  M2=M2+(M1 ** 2)
970  M1=0
980  RETURN
990  REM SUM( TOTAL FOR EACH TREATMENT WITHIN AN EXPERIMENT )**2
1000  MAT E=ZER[1,R/M]
1010  T1=T2=V=0
1020  FOR J=1 TO R STEP M
1030  V=V+1
1040  FOR J1=J TO J+(M-1)
1050  T1=T1+D[1,J1]
1060  NEXT J1
```

```
1070  GOSUB 1100
1080  NEXT J
1090  GOTO 1140
1100  E[1,V]=T1
1110  T2=T2+(T1 ** 2)
1120  T1=0
1130  RETURN
1140  REM SUM( TOTAL FOR EACH EXPERIMENT )**2
1150  MAT F=ZER[1,R/(T*M)]
1160  V=E1=E2=0
1170  FOR J=1 TO R/M STEP T
1180  V=V+1
1190  FOR J1=J TO J+(T-1)
1200  E1=E1+E[1,J1]
1210  NEXT J1
1220  GOSUB 1250
1230  NEXT J
1240  GOTO 1290
1250  F[1,V]=E1
1260  E2=E2+(E1 ** 2)
1270  E1=0
1280  RETURN
1290  REM SUM OF SQUARES (DEVIANCE)
1300  IF C$="one" THEN 1360
1310  Z[1,3]=(E2/(T*M*C))-C2
1320  Z[2,3]=(T2/(M*C))-(E2/(T*M*C))
1330  Z[3,3]=(M2/C)-(T2/(M*C))
1340  Z[4,3]=R1-(M2/C)
1350  GOTO 1400
1360  MAT Z=ZER[3,5]
1370  Z[1,3]=(T2/(M*C))-C2
1380  Z[2,3]=(M2/C)-(T2/(M*C))
1390  Z[3,3]=R1-(M2/C)
1400  REM DEGREES OF FREEDOM
1410  IF C$="one" THEN 1470
1420  Z[1,2]=(R/(T*M))-1
1430  Z[2,2]=(T-1)*(R/(T*M))
1440  Z[3,2]=(M-1)*(R/(T*M))*T
1450  Z[4,2]=R*(C-1)
1460  GOTO 1500
1470  Z[1,2]=T-1
1480  Z[2,2]=(M-1)*T
1490  Z[3,2]=(C-1)*(T*M)
1500  REM MEAN SQUARES
1510  Z[1,4]=Z[1,3]/Z[1,2]
1520  Z[2,4]=Z[2,3]/Z[2,2]
1530  Z[3,4]=Z[3,3]/Z[3,2]
1540  IF C$="one" THEN 1560
1550  Z[4,4]=Z[4,3]/Z[4,2]
1560  REM VARIANCE RATIOS
1570  Z[1,5]=Z[1,4]/Z[2,4]
1580  Z[2,5]=Z[2,4]/Z[3,4]
1590  IF C$="one" THEN 1610
1600  Z[3,5]=Z[3,4]/Z[4,4]
```

```
1610  REM MEANS
1620  MAT G=ZER[1,R]
1630  MAT G=(1/C)*D
1640  MAT H=ZER[1,R/M]
1650  MAT H=(1/(C*M))*E
1660  MAT I=ZER[1,R/(M*T)]
1670  MAT I=(1/(C*M*T))*F
1680  REM STANDARD DEVN.,COEFF. OF VARIATION AND %DIFF. DETECTABLE
1690  D1=D2=D5=0
1700  FOR J=1 TO R
1710  D1=D1+(G[1,J] ** 2)
1720  D2=D2+G[1,J]
1730  NEXT J
1740  D3=D1-((D2 ** 2)/R)
1750  D4=SQR(D3/(R-1))
1760  FOR J=1 TO R/6
1770  D5=D5+I[1,J]
1780  NEXT J
1790  D6=D5/(R/6)
1800  D7=(D4/D6)*100
1810  D8=(2*D7*SQR(2))/(SQR(C))
1820  REM PRINT OUT
1830  PRINT
1840  PRINT "RAW DATA:"
1850  PRINT #2;"RAW DATA:"
1860  PRINT
1870  PRINT #2;" "
1880  PRINT " "," 1 ","REPLICATES"," 3 "
1890  PRINT #2;" "," 1 ","REPLICATES"," 3 "
1900  PRINT "SAMPLE NO.","MICROG./ML.","MICROG./ML.","MICROG./ML."
1910  PRINT #2;"SAMPLE NO.","MICROG./ML.","MICROG./ML.","MICROG./M
1920  MAT  PRINT B
1930  MAT  PRINT #2;B
1940  PRINT
1950  PRINT #2;" "
1960  PRINT "SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
1970  PRINT #2;"SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
1980  PRINT "VARIATION","FREEDOM","SQUARES","SQUARE","RATIO"
1990  PRINT #2;"VARIATION","FREEDOM","SQUARES","SQUARE","RATIO"
2000  PRINT
2010  PRINT #2;" "
2020  IF C$="one" THEN 2120
2030  PRINT "EXPERIMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2040  PRINT #2;"EXPERIMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2050  PRINT "TREATMENTS",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2060  PRINT #2;"TREATMENTS",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2070  PRINT "MICE",Z[3,2],Z[3,3],Z[3,4],Z[3,5]
2080  PRINT #2;"MICE",Z[3,2],Z[3,3],Z[3,4],Z[3,5]
2090  PRINT "REPLICATES",Z[4,2],Z[4,3],Z[4,4]," - "
2100  PRINT #2;"REPLICATES",Z[4,2],Z[4,3],Z[4,4]," - "
2110  GOTO 2180
2120  PRINT "TREATMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2130  PRINT #2;"TREATMENTS",Z[1,2],Z[1,3],Z[1,4],Z[1,5]
2140  PRINT "MICE",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
```

```
2150  PRINT #2;"MICE",Z[2,2],Z[2,3],Z[2,4],Z[2,5]
2160  PRINT "REPLICATES",Z[3,2],Z[3,3],Z[3,4]," - "
2170  PRINT #2;"REPLICATES",Z[3,2],Z[3,3],Z[3,4]," - "
2180  PRINT
2190  PRINT #2;" "
2200  PRINT " ","MICE","TREATMENT","EXPERIMENTAL"
2210  PRINT #2;" ","MICE","TREATMENT","EXPERIMENTAL"
2220  PRINT "SAMPLE NO.","MEANS","MEANS","MEANS"
2230  PRINT #2;"SAMPLE NO.","MEANS","MEANS","MEANS"
2240  V=-1
2250  X=0
2260  FOR J=1 TO R STEP 6
2270  V=V+2
2280  X=X+1
2290  PRINT B[J,1],G[1,J]
2300  PRINT #2;B[J,1],G[1,J]
2310  PRINT B[J+1,1],G[1,J+1],H[1,V]
2320  PRINT #2;B[J+1,1],G[1,J+1],H[1,V]
2330  PRINT B[J+2,1],G[1,J+2]
2340  PRINT #2;B[J+2,1],G[1,J+2]
2350  PRINT " "," "," ",I[1,X]
2360  PRINT #2;" "," "," ",I[1,X]
2370  PRINT B[J+3,1],G[1,J+3]
2380  PRINT #2;B[J+3,1],G[1,J+3]
2390  PRINT B[J+4,1],G[1,J+4],H[1,V+1]
2400  PRINT #2;B[J+4,1],G[1,J+4],H[1,V+1]
2410  PRINT B[J+5,1],G[1,J+5]
2420  PRINT #2;B[J+5,1],G[1,J+5]
2430  IF C$="one" THEN 2460
2440  PRINT "**********************************************************
2450  PRINT #2;"**********************************************************
2460  NEXT J
2470  PRINT
2480  PRINT "COEFFICIENT OF VARIATION FOR MICE :";D7
2490  PRINT #2;"COEFFICIENT OF VARIATION FOR MICE :";D7
2500  PRINT "% DIFFERENCE DETECTABLE BETWEEN MICE :";D8
2510  PRINT #2;"% DIFFERENCE DETECTABLE BETWEEN MICE :";D8
2520  PRINT
2530  PRINT "ANOVAR STORED IN FILE ";B$;" FOR PRINTOUT"
2540  DIM M[10,2]
2550  PRINT "IS ANOVAR OF TREATMENTS AS BLOCKS REQUIRED? Y OR N?"
2560  INPUT D$
2570  IF D$="n" THEN 2730
2580  MAT M=ZER[R/(T*M),T]
2590  V=0
2600  FOR J=1 TO R/M STEP T
2610  V=V+1
2620  X=0
2630  FOR J1=J TO J+(T-1)
2640  X=X+1
2650  M[V,X]=H[1,J1]
2660  NEXT J1
2670  NEXT J
2680  CREATE V1,"XXXXXX",1
2690  ASSIGN "XXXXXX",3,V2
2700  PRINT #3;R,T,M
2710  MAT  PRINT #3;M
2720  CHAIN "AVPMNS"
2730  END
```

AVPMNS

```
10  REM ****************************************
20  REM * TWO WAY ANALYSIS OF VARIANCE         *
30  REM * FOR COMBINED PANC. EXPTS. AS MEANS *
40  REM ****************************************
50  DIM M[10,2],X[4,5]
60  DIM A$[6]
70  FILES xxxxxx,*
80  PRINT "ENTER NAME OF FILE FOR STORAGE"
90  INPUT A$
100  ASSIGN A$,2,V1
110  READ #1,1
120  READ #1;R,T,M
130  MAT  READ #1;M
140  ASSIGN *,1,V2
150  PURGE V1,"XXXXXX"
160  PRINT "V1 =";V1
170  X=R/(T*M)
180  REM CORRECTION FACTOR
190  C3=S1=C4=0
200  FOR I=1 TO X
210  FOR J=1 TO T
220  C3=C3+M[I,J]
230  S1=S1+(M[I,J] ** 2)
240  NEXT J
250  NEXT I
260  C4=(C3 ** 2)/(T*X)
270  REM SUM( TREATMENT TOTALS )**2
280  T3=T4=0
290  FOR J=1 TO T
300  FOR I=1 TO X
310  T3=T3+M[I,J]
320  NEXT I
330  T4=T4+(T3 ** 2)
340  T3=0
350  NEXT J
360  REM SUM (EXPERIMENT TOTALS )**2
370  E3=E4=0
380  FOR I=1 TO X
390  FOR J=1 TO T
400  E3=E3+M[I,J]
410  NEXT J
420  E4=E4+(E3 ** 2)
430  E3=0
440  NEXT I
450  REM SUM OF SQUARES
460  X[1,3]=S1-C4
470  X[2,3]=(T4/X)-C4
480  X[3,3]=(E4/T)-C4
490  X[4,3]=X[1,3]-(X[2,3]+X[3,3])
500  REM DEGREES OF FREEDOM
510  X[1,2]=(X*T)-1
520  X[2,2]=T-1
```

```
530  X[3,2]=X-1
540  X[4,2]=X[1,2]-(X[2,2]+X[3,2])
550  REM MEAN SQUARES
560  X[2,4]=X[2,3]/X[2,2]
570  X[3,4]=X[3,3]/X[3,2]
580  X[4,4]=X[4,3]/X[4,2]
590  REM VARIANCE RATIOS
600  X[2,5]=X[2,4]/X[4,4]
610  X[3,5]=X[3,4]/X[4,4]
620  REM PRINT OUT
630  PRINT "RAW DATA :"
640  PRINT #2;"RAW DATA :"
650  PRINT
660  PRINT #2;" "
670  PRINT "EXPERIMENT","MEANS OF","MEANS OF"
680  PRINT #2;"EXPERIMENT","MEANS OF","MEANS OF"
690  PRINT "NUMBER","TREATMENT 1","TREATMENT 2"
700  PRINT #2;"NUMBER","TREATMENT 1","TREATMENT 2"
710  PRINT
720  PRINT #2;" "
730  V=0
740  FOR I=1 TO X
750  V=V+1
760  PRINT V,M[I,1],M[I,2]
770  PRINT #2;V,M[I,1],M[I,2]
780  NEXT I
790  PRINT
800  PRINT #2;" "
810  PRINT "ANOVAR :"
820  PRINT #2;"ANOVAR :"
830  PRINT
840  PRINT #2;" "
850  PRINT "SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
860  PRINT #2;"SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
870  PRINT "VARIATION","FREEDOM","SQUARES","SQUARES","RATIOS"
880  PRINT #2;"VARIATION","FREEDOM","SQUARES","SQUARES","RATIOS"
890  PRINT
900  PRINT #2;" "
910  PRINT "TOTAL",X[1,2],X[1,3]," - "," - "
920  PRINT #2;"TOTAL",X[1,2],X[1,3]," - "," - "
930  PRINT "TREATMENTS",X[2,2],X[2,3],X[2,4],X[2,5]
940  PRINT #2;"TREATMENTS",X[2,2],X[2,3],X[2,4],X[2,5]
950  PRINT "EXPERIMENTS",X[3,2],X[3,3],X[3,4],X[3,5]
960  PRINT #2;"EXPERIMENTS",X[3,2],X[3,3],X[3,4],X[3,5]
970  PRINT "ERROR",X[4,2],X[4,3],X[4,4]," - "
980  PRINT #2;"ERROR",X[4,2],X[4,3],X[4,4]," - "
990  PRINT
1000  PRINT "ANOVAR STORED IN FILE ";A$;" FOR PRINTOUT"
1010  END
```

TIMINP

```
10  REM ****************************************
20  REM * DATA INPUT FOR TIMTRT                *
30  REM * INPUT AS DPM FROM B-COUNTER PRINTOUT *
40  REM ****************************************
50  PRINT "ENTER NAME OF FILE FOR STORAGE OF DATA"
60  INPUT A$
70  DIM A$[6]
80  FILES *
90  ASSIGN A$,1,V1
100  B=A=S=0
110  PRINT "ENTER NO. OF BLANKS"
120  INPUT B
130  PRINT "ENTER NO. OF STANDARDS"
140  INPUT S
150  PRINT "ENTER NO. OF SAMPLES"
160  INPUT A
170  MAT C=CON[A,5]
180  MAT C=(-1)*C
190  MAT A=ZER[B]
200  MAT B=ZER[S]
210  PRINT #1;B,S,A
220  MAT  PRINT #1;A,B,C
230  DIM A[65],B[10],C[500,5]
240  PRINT "ENTER BLANKS"
250  MAT  INPUT A[B]
260  PRINT "ENTER STANDARDS"
270  MAT  INPUT B[S]
280  S1=0
290  FOR S2=1 TO A STEP A/2
300  S1=S1+1
310  P=0
320  FOR X=S2 TO S2+A/2-1 STEP 15
330  N=0
340  P=P+1
350  FOR Y=X TO X+14 STEP 3
360  N=N+10
370  IF N=40 THEN 410
380  IF N=50 THEN 430
390  N1=N
400  GOTO 440
410  N1=45
420  GOTO 440
430  N1=60
440  Z1=0
450  FOR Z=Y TO Y+2
460  Z1=Z1+1
470  C[Z,1]=(S1*10000)+(P*1000)+(N1*10)+Z1
480  NEXT Z
490  NEXT Y
500  NEXT X
510  NEXT S2
520  PRINT "ENTER SAMPLE DPM's"

530  FOR I=1 TO A
540  INPUT C[I,2]
550  NEXT I
560  READ #1,1
570  PRINT #1;B,S,A
580  MAT  PRINT #1;A,B,C
590  MAT  PRINT C
600  END
```

TIMTRT

```
10  REM ****************************************
20  REM * TREATMENT OF PANC. TIME COURSE DATA *
30  REM * CONVERTS DPM TO MICROG./ML. T.       *
40  REM ****************************************
50  PRINT "ENTER NAME OF DATA FILE"
60  INPUT A$
70  PRINT "ENTER NAME OF PRINTOUT FILE"
80  INPUT B$
90  PRINT "ENTER NAME OF ANALYSIS FILE"
100  INPUT C$
110  DIM A$[6],B$[6]
120  DIM C$[6]
130  FILES *,*,*
140  ASSIGN A$,1,V1
150  ASSIGN B$,2,V1
160  ASSIGN C$,3,V1
170  B=S=A=T=U=V=W=Z=Y=B1=B2=B3=S1=S2=S3=A1=A2=A3=A4=0
180  P=H=0
190  READ #1;B,S,A
200  PRINT "NO. OF BLANKS";B
210  PRINT #2;"NO. OF BLANKS",B
220  PRINT "NO. OF STANDARDS";S
230  PRINT #2;"NO. OF STANDARDS",S
240  PRINT "NO. OF SAMPLES";A
250  PRINT #2;"NO. OF SAMPLES",A
260  DIM A[40],B[20],C[500,5]
270  MAT A=ZER[B]
280  MAT B=ZER[S]
290  MAT C=ZER[A,5]
300  MAT  READ #1;A,B,C
310  FOR U=1 TO B
320  LET B1=A[U]
330  B2=B2+B1
340  NEXT U
350  B3=B2/B
360  FOR T=1 TO S
370  LET S1=B[T]
380  S2=S2+S1
390  NEXT T
400  S3=S2/S
410  PRINT "BLANK MEAN:";B3
420  PRINT #2;"BLANK MEAN :",B3
430  PRINT "STANDARD MEAN:";S3
440  PRINT #2;"STANDARD MEAN :",S3
450  FOR W=1 TO A
460  LET A1=C[W,2]
470  IF A1<B3 THEN 500
480  A2=((A1-B3)/(S3-B3))*1.1
490  GOTO 510
500  LET A2=0
510  Z=Z+1
520  GOSUB 550
```

```
530  NEXT W
540  GOTO 580
550  LET C[Z,3]=A2
560  A2=0
570  RETURN
580  FOR V=1 TO A
590  LET A3=C[V,3]
600  LET A4=A3*1000
610  LET Y=Y+1
620  GOSUB 650
630  NEXT V
640  GOTO 680
650  LET C[Y,4]=A4
660  LET A4=0
670  RETURN
680  FOR H=1 TO A
690  A5=C[H,4]
700  A6=A5*20
710  P=P+1
720  GOSUB 750
730  NEXT H
740  GOTO 780
750  C[P,5]=A6
760  A6=0
770  RETURN
780  PRINT
790  PRINT " "," ","COLCH. BOUND","TUBULIN CONC.","TUBULIN"
800  PRINT #2;" "," ","COLCH. BOUND","TUBULIN CONC.","TUBULIN"
810  PRINT "SAMPLE NO.","DPM","NM./110MICROL.","MICROG./ML.","MICRO
820  PRINT #2;"SAMPLE NO.","DPM","NM./110MICROL.","MICROG./ML.","MI
830  MAT  PRINT C
840  MAT  PRINT #2;C
850  DIM D[170,4]
860  MAT D=ZER[A/3,4]
870  X=0
880  FOR D1=1 TO A/2 STEP 3
890  X=X+1
900  D[X,2]=C[D1,4]
910  NEXT D1
920  X=0
930  FOR D2=2 TO A/2 STEP 3
940  X=X+1
950  D[X,3]=C[D2,4]
960  NEXT D2
970  X=0
980  FOR D3=3 TO A/2 STEP 3
990  X=X+1
1000  D[X,4]=C[D3,4]
1010  NEXT D3
1020  X=A/6
1030  FOR D4=((A/2)+1) TO A STEP 3
1040  X=X+1
1050  D[X,2]=C[D4,4]
1060  NEXT D4
```

```
1070  X=A/6
1080  FOR D5=((A/2)+2) TO A STEP 3
1090  X=X+1
1100  D[X,3]=C[D5,4]
1110  NEXT D5
1120  X=A/6
1130  FOR D6=((A/2)+3) TO A STEP 3
1140  X=X+1
1150  D[X,4]=C[D6,4]
1160  NEXT D6
1170  X=0
1180  FOR D7=1 TO A STEP 3
1190  X=X+1
1200  D[X,1]=INT((C[D7,1])/10)
1210  NEXT D7
1220  PRINT #3;A
1230  MAT  PRINT #3;D
1240  PRINT
1250  PRINT " ","1","REPLICATES","3"
1260  PRINT "SAMPLE NO.","MICROG./ML.","MICROG./ML."
1270  MAT  PRINT D
1280  PRINT "DATA STORED IN ";B$;" FOR PRINTOUT,AND ";C$;" FOR ANAL
1290  END
```

AVORT2

```
10  REM ************************************
20  REM * TWO WAY ANALYSIS OF VARIANCE    *
30  REM * USING ORTHOGONAL POLYNOMIALS    *
35  REM * TO PARTITION TREATMENT EFFECTS *
40  REM ************************************
50  DIM A[60,4]
60  DIM B[20,4]
70  DIM C[5,12],D[5,12]
80  DIM E[4,7],F[4,7]
90  DIM G[9,11],H[9,11]
100  DIM I[9,4],J[9,4]
110  DIM Y[15,5],Z[15,5]
120  DIM P[11,10]
130  FILES *,*
140  PRINT "ENTER NAME OF FILE FOR ANOVAR USING ORTHOGONAL POLYNOMIA
150  INPUT Z$
160  DIM Z$[6]
170  ASSIGN Z$,1,V1
180  READ #1,1
190  READ #1;A
200  MAT A=ZER[A/3,4]
210  MAT  READ #1;A
220  M=A/3
230  MAT B=ZER[M/3,4]
240  V=0
250  FOR I=1 TO M STEP 3
260  V=V+1
270  B[V,1]=INT(A[I,1]/10)
280  FOR I1=0 TO 2
290  I2=I+I1
300  A1=0
310  FOR J=2 TO 4
320  A1=A1+A[I2,J]
330  NEXT J
340  B[V,I1+2]=A1/3
350  NEXT I1
360  NEXT I
370  T=M/3
380  MAT C=ZER[5,(T/2)+2]
390  MAT D=ZER[5,(T/2)+2]
400  FOR I=1 TO T/2
410  FOR J=1 TO 4
420  C[J,I+1]=B[I,J]
430  D[J,I+1]=B[I+10,J]
440  NEXT J
450  NEXT I
460  FOR I=1 TO 3
470  C[I+1,1]=D[I+1,1]=I
480  NEXT I
490  T1=T2=S1=S2=0
500  FOR I=2 TO 4
510  R1=R2=0
```

```
520  FOR J=2 TO 11
530  R1=R1+C[I,J]
540  R2=R2+D[I,J]
550  T1=T1+C[I,J]
560  T2=T2+D[I,J]
570  S1=S1+(C[I,J] ** 2)
580  S2=S2+(D[I,J] ** 2)
590  NEXT J
600  C[I,12]=R1
610  D[I,12]=R2
620  NEXT I
630  S3=S4=0
640  FOR J=2 TO 11
650  C1=C2=0
660  FOR I=2 TO 4
670  C1=C1+C[I,J]
680  C2=C2+D[I,J]
690  NEXT I
700  C[5,J]=C1
710  D[5,J]=C2
720  S3=S3+(C1 ** 2)
730  S4=S4+(C2 ** 2)
740  NEXT J
750  REM TOTAL S.S.
760  MAT Y=ZER
770  MAT Z=ZER
780  C[5,12]=T1
790  D[5,12]=T2
800  X8=(T1 ** 2)/(M/2)
810  X9=(T2 ** 2)/(M/2)
820  Y[2,3]=S1-X8
830  Z[2,3]=S2-X9
840  REM TOTAL TRT. S.S.
850  Y[3,3]=(S3/3)-X8
860  Z[3,3]=(S4/3)-X9
870  REM ERROR S.S.
880  Y[15,3]=Y[2,3]-Y[3,3]
890  Z[15,3]=Z[2,3]-Z[3,3]
900  REM PARTITIONED TOTAL TRT. S.S.
910  MAT E=ZER[4,(T/4)+2]
920  MAT F=ZER[4,(T/4)+2]
930  C1=C2=0
940  FOR J=2 TO 6
950  E[1,J]=C[1,J]-1100
960  F[1,J]=D[1,J]-2100
970  E[2,J]=T1=C[5,J]
980  E[3,J]=T2=C[5,J+5]
990  F[2,J]=T3=D[5,J]
1000  F[3,J]=T4=D[5,J+5]
1010  E[4,J]=T5=T1+T2
1020  F[4,J]=T6=T3+T4
1030  E[2,7]=E[2,7]+T1
1040  E[3,7]=E[3,7]+T2
1050  F[2,7]=F[2,7]+T3
```

```
1060  F[3,7]=F[3,7]+T4
1070  C1=C1+(T5 ** 2)
1080  C2=C2+(T6 ** 2)
1090  NEXT J
1100  R1=R2=0
1110  FOR I=2 TO 3
1120  E[4,7]=E[4,7]+E[I,7]
1130  F[4,7]=F[4,7]+F[I,7]
1140  R1=R1+(E[I,7] ** 2)
1150  R2=R2+(F[I,7] ** 2)
1160  NEXT I
1170  REM TRT. S.S.
1180  Y[4,3]=(R1/15)-X8
1190  Z[4,3]=(R2/15)-X9
1200  REM TIMES S.S.
1210  Y[5,3]=(C1/6)-X8
1220  Z[5,3]=(C2/6)-X9
1230  REM TRT. x TIMES S.S.
1240  Y[10,3]=Y[3,3]-(Y[4,3]+Y[5,3])
1250  Z[10,3]=Z[3,3]-(Z[4,3]+Z[5,3])
1260  REM POLYNOMIALS
1270  L=T/4
1280  MAT A=ZER[L,5]
1290  MAT P=ZER[L,5]
1300  L1=L2=L3=0
1310  FOR I=1 TO L
1320  A[I,1]=C[1,I+1]-1100
1330  NEXT I
1340  FOR I=1 TO L
1350  A[I,2]=A[I,1]-A[1,1]
1360  NEXT I
1370  FOR I=L TO 2 STEP -1
1380  A[I,2]=A[I,2]/A[2,2]
1390  NEXT I
1400  FOR I=1 TO L
1410  L1=L1+A[I,2]
1420  L2=L2+(A[I,2] ** 2)
1430  L3=L3+(A[I,2] ** 3)
1440  NEXT I
1450  REM LINEAR POLYNOMIALS
1460  A1=-L1/L
1470  FOR I=1 TO L
1480  A[I,3]=A1+A[I,2]
1490  NEXT I
1500  IF L<3 THEN 900
1510  REM QUADRATIC POLYNOMIALS
1520  Q1=Q2=0
1530  FOR I=1 TO L
1540  Q1=Q1+(A[I,2]*A[I,3])
1550  Q2=Q2+((A[I,2] ** 2)*A[I,3])
1560  NEXT I
1570  B1=-Q2/Q1
1580  A2=((-(L1*B1)-L2)/L
1590  FOR I=1 TO L
```

```
1600  A[I,4]=A2+(A[I,2]*B1)+(A[I,2] ** 2)
1610  NEXT I
1620  IF L<4 THEN 900
1630  REM CUBIC POLYNOMIALS
1640  C1=C2=C3=0
1650  FOR I=1 TO L
1660  C1=C1+((A[I,2] ** 3)*A[I,3])
1670  C2=C2+((A[I,2] ** 2)*A[I,4])
1680  C3=C3+((A[I,2] ** 3)*A[I,4])
1690  NEXT I
1700  C4=-C3/C2
1710  B2=((-(Q2*C4))-C1)/Q1
1720  A3=(-(L1*B2)-(L2*C4)-L3)/L
1730  FOR I=1 TO L
1740  A[I,5]=A3+(B2*A[I,2])+(C4*(A[I,2] ** 2))+(A[I,2] ** 3)
1750  NEXT I
1760  MAT P=ZER[5,5]
1770  MAT P=A
1780  REM PARTITIONED TIME S.S. & TRT. x TIMES S.S.
1790  MAT G=ZER[9,(T/2)+1]
1800  MAT H=ZER[9,(T/2)+1]
1810  FOR J=2 TO 11
1820  G[1,J]=C[1,J]
1830  H[1,J]=D[1,J]
1840  G[2,J]=C[5,J]
1850  H[2,J]=D[5,J]
1860  NEXT J
1870  FOR J=2 TO 6
1880  G[3,J]=H[3,J]=-1
1890  G[3,J+5]=H[3,J+5]=1
1900  FOR I=4 TO 6
1910  G[I,J]=G[I,J+5]=H[I,J]=H[I,J+5]=P[J-1,I-1]
1920  G[I+3,J]=H[I+3,J]=G[I,J]*G[3,J]
1930  G[I+3,J+5]=H[I+3,J+5]=G[I,J+5]*G[3,J+5]
1940  NEXT I
1950  NEXT J
1960  MAT I=ZER[9,4]
1970  MAT J=ZER[9,4]
1980  FOR J=2 TO 11
1990  FOR I=3 TO 9
2000  I[I,2]=I[I,2]+(G[2,J]*G[I,J])
2010  J[I,2]=J[I,2]+(H[2,J]*H[I,J])
2020  I[I,3]=I[I,3]+(G[I,J] ** 2)
2030  J[I,3]=J[I,3]+(H[I,J] ** 2)
2040  NEXT I
2050  NEXT J
2060  I[2,2]=C[5,12]
2070  J[2,2]=D[5,12]
2080  I[2,3]=J[2,3]=T/2
2090  FOR I=4 TO 9
2100  IF I>6 THEN 2130
2110  I1=I+2
2120  GOTO 2140
2130  I1=I+4
```

```
2140  Y[I1,3]=(I[I,2] ** 2)/(3*I[I,3])
2150  Ż[I1,3]=(J[I,2] ** 2)/(3*J[I,3])
2160  NEXT I
2170  FOR I=9 TO 14 STEP 5
2180  Y[I,3]=Y[I-4,3]
2190  Z[I,3]=Z[I-4,3]
2200  NEXT I
2210  FOR I=6 TO 8
2220  Y[9,3]=Y[9,3]-Y[I,3]
2230  Y[14,3]=Y[14,3]-Y[I+5,3]
2240  Z[9,3]=Z[9,3]-Z[I,3]
2250  Z[14,3]=Z[14,3]-Z[I+5,3]
2260  NEXT I
2270  REM DEG. FREEDOM
2280  Y[2,2]=Z[2,2]=(M/2)-1
2290  Y[3,2]=Z[3,2]=(T/2)-1
2300  Y[4,2]=Z[4,2]=1
2310  Y[5,2]=Z[5,2]=Y[10,2]=Z[10,2]=4
2320  FOR I=6 TO 9
2330  Y[I,2]=Z[I,2]=Y[I+5,2]=Z[I+5,2]=1
2340  NEXT I
2350  Y[15,2]=Z[15,2]=Y[2,2]-Y[3,2]
2360  REM MEAN SQUARES
2370  FOR I=3 TO 15
2380  Y[I,4]=Y[I,3]/Y[I,2]
2390  Z[I,4]=Z[I,3]/Z[I,2]
2400  NEXT I
2410  REM VARIANCE RATIOS
2420  FOR I=3 TO 14
2430  Y[I,5]=Y[I,4]/Y[15,4]
2440  Z[I,5]=Z[I,4]/Z[15,4]
2450  NEXT I
2460  REM MEAN EFFECTS
2470  FOR I=2 TO 9
2480  I[I,4]=I[I,2]/(3*I[I,3])
2490  J[I,4]=J[I,2]/(3*J[I,3])
2500  NEXT I
2510  REM PRINT OUT
2520  PRINT "**********************************************************************
2530  PRINT
2540  PRINT "RAW DATA :"
2550  PRINT
2560  A$="REP. 's"
2570  B$="T10"
2580  C$="T20"
2590  D$="T30"
2600  E$="T45"
2610  F$="T60"
2620  DIM A$[6],B$[6],C$[6],D$[6],E$[6],F$[6],G$[6],H$[11]
2630  PRINT "TREATMENTS","TIMES"," ","REPLICATES"
2640  PRINT " "," "," 1."," 2."," 3."
2650  PRINT "-------------------------------------------------------------------
2660  PRINT
2670  MAT A=ZER[5,12]
```

```
2680  MAT A=C
2690  PRINT "SN-I's :"
2700  PRINT "-------"
2710  GOSUB 2780
2720  MAT A=D
2730  PRINT "SN-II's :"
2740  PRINT "--------"
2750  GOSUB 2780
2760  GOTO 3130
2770  V=0
2780  FOR J=2 TO 11
2790  IF J=2 THEN 2900
2800  IF J=3 THEN 2920
2810  IF J=4 THEN 2940
2820  IF J=5 THEN 2960
2830  IF J=7 THEN 2900
2840  IF J=8 THEN 2920
2850  IF J=9 THEN 2940
2860  IF J=10 THEN 2960
2870  G$=F$
2880  V=1
2890  GOTO 2970
2900  G$=B$
2910  GOTO 2970
2920  G$=C$
2930  GOTO 2970
2940  G$=D$
2950  GOTO 2970
2960  G$=E$
2970  IF J=4 THEN 3000
2980  IF J=9 THEN 3020
2990  GOTO 3040
3000  H$="SALINE"
3010  GOTO 3050
3020  H$="PILOCARPINE"
3030  GOTO 3050
3040  H$="      "
3050  PRINT H$,G$,A[2,J],A[3,J],A[4,J]
3060  IF V=1 THEN 3080
3070  GOTO 3100
3080  PRINT
3090  V=0
3100  NEXT J
3110  PRINT
3120  RETURN
3130  PRINT "*****************************************************************
3140  PRINT
3150  PRINT "MEANS :"
3160  PRINT
3170  PRINT "      "," TREATMENTS"
3180  PRINT "TIMES","SALINE","PILOCARPINE","TOTALS"
3190  PRINT "-------------------------------------------------------------------
3200  PRINT
3210  MAT A=ZER[4,7]
```

```
3220  MAT A=(1/3)*E
3230  PRINT "SN-I's :"
3240  PRINT "-------"
3250  GOSUB 3310
3260  MAT A=(1/3)*F
3270  PRINT "SN-II's :"
3280  PRINT "--------"
3290  GOSUB 3310
3300  GOTO 3530
3310  FOR J=2 TO 7
3320  IF J=2 THEN 3400
3330  IF J=3 THEN 3420
3340  IF J=4 THEN 3440
3350  IF J=5 THEN 3460
3360  IF J=6 THEN 3480
3370  PRINT
3380  PRINT "TOTALS",A[2,J],A[3,J],A[4,J]
3390  GOTO 3500
3400  A$="T10"
3410  GOTO 3490
3420  A$="T20"
3430  GOTO 3490
3440  A$="T30"
3450  GOTO 3490
3460  A$="T45"
3470  GOTO 3490
3480  A$="T60"
3490  PRINT A$,A[2,J],A[3,J],A[4,J]
3500  NEXT J
3510  PRINT
3520  RETURN
3530  PRINT "*****************************************************************
3540  PRINT
3550  PRINT "ORTHOGONAL POLYNOMIALS :"
3560  PRINT
3570  PRINT " "," "," ","COEFFICIENTS"
3580  PRINT "TIME PERIODS","REDUCED LEVELS","LINEAR","QUADRATIC","C
3590  PRINT "-------------------------------------------------------------------
3600  PRINT
3610  MAT  PRINT P
3620  PRINT
3630  PRINT "TREATMENT COEFFICIENTS :"
3640  PRINT "                                SALINE  -1"
3650  PRINT "                             PILOCARPINE  +1"
3660  PRINT
3670  PRINT "*****************************************************************
3680  PRINT
3690  PRINT "ANALYSIS OF VARIANCE :"
3700  PRINT
3710  PRINT "SOURCE OF","DEGREES OF","SUM OF","MEAN","VARIANCE"
3720  PRINT "VARIATION","FREEDOM","SQUARES","SQUARES","RATIOS"
3730  PRINT "-------------------------------------------------------------------
3740  PRINT
3750  DIM N[15,5]
```

```
3760  MAT N=Y
3770  PRINT "SN-I's :"
3780  PRINT "-------"
3790  GOSUB 3850
3800  MAT N=Z
3810  PRINT "SN-II's :"
3820  PRINT "--------"
3830  GOSUB 3850
3840  GOTO 4050
3850  PRINT "TOTAL",N[2,2],N[2,3]," - "," - "
3860  PRINT
3870  PRINT "TOTAL TRT. ",N[3,2],N[3,3],N[3,4],N[3,5]
3880  PRINT "TRT. ",N[4,2],N[4,3],N[4,4],N[4,5]
3890  PRINT
3900  PRINT "TIMES :",N[5,2],N[5,3],N[5,4],N[5,5]
3910  PRINT "T-L. ",N[6,2],N[6,3],N[6,4],N[6,5]
3920  PRINT "T-Q. ",N[7,2],N[7,3],N[7,4],N[7,5]
3930  PRINT "T-C. ",N[8,2],N[8,3],N[8,4],N[8,5]
3940  PRINT "T-RES. ",N[9,2],N[9,3],N[9,4],N[9,5]
3950  PRINT
3960  PRINT "TRT. x TIMES :",N[10,2],N[10,3],N[10,4],N[10,5]
3970  PRINT "TRT. x T-L. ",N[11,2],N[11,3],N[11,4],N[11,5]
3980  PRINT "TRT. x T-Q. ",N[12,2],N[12,3],N[12,4],N[12,5]
3990  PRINT "TRT. x T-C. ",N[13,2],N[13,3],N[13,4],N[13,5]
4000  PRINT "TRT. x T-RES. ",N[14,2],N[14,3],N[14,4],N[14,5]
4010  PRINT
4020  PRINT "ERROR",N[15,2],N[15,3],N[15,4]," - "
4030  PRINT
4040  RETURN
4050  PRINT "ARE CURVE COORDINATES REQUIRED ? ENTER Y OR N."
4060  INPUT Z$[1]
4070  IF Z$="N" THEN 4170
4080  E8=Y[15,4]
4090  E9=Z[15,4]
4100  D9=Y[15,2]
4110  CREATE V1,"CURVFL",10
4120  ASSIGN "CURVFL",2,V2
4130  READ #2,1
4140  PRINT #2;E8,E9,D9
4150  MAT  PRINT #2;G,H,I,J
4160  CHAIN "CURVE"
4170  END
```

RIAINP

```
10  REM ****************************************
20  REM * DATA INPUT FOR RIATRT                *
30  REM * INPUT AS CPM FROM G-COUNTER PRINTOUT *
40  REM ****************************************
50  FILES *
60  DIM A$[6],A[39,2],B[3],C[3],D[3],E[100]
70  PRINT "ENTER NAME OF FILE FOR STORAGE"
80  INPUT A$
90  ASSIGN A$,1,V1
100  PRINT "ENTER NO. OF REPLICATES USED"
110  INPUT R
120  PRINT "ENTER NO. OF STANDARDS"
130  INPUT S
140  PRINT "ENTER NO. OF TOTALS"
150  INPUT T
160  PRINT "ENTER NO. OF BLANKS"
170  INPUT O
180  PRINT "ENTER NO. OF BACKGROUNDS"
190  INPUT B
200  PRINT "ENTER NO. OF REPLICATE SAMPLES"
210  INPUT A
220  PRINT "ENTER NO. PROTEIN/100 MICROL. IN FIRST STANDARD"
230  INPUT P
240  MAT A=ZER[S*R,2]
250  MAT B=ZER[T]
260  MAT C=ZER[O]
270  MAT D=ZER[B]
280  MAT E=ZER[A]
290  PRINT #1;R,S,T,O,B,A,P
300  MAT  PRINT #1;A,B,C,D,E
310  P1=P*2
320  FOR I=1 TO S*R STEP R
330  P1=P1/2
340  A[I,1]=A[I+1,1]=A[I+2,1]=P1
350  NEXT I
360  PRINT "ENTER STANDARDS"
370  FOR I=1 TO S*R
380  INPUT A[I,2]
390  NEXT I
400  PRINT "ENTER TOTALS"
410  MAT  INPUT B
420  PRINT "ENTER BLANKS"
430  MAT  INPUT C
440  PRINT "ENTER BACKGROUNDS"
450  MAT  INPUT D
460  PRINT "ENTER SAMPLES"
470  MAT  INPUT E
480  READ #1,1
490  PRINT #1;R,S,T,O,B,A,P
500  MAT  PRINT #1;A,B,C,D,E
510  PRINT "DATA STORED IN ";A$;" FOR TREATMENT"
520  END
```

RIATRT

```
10  REM ********************************************
20  REM * TREATMENT OF RIA DATA                     *
30  REM * CONVERTS CPM TO MICROG./ML. T.  OR MAP.   *
40  REM ********************************************
50  FILES *,*
60  DIM A$[6],B$[6],C$[9]
70  DIM A[39,2],B[3],C[3],D[3],E[100]
80  DIM F[39],G[100]
90  DIM H[13,2],I[13,2],J[13,2],K[13,2]
100  DIM L[13,2],M[100],N[100],O[100]
110  PRINT "ENTER NAME OF FILE FOR TREATMENT"
120  INPUT A$
130  ASSIGN A$,1,V1
140  READ #1,1
150  READ #1;R,S,T,O,B,A,P
160  MAT A=ZER[S*R,2]
170  MAT B=ZER[T]
180  MAT C=ZER[O]
190  MAT D=ZER[B]
200  MAT E=ZER[A]
210  MAT  READ #1;A,B,C,D,E
220  REM TOT.,BLK. and BKG. means
230  T1=0
240  FOR I=1 TO T
250  T1=T1+B[I]
260  NEXT I
270  M1=T1/T
280  O1=0
290  FOR I=1 TO O
300  O1=O1+C[I]
310  NEXT I
320  M2=O1/O
330  B1=0
340  FOR I=1 TO B
350  B1=B1+D[I]
360  NEXT I
370  M3=B1/B
380  REM % of total bound in BLK.
390  MAT F=ZER[S*R]
400  FOR I=1 TO S*R
410  F[I]=((A[I,2]-M3)/(M2-M3))*100
420  NEXT I
430  MAT G=ZER[A]
440  FOR I=1 TO A
450  G[I]=((E[I]-M3)/(M2-M3))*100
460  NEXT I
470  REM Mean STND. values
480  MAT H=ZER[S,2]
490  X=0
500  FOR I=1 TO S*R STEP R
510  T2=0
520  X=X+1
```

```
530  FOR Z=I TO I+R-1
540  T2=T2+F[Z]
550  NEXT Z
560  H[X,1]=LOG(A[I,1])
570  H[X,2]=T2/R
580  NEXT I
590  REM Raw STND. curves
600  MAT I=ZER[S,2]
610  MAT J=ZER[S,2]
620  MAT K=ZER[S,2]
630  FOR I=1 TO S
640  I[I,1]=J[I,1]=K[I,1]=H[I,1]
650  NEXT I
660  X=0
670  FOR I=1 TO S*R STEP R
680  X=X+1
690  I[X,2]=F[I]
700  J[X,2]=F[I+1]
710  K[X,2]=F[I+2]
720  NEXT I
730  REM STND. curve formulae
740  MAT L=ZER[S,2]
750  MAT L=H
760  GOSUB 1080
770  B3=B2
780  A3=A2
790  R3=R2
800  S3=X4
810  T3=X1
820  Q3=Q2
830  MAT L=I
840  GOSUB 1080
850  B4=B2
860  A4=A2
870  R4=R2
880  S4=X4
890  T4=X1
900  Q4=Q2
910  MAT L=J
920  GOSUB 1080
930  B5=B2
940  A5=A2
950  R5=R2
960  S5=X4
970  T5=X1
980  Q5=Q2
990  MAT L=K
1000  GOSUB 1080
1010  B6=B2
1020  A6=A2
1030  R6=R2
1040  S6=X4
1050  T6=X1
1060  Q6=Q2
```

```
1070  GOTO 1300
1080  Y1=Y2=X1=X2=Z1=Z2=0
1090  FOR I=1 TO S
1100  Y1=Y1+L[I,2]
1110  Y2=Y2+(L[I,2] ** 2)
1120  X1=X1+L[I,1]
1130  X2=X2+(L[I,1] ** 2)
1140  Z2=Z2+(L[I,1]*L[I,2])
1150  NEXT I
1160  Z1=Y1*X1
1170  Y3=(Y1 ** 2)/S
1180  X3=(X1 ** 2)/S
1190  Z3=Z1/S
1200  Y4=Y2-Y3
1210  X4=X2-X3
1220  Z4=Z2-Z3
1230  B2=Z4/X4
1240  A2=(Y1/S)-(B2*(X1/S))
1250  R1=B2*Z4
1260  R2=SQR(R1/Y4)
1270  Q1=Y4-R1
1280  Q2=Q1/(S-2)
1290  RETURN
1300  REM Calculation of sample conc. using best curve
1310  IF R3>R4 THEN 1350
1320  IF R4>R5 THEN 1480
1330  IF R5>R6 THEN 1500
1340  GOTO 1660
1350  IF R3>R5 THEN 1380
1360  IF R5>R6 THEN 1500
1370  GOTO 1660
1380  IF R3>R6 THEN 1400
1390  GOTO 1660
1400  B7=B3
1410  A7=A3
1420  R7=R3
1430  S7=S3
1440  T7=T3
1450  Q7=Q3
1460  C$="MEAN      "
1470  GOTO 1730
1480  IF R4>R6 THEN 1580
1490  GOTO 1660
1500  B7=B5
1510  A7=A5
1520  R7=R5
1530  S7=S5
1540  T7=T5
1550  Q7=Q5
1560  C$="CURVE II "
1570  GOTO 1730
1580  B7=B4
1590  A7=A4
1600  R7=R4
```

```
1610  S7=S4
1620  T7=T4
1630  Q7=Q4
1640  C$="CURVE I   "
1650  GOTO 1730
1660  B7=B6
1670  A7=A6
1680  R7=R6
1690  S7=S6
1700  T7=T6
1710  Q7=Q6
1720  C$="CURVE III"
1730  MAT M=ZER[A]
1740  FOR I=1 TO A
1750  M[I]=(G[I]-A7)/B7
1760  NEXT I
1770  REM Confidence limits for sample values
1780  PRINT "ENTER VALUE OF T FOR N-2 =";S-2;"DEG. OF FREEDOM, P=0.
1790  INPUT T9
1800  MAT N=ZER[A]
1810  MAT O=ZER[A]
1820  D1=(T9*SQR(Q7))/B7
1830  D2=(D1 ** 2)/S7
1840  D3=1-(D2 ** 2)
1850  D6=(S+1)/S
1860  FOR I=1 TO A
1870  D4=M[I]-(T7/S)
1880  D5=(D4 ** 2)/S7
1890  D7=(D1*SQR((D6*D3)+D5))/D3
1900  N[I]=(T7/S)+(D4/D3)+D7
1910  O[I]=(T7/S)+(D4/D3)-D7
1920  NEXT I
1930  GOTO 2290
1940  REM Printout
1950  PRINT LIN(4)
1960  PRINT "STANDARD CURVE VALUES :"
1970  PRINT
1980  PRINT "% BOUND = [(STND. - BKG.) x 100] / [BLK. - BKG.]"
1990  PRINT
2000  PRINT "PROTEIN CONC.","CURVE I","CURVE II","CURVE III","MEAN
2010  PRINT "NG./100 MICROL.","% BOUND","% BOUND","% BOUND","% BOUN
2020  PRINT
2030  FOR I=1 TO S
2040  PRINT EXP(H[I,1]),I[I,2],J[I,2],K[I,2],H[I,2]
2050  NEXT I
2060  PRINT
2070  PRINT "TOTAL MEAN :";M1;"COUNTS"
2080  PRINT " BLK. MEAN :";M2;"COUNTS"
2090  PRINT " BKG. MEAN :";M3;"COUNTS"
2100  PRINT
2110  PRINT "CURVE FORMULAE :"
2120  PRINT
2130  PRINT "CURVE I   :";"Y =";B4;"X +";A4;"  .   R =";R4
2140  PRINT "CURVE II  :";"Y =";B5;"X +";A5;"  .   R =";R5
```

```
2150  PRINT "CURVE III :";"Y =";B6;"X +";A6;"  .   R =";R6
2160  PRINT
2170  PRINT "MEAN       :";"Y =";B3;"X +";A3;"  .   R =";R3
2180  PRINT
2190  PRINT "CURVE WITH GREATEST CORRELATION COEFFICIENT USED TO CA
2200  PRINT "PROTEIN CONCENTRATIONS :    ";C$
2210  PRINT
2220  PRINT "SAMPLE VALUES :"
2230  PRINT
2240  PRINT "RAW COUNTS","% BOUND","PROTEIN CONC.","    CONFIDENCE
2250  PRINT " "," ","MICROG./ML."
2260  FOR I=1 TO A
2270  PRINT E[I],G[I],(EXP(M[I]))/100,(EXP(N[I]))/100,(EXP(O[I]))/1
2280  NEXT I
2290  FOR I=1 TO A
2300  M[I]=(EXP(M[I]))/100
2310  NEXT I
2320  DIM Q$[6]
2330  PRINT "DO TREATED SAMPLES REQUIRE STORAGE : Y OR N."
2340  INPUT Q$
2350  IF Q$="N" THEN 2420
2360  CREATE Q1,"SAMPLE",10
2370  ASSIGN "SAMPLE",2,V2
2380  READ #2,1
2390  PRINT #2;A,R
2400  MAT  PRINT #2;M
2410  CHAIN "AVMGIN"
2420  END
```

ACV2IN

```
10  REM ************************************************
20  REM * INPUT OF DATA FOR ANCOV2                      *
30  REM * DATA ENTRY AS X-PANC. WT.;Y-T. CONTENT  *
40  REM ************************************************
50  FILES *,*
60  DIM A[8,10]
70  DIM A$[6]
80  PRINT "ENTER NO. OF TREATMENTS(PAIRS OF ROWS)"
90  INPUT T
100  PRINT "ENTER NO. OF REPLICATES(COLUMNS)"
110  INPUT R
120  MAT A=ZER[(2*T)+2,R+1]
130  V=0
140  FOR I=1 TO 2*T STEP 2
150  V=V+1
160  PRINT "FOR TREATMENT ";V;" :"
170  PRINT "ENTER X VALUES - INDEPENDENT VARIABLE"
180  FOR J=1 TO R
190  INPUT A[I,J]
200  NEXT J
210  PRINT "ENTER Y VALUES - DEPENDENT VARIABLE"
220  FOR J=1 TO R
230  INPUT A[I+1,J]
240  NEXT J
250  NEXT I
260  PRINT "FILE NAME FOR STORAGE ?"
270  INPUT A$
280  ASSIGN A$,1,V1
290  READ #1,1
300  PRINT #1;T,R
310  MAT  PRINT #1;A
320  END
```

ANCOV2

```
10  REM ************************************
20  REM * TWO WAY ANALYSIS OF COVARIANCE *
30  REM * DATA FROM ACV2IN               *
40  REM ************************************
50  PRINT "2 WAY ANALYSIS OF COVARIANCE"
60  FILES *,*
70  DIM A[8,100],B[5,9]
80  DIM A$[6]
90  GOTO 280
100  PRINT "ENTER NO. OF TREATMENTS(PAIRS OF ROWS)"
110  INPUT T
120  PRINT "ENTER NO. OF REPLICATES(COLUMNS)"
130  INPUT R
140  MAT A=ZER[(2*T)+2,R+1]
150  V=0
160  FOR I=1 TO T*2 STEP 2
170  V=V+1
180  PRINT "FOR TREATMENT ";V;" :"
190  PRINT "ENTER X VALUES - INDEPENDENT VARIABLE"
200  FOR J=1 TO R
210  INPUT A[I,J]
220  NEXT J
230  PRINT "ENTER Y VALUES - DEPENDENT VARIABLE"
240  FOR J=1 TO R
250  INPUT A[I+1,J]
260  NEXT J
270  NEXT I
280  PRINT "ENTER FILE FOR ANCOV"
290  INPUT A$
300  ASSIGN A$,1,V1
310  READ #1,1
320  READ #1;T,R
330  MAT A=ZER[(T*2)+2,R+1]
340  MAT  READ #1;A
350  REM TOTALS
360  X2=Y2=P1=0
370  FOR J=1 TO R
380  X1=Y1=0
390  FOR I=1 TO T*2 STEP 2
400  X1=X1+A[I,J]
410  Y1=Y1+A[I+1,J]
420  X2=X2+(A[I,J]^2)
430  Y2=Y2+(A[I+1,J]^2)
440  P1=P1+(A[I,J]*A[I+1,J])
450  NEXT I
460  A[(T*2)+1,J]=X1
470  A[(T*2)+2,J]=Y1
480  NEXT J
490  FOR I=1 TO (T*2)+2
500  T1=0
510  FOR J=1 TO R
520  T1=T1+A[I,J]
```

```
530  NEXT J
540  A[I,R+1]=T1
550  NEXT I
560  REM CORRECTION FACTOR
570  C1=(A[(T*2)+1,R+1]^2)/(T*R)
580  C2=(A[(T*2)+2,R+1]^2)/(T*R)
590  C3=(A[(T*2)+1,R+1]*A[(T*2)+2,R+1])/(T*R)
600  REM TOTAL SSx,SSy,SPxy
610  MAT B=CON
620  MAT B=(-1)*B
630  B[4,2]=X2-C1
640  B[4,3]=Y2-C2
650  B[4,4]=P1-C3
660  REM REPLICATE SSx,SSy,SPxy
670  R1=R2=R3=0
680  FOR J=1 TO R
690  R1=R1+(A[(T*2)+1,J]^2)
700  R2=R2+(A[(T*2)+2,J]^2)
710  R3=R3+(A[(T*2)+1,J]*A[(T*2)+2,J])
720  NEXT J
730  B[1,2]=(R1/T)-C1
740  B[1,3]=(R2/T)-C2
750  B[1,4]=(R3/T)-C3
760  REM TREATMENT SSx,SSy,SPxy
770  T1=T2=T3=0
780  FOR I=1 TO T*2 STEP 2
790  T1=T1+(A[I,R+1]^2)
800  T2=T2+(A[I+1,R+1]^2)
810  T3=T3+(A[I,R+1]*A[I+1,R+1])
820  NEXT I
830  B[2,2]=(T1/R)-C1
840  B[2,3]=(T2/R)-C2
850  B[2,4]=(T3/R)-C3
860  REM D.F.
870  B[1,1]=R-1
880  B[2,1]=T-1
890  B[4,1]=(T*R)-1
900  REM ERROR SSx,SSy,SPxy,D.F.
910  FOR J=1 TO 4
920  B[3,J]=B[4,J]-(B[1,J]+B[2,J])
930  NEXT J
940  REM LINEARITY CHECK
950  B[3,5]=(B[3,4]^2)/B[3,2]
960  B[3,6]=B[3,3]-B[3,5]
970  B[3,7]=B[3,1]-1
980  B[3,8]=B[3,6]/B[3,7]
990  B=B[3,4]/B[3,2]
1000  E=SQR(B[3,8]/B[3,2])
1010  T9=B/E
1020  GOTO 1060
1030  PRINT "ENTER VALUE OF T FOR ";B[3,7];" D.F.,P=0.05."
1040  INPUT TO
1050  IF T9<TO THEN 1270
1060  PRINT "B = ";B;" WITH S.E. ";E;" AND"
```

```
1070  PRINT "T = ";T9;" WITH ";B[3,7];" D.F."
1080  REM F-TEST
1090  FOR J=1 TO 4
1100  B[5,J]=B[2,J]+B[3,J]
1110  NEXT J
1120  B[5,5]=(B[5,4]^2)/B[5,2]
1130  B[5,6]=B[5,3]-B[5,5]
1140  B[2,6]=B[5,6]-B[3,6]
1150  B[5,7]=B[5,1]-1
1160  B[2,7]=B[5,7]-B[3,7]
1170  B[2,8]=B[2,6]/B[2,7]
1180  B[2,9]=B[2,8]/B[3,8]
1190  PRINT LIN(2)
1200  MAT  PRINT A
1210  PRINT LIN(2)
1220  PRINT "FOR TREATMENTS, ADJUSTED F FOR ";B[2,7];" AND ";B[3,7]
1230  PRINT LIN(3)
1240  MAT  PRINT  USING 1250;B
1250  IMAGE9(S4D.2DX)/
1260  GOTO 1280
1270  PRINT "NO LINEAR RELATIONSHIP, ANALYSIS OF COVARIANCE INVALID
1280  END
```

An in vivo study of the effect of Pilocarpine on the exocrine pancreas.

As was noted in the introduction section Pilocarpine is known to be a potent exocrine pancreatic secretagogue. In order to examine its effects in vivo under the experimental conditions used to measure pancreatic T. levels this study was made in collaboration with Mr. Paul Wickstead, an undergraduate project student.

The experimental mice used were treated in exactly the same way as for the pancreas time course experiments ('PANC : 19 to 22'). One mouse was used per time interval period after injection, seven mice for examining the effect of Pilocarpine injection and three as controls injected with the vehicle-phosphate buffered saline. The mice were each killed at different times, the Pilocarpine-treated mice 5, 10, 15, 20, 25, 30 and 60 minutes after injection and the saline-treated mice 5, 15 and 60 minutes after injection. The pancreas from these mice were then removed and cut longitudinally into two halves for fixation.

The one pancreas half was fixed and dehydrated for electron microscopy as outlined in the methods section. The other half was fixed for conventional light microscopy. The procedure for fixation, dehydration and wax embedding is tabulated below (Sumner and Sumner 1969, Drury and Wallington, 1980):

Solution in which tissue was immersed	Duration of immersion
1. 10% formal saline	4 days
2. 70% ethanol (4 changes)	4 days
3. Absolute ethanol (3 changes)	1 day
4. 50% ABS ethanol : 50% chloroform	1 hour
5. Chloroform	15 hours
6. 50% wax : 50% chloroform	1 hour
7. Wax (3 changes)	6 hours
8. Wax (embedding)	

Sections (5µm) from the embedded specimens were stained either with Ehrlich's Haemotoxylin and Eosin (Sumner and Sumner 1969) or cleared in

xylene and rehydrated. Counts were then made on the sections of the number of zymogen granules per pancreatic acinus. The H + E stained sections being observed at x 1,250 using conventional light microscopy and the cleared sections at x 1,250 using phase contrast microscopy.

The zymogen granule counts were made per acinus selected at random from a randomly selected slide. Thirty acini per pancreas were counted using phase contrast microscopy while ten were counted per pancreas using H + E staining. The mean counts with standard errors of the mean (SEM) are shown following :

Table A1 : Mean numbers of zymogen granules per acinus counted from H + E stained pancreas sections taken from mice under different secretory states. Means calculated from 10 acini.

Treatment	Period after injection (mins)	Zymogen granules per acinus Mean	S.E.M.
Saline :	5	86.63	9.46
	15	87.67	7.57
	60	80.42	6.49
Pilocarpine :	5	** 62.17	3.42
	10	58.83	5.96
	15	** 60.60	6.36
	20	72.33	5.02
	25	56.00	10.10
	30	43.57	12.13
	60	** 9.00	4.77

** Significant differences shown by paired t-tests.

Table A2 : Mean numbers of zymogen granules per acinus counted from xylene cleared and rehydrated pancreas sections taken from mice under different secretory states. Means calculated from 30 acini.

Treatment	Period after injection (mins)	Zymogen granules per acinus Mean	S.E.M.
Saline :	5	95.64	5.97
	15	90.85	6.15
	60	84.47	5.05
Pilocarpine :	5	** 62.43	2.62
	10	62.13	2.53
	15	** 65.88	1.86
	20	65.00	5.30
	25	33.39	4.03
	30	37.03	2.78
	60	** 30.76	2.83

** Significant differences shown by paired t-tests.

The results of this study and the accompanying photographs indicated that Pilocarpine injection induced the rapid and almost complete loss of zymogen granules from exocrine pancreatic acinar cells over the time period during which studies were made on the T. levels of the pancreas. The reduction in acini zymogen granules was most marked in the period from 20 to 60 minutes after Pilocarpine injection. This corresponds with the 10 to 60 minute post-injection period over which significant alterations were found in the MT-free T. equilibrium of the pancreas. Thus it seems that zymogen granule secretion in response to the Pilocarpine secretagogue was accompanied by alterations in the MT-free T. equilibrium of the acini. However the onset of rapid secretion seemed to lag about 10 minutes behind the commencement of alterations in the MT. state of the pancreas.

Photographs P34 to P38 : The effect of Pilocarpine on exocrine pancreatic granule content in vivo.

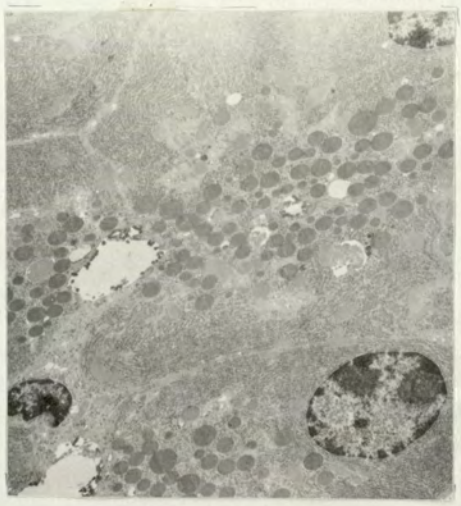
P34: Overall view of pancreatic acinar cells from an animal injected with saline alone.Note the abundance of dense zymogen granules near the apex of the cels and the large lumen with numerous microvilli.Compare with Figure 1.1;x 3.6K.

P35: Overall view of pancreatic acinar cells from an animal injected with Pilocarpine 60 minutes before sacrifice.Note the almost complete absence of zymogen granules around the lumen at the centre of the photograph; x 3.8K.

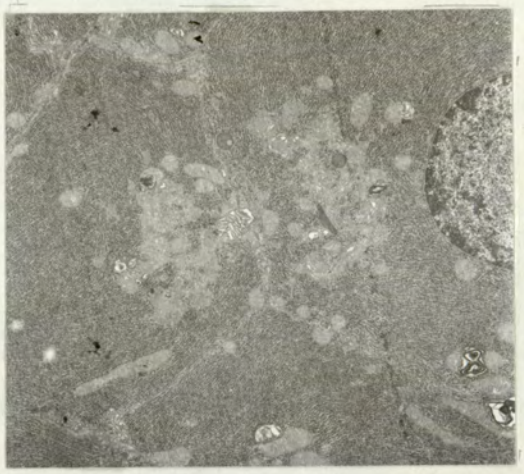
P36: Dense zymogen granules in the supranuclear Golgi zone of two pancreatic acinar cells;x 8.8K.

P37: Higher magnification of the area indicated in photograph P36.A MT. is seen in t.s. in close juxtaposition to a zymogen granule.A second MT. can be seen in the lower right-hand corner within the granular endoplasmic reticulum;x 40.6K.

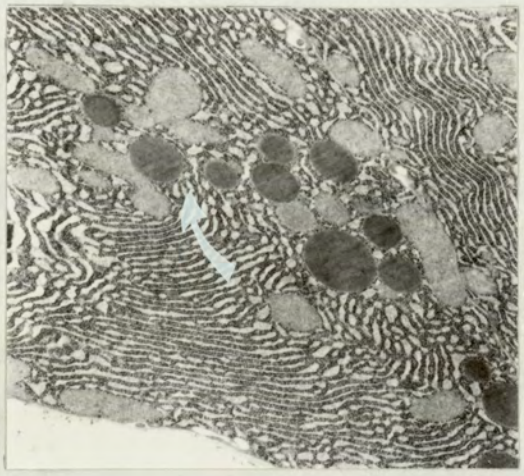
P38: A MT. seen in l.s. running between four closely positioned zymogen granules;x 88.2K.



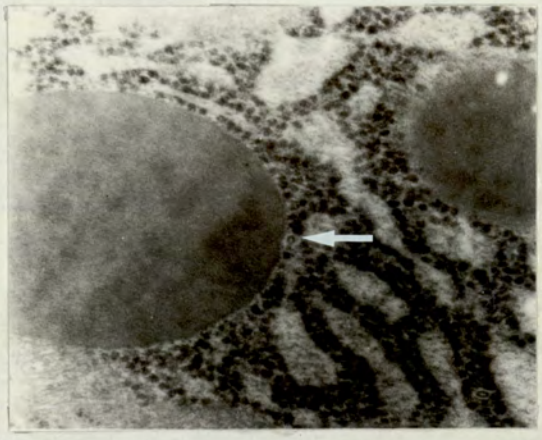
P34.



P35.



P36.



P37.

P38.

Examination of the validity of the ^{3}H-colchicine binding assay for T.

Before applying the ^{3}H-colchicine binding assay to a wide variety of applications involving the estimation of sample T. it was necessary to determine the variable parameters of the assay. This involved comparing the effects of sample dilution, concentrations of charcoal, BSA and glycerol and the time course for binding on the determined T. concentration. Each test was carried out in MT. isolation buffer.

1. Effect of sample dilution on the ^{3}H-colchicine binding assay :

A stock solution of 'two cycle' MT. proteins isolated as usual was diluted serially with MT. isolation buffer and then the serial dilutions assayed using the ^{3}H-colchicine binding assay according to the procedure outlined in the methods section. Each sample, blank and standard, contained 0.1% BSA and 4m glycerol. The incubation period with the ^{3}H-colchicine was 90 minutes at 37^{o}C, unbound colchicine being removed by 1 ml of a 2mg/ml charcoal suspension per sample for 10 minutes.

2. Effect of charcoal, BSA and glycerol concentrations on the ^{3}H-colchcine binding assay :

A serially diluted solution of BSA in MT. isolation buffer was mixed one to one with a standard MT. protein solution to produce a series of T. samples containing the same concentration of T. but BSA in the range 0.1 - 10%. These samples were then assayed using the ^{3}H-colchicine binding assay for T.

To examine the effect of sample glycerol concentration on the assay a stock MT. protein solution was diluted one to one with a series of glycerol solution in MT. isolation buffer of different concentrations. This produced a series of samples with the same T. concentration but glycerol concentrations in the range 2M - 18 M . These samples were then assayed for T. using the ^{3}H-colchicine binding assay.

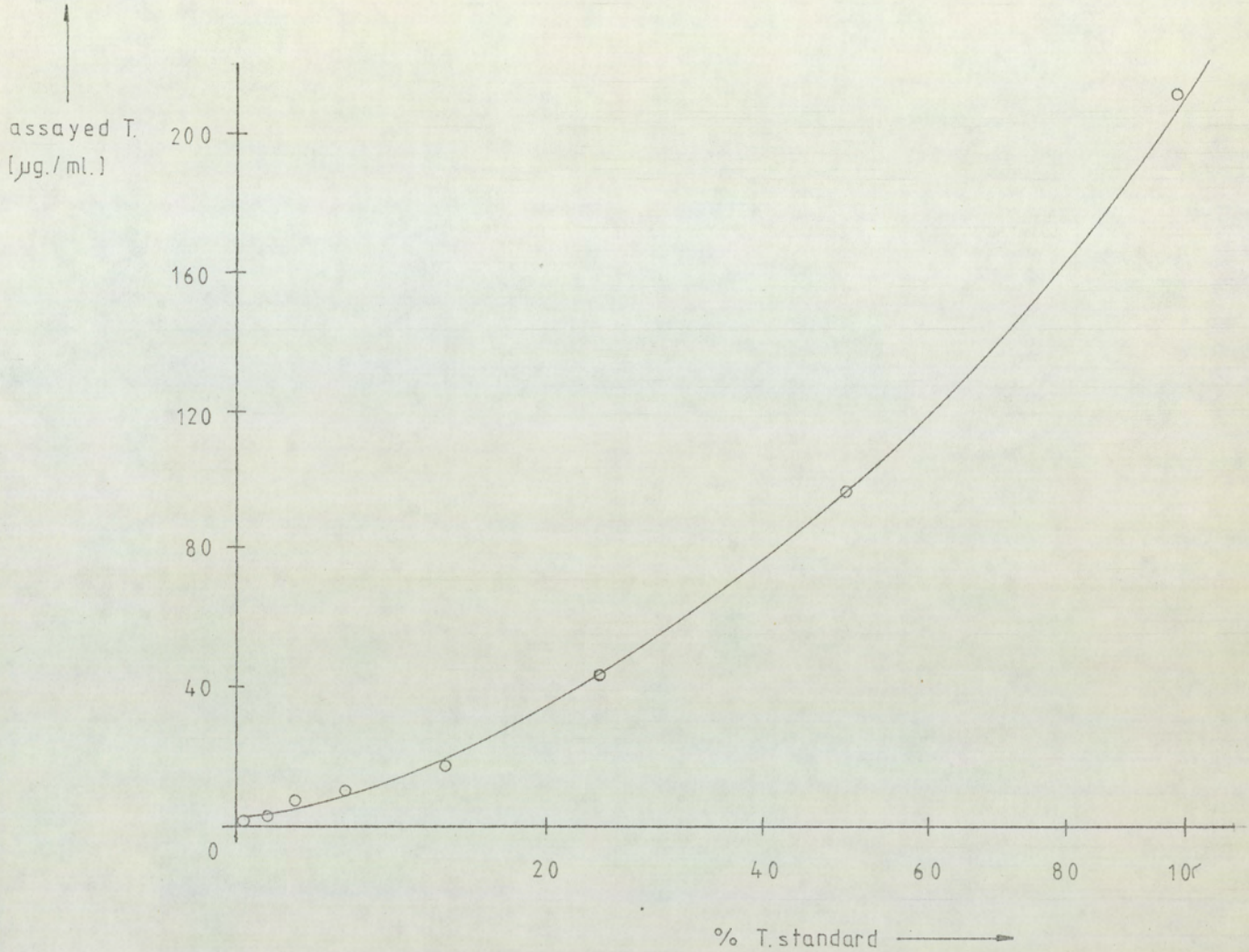


Figure A1 : The effect of sample dilution on the ^{3}H-colchicine binding assay for tubulin.

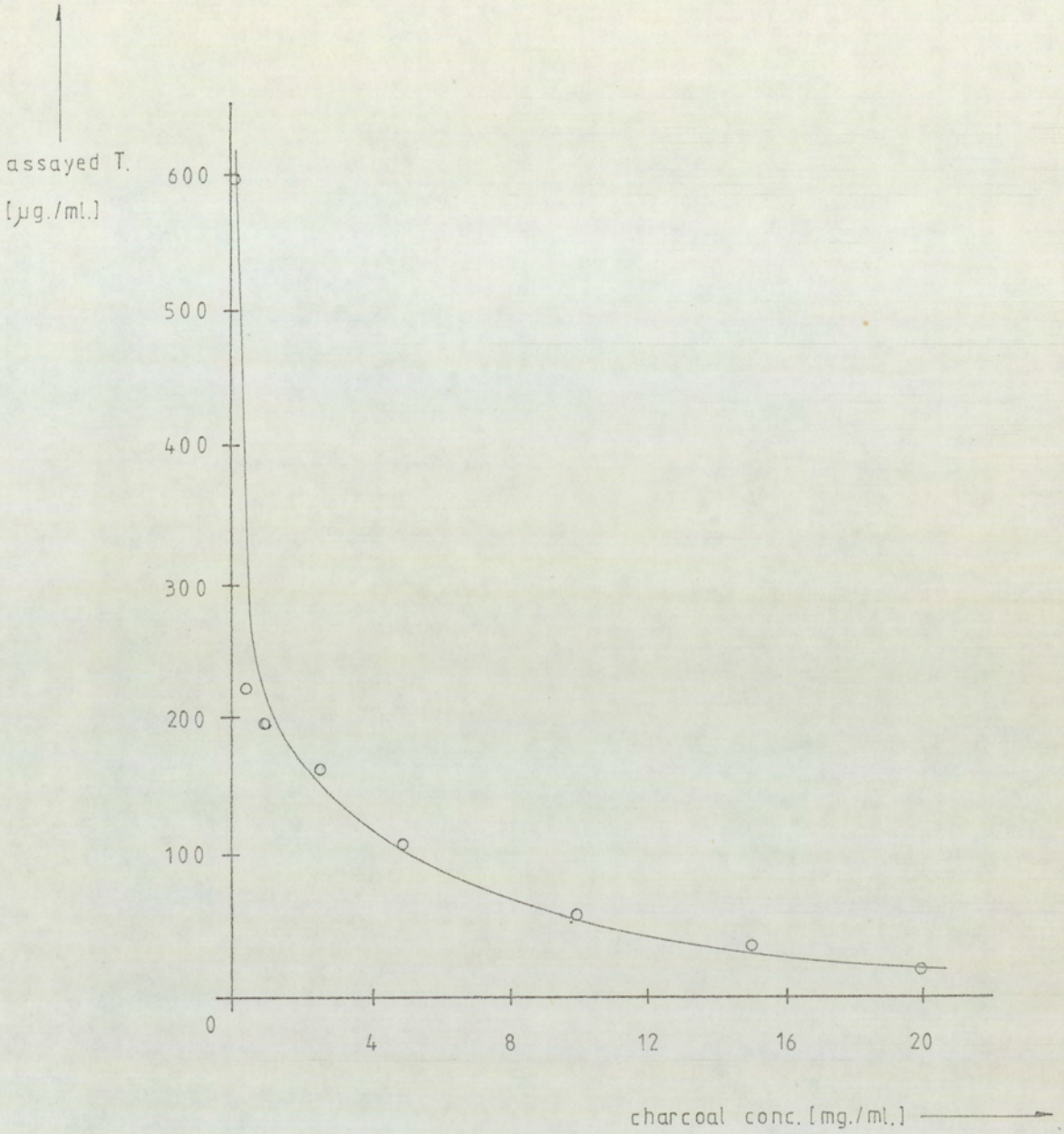


Figure A2 : The effect of charcoal concentration on the ^{3}H-colchicine binding assay for tubulin.

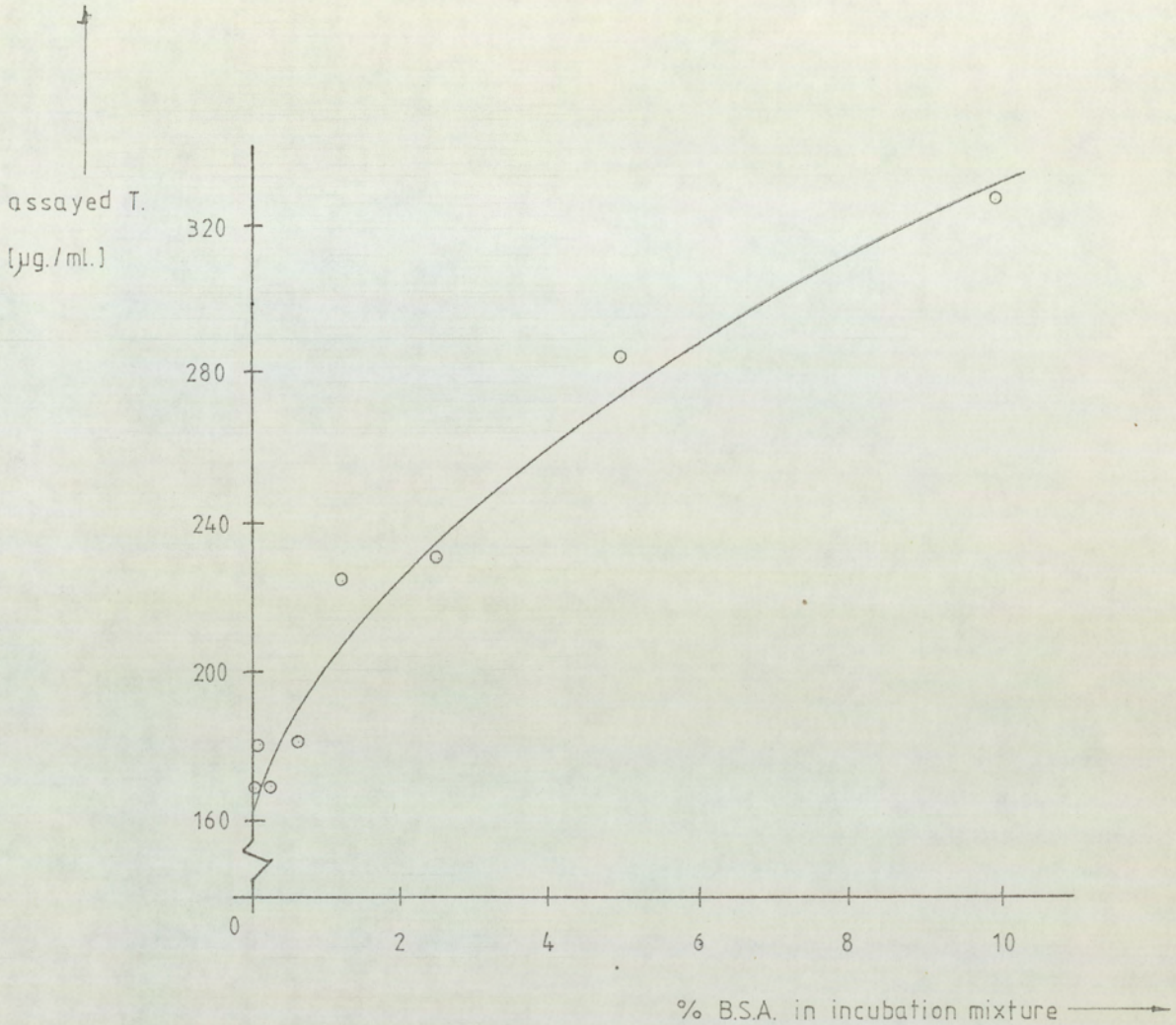


Figure A3 : The effect of Bovine serum albumin concentration on the ^{3}H-colchicine binding assay for tubulin.

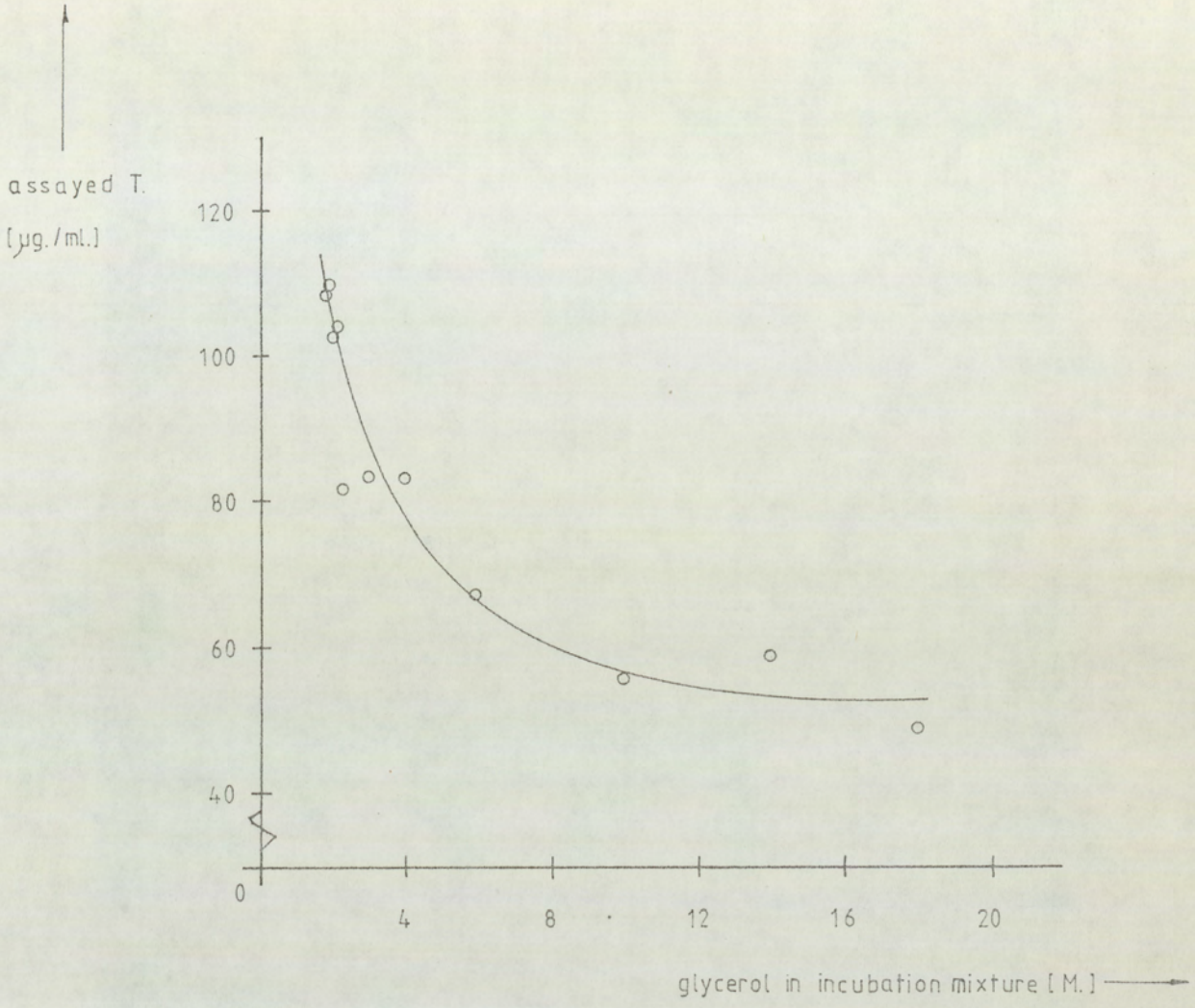


Figure A4 : The effect of glycerol concentration on the ^{3}H-colchicine binding assay for tubulin.

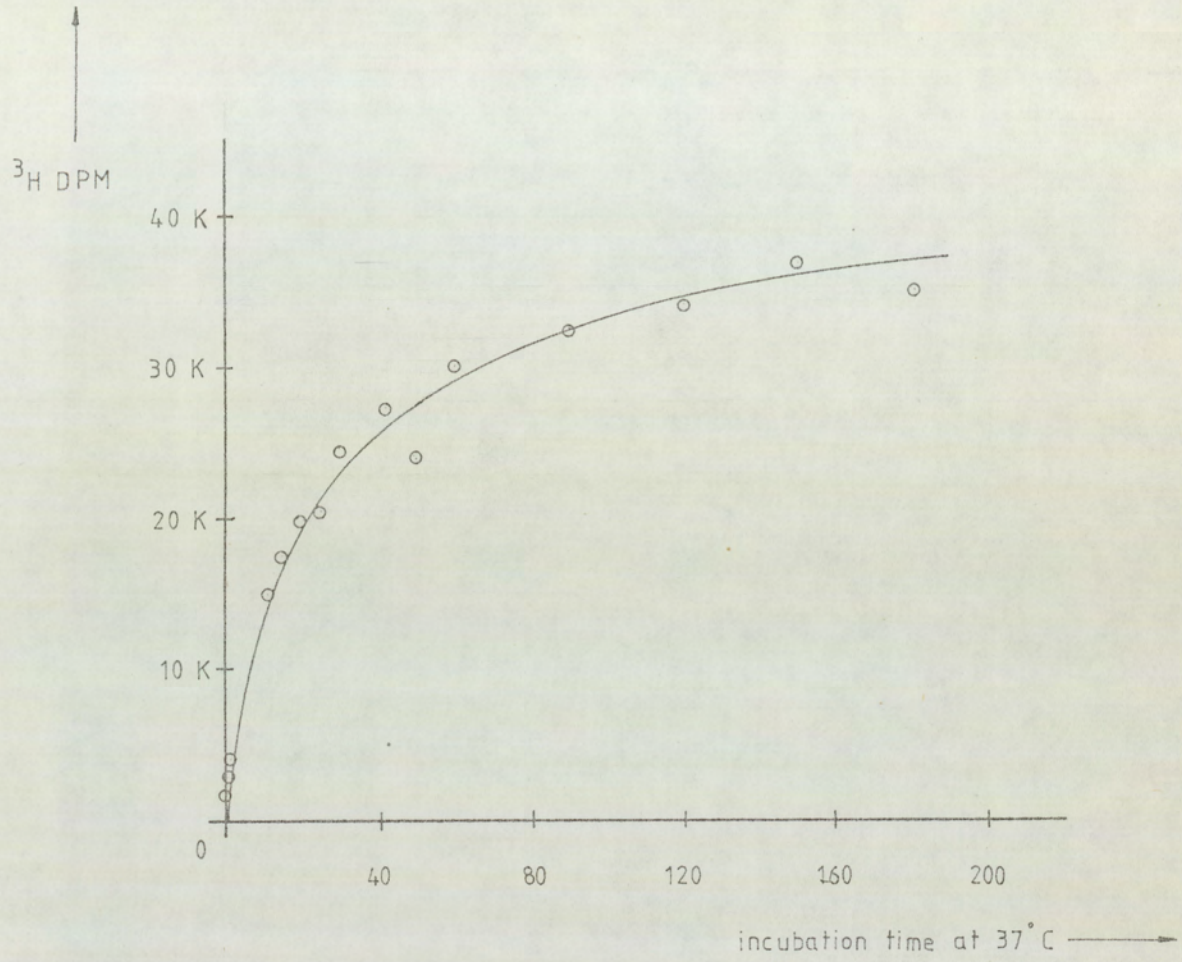


Figure A5 : The effect of incubation period on the ^{3}H-colchicine binding assay for tubulin.

The concentration of charcoal used to bind free colchicine was varied in the final test in this group. A range of charcoal suspensions from 0.1 to 20mg/ml were prepared and used to bind the free colchicine from a series of samples each containing the same T. concentration. All the samples in this section were assayed together, unless mentioned otherwise each contained 0.1% BSA and 4M glycerol and had their free colchicine removed by a 2mg/ml suspension of charcoal.

3. Effect of incubation time on the ^{3}H-colchicine binding assay :

A standard T. solution was duplicated 15 times and incubated with ^{3}H-colchicine at 37°C for different lengths of time. Each sample then had its free colchicine removed with a charcoal suspension (2mg/ml) and was scintillation counted in the usual manner. Every sample contained 0.1% BSA and 4M glycerol in addition to its T. content.

The validity tests presented in the section were used to determine the optimum conditions required for the ^{3}H-colchicine binding assay of T. These conditions were those already outlined in the standard procedures section.

Initial tests of radioimmunoassays - standard curves.

In this section are the standard R.I.A. curves produced during the initial tests of the R.I.A. for T. and M.A.P. These show the number of attempts necessary before a standard curve of sufficient quality was produced to measure unknown sample protein concentrations. Most of the problems associated with these curves were the result of incorrect antiserum dilution. The dilutions quoted for first antiserum (anti-T or anti-M.A.P.) are for initial dilution prior to adding 50µl of diluted antisera to each standard. Similarly the goat antirabbit serum (GARS) dilutions are for pre-addition dilutions, 200µl of this stock being added per standard.

Table A3 : R.I.A. standard curves for M.A.P; antiserum dilution

1 : 499 (GARS - 1 : 49)

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
800	238.216	186.674	157.599	194.163
400	77.9735	186.674	215.749	160.132
200	44.6035	222.357	73.0176	113.326
100	48.5683	89.207	-72.6872	21.696
50	182.379	127.863	141.079	150.441
25	136.123	34.6916	78.3039	83.0396
12.5	182.709	130.837	156.277	156.608
6.25	309.251	128.524	-28.7445	136.344
3.125	243.172	141.41	88.5462	157.709
1.5625	301.322	243.502	37.3348	194.053
.78125	311.564	180.727	207.159	233.15
.390625	183.7	183.7	201.872	189.758
.195312	192.291	138.766	110.352	147.137

Total mean : 47571 counts
BLK mean : 1826.33 counts
BKG mean : 2129 counts

Curve formulae :

Curve I : Y = -18.8176 X + 236.134 R = .54757 NS
Curve II : Y = -.911425 X + 155.758 R = 4.42465E-02 NS
Curve III : Y = 2.39117 X + 111.105 R = 7.25998E-02 NS
Mean : Y = -7.37342 X + 167.666 R = .368209 NS

Table A4 : R.I.A. standard curve for T.,antiserum dilution 1 : 499 (GARS - 1 : 19).

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
400	32.3529	85.2315	-180.816	-21.077
200	-38.683	25.3488	-41.0123	-18.1155
100	-67.6457	-2.08532	11.8299	-19.3004
50	-75.6689	38.0144	-49.6744	-29.1096
25	-94.8938	48.2092	-62.429	-36.3712
12.5	-166.73	-54.5959	-111.523	-110.95
6.25	-114.628	-62.9912	-92.1965	-89.9386
3.125	-78.3581	75.4654	111.582	36.2297
1.5625	159.41	213.279	-74.4315	99.419
.78125	-31.2705	103.866	-70.796	.599864
.390625	183.006	-37.943	184.502	109.855
.195312	-62.4897	-51.6277	-30.2554	-48.1243
9.76562E-02	-60.8519	-76.3119	-83.963	-73.709

Total mean : 470401 counts

BLK mean : 87935 counts

BKG mean : 63206.7 counts

Curve formulae :

Curve I : Y = -8.35906 X + -16.7161 R = .222809 NS

Curve II : Y = 6.65714 X + 11.174 R = .215525 NS

Curve III : Y = -11.5838 X + -16.4012 R = .32897 NS

Mean : Y = -4.42856 X + -7.31443 R = .182247 NS

Table A5 : R.I.A. standard curve for T.,antiserum dilution 1 : 249 (GARS - 1 : 19).

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
400	-1.97591	12.3053	13.6463	7.99192
200	23.1871	22.1877	25.7153	23.6967
100	42.8494	28.5169	19.3093	30.2252
50	49.9388	45.446	47.5301	47.6383
25	56.9769	65.5012	71.5913	64.6898
12.5	71.6596	83.9422	80.1156	78.5725
6.25	70.0538	80.6281	24.0754	58.2524
3.125	51.8179	13.467	69.8574	45.0474
1.5625	62.2555	86.3594	85.0952	77.9034
.78125	105.244	47.1543	21.3592	57.9193
.390625	-12.7295	86.8975	113.205	62.4577
.195312	104.988	80.4743	-15.7447	56.5726
9.76562E-02	68.4651	102.759	98.4796	89.9012

Total mean : 137059 counts
BLK mean : 19266 counts
BKG mean : 7558.33 counts

Curve formulae :

Curve I	: Y = -6.07175	X + 64.414	R = .467399	NS
Curve II	: Y = -8.27602	X + 73.2925	R = .709816	**
Curve III	: Y = -4.87916	X + 59.2672	R = .339911	NS
Mean	: Y = -6.40898	X + 65.6579	R = .748508	**

Table A6 : Standard curve for M.A.P, antiserum dilution 1 : 249 (GARS - 1 : 19).

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
800	-39.2157	75.4902	-9.80392	8.82353
400	-36.2745	3.92157	25.4902	-2.28758
200	-43.1373	-17.6471	57.8431	-.98039
100	2.94118	63.7255	28.4314	31.6994
50	66.6667	107.843	45.098	73.2026
25	10.7843	74.5098	17.6471	34.3137
12.5	89.2157	100.98	-103.922	28.7582
6.25	-118.627	156.863	47.0588	28.4314
3.125	14.7059	80.3922	68.6275	54.5752
1.5625	68.6275	17.6471	87.2549	57.8431
.78125	125.49	23.5294	57.8431	68.9542
.390625	70.5882	45.098	83.3333	66.3399
.195312	-3.92157	91.1765	87.2549	58.1699

Total mean : 9208.33 counts

BLK mean : 390 counts

BKG mean : 288 counts

Curve formulae :

Curve I : Y = -10.9733 X + 43.7035 R = .44295 NS

Curve II : Y = -2.80551 X + 70.4344 R = .157979 NS

Curve III : Y = -8.91388 X + 60.3723 R = .46703 NS

Mean : Y = -7.56423 X + 58.1701 R = .777096 **

Table A7 : R.I.A. standard curve for T., antiserum dilution 1 : 249 (GARS - 1 : 19).

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
2000	-3.23948	2.71276	1.9326	.468628
1000	.796008	4.32854	2.71672	2.61376
500	5.93244	15.738	10.2451	10.6385
250	11.552	7.18387	16.2013	11.6457
125	20.5576	21.314	31.7888	24.5535
62.5	23.3139	31.3691	43.4636	32.7155
31.25	30.3038	30.9295	49.796	37.0098
15.625	7.71851	43.1072	67.6528	39.4928
7.8125	42.6874	61.2807	84.9352	62.9678
3.90625	63.649	72.1397	62.2074	65.9987
1.95313	21.6506	90.5588	95.4259	69.2118
.976563	76.3059	73.1615	90.0994	79.8556
.488281	95.1012	85.2085	96.6061	92.3053

Total mean : 291148 counts

BLK mean : 30980 counts

BKG mean : 5729 counts

Curve formulae :

Curve I : Y = -9.63451 X + 63.649 R = .849405 ***

Curve II : Y = -11.2955 X + 80.3434 R = .964158 ***

Curve III : Y = -12.7954 X + 94.2783 R = .97154 ***

Mean : Y = -11.2418 X + 79.2436 R = .988846 ***

Table A8 : R.I.A. standard curve for M.A.P., antiserum dilution 1 : 249 (GARS - 1 : 19).

% Bound = ((Stnd - BKG) x 100) / (BLK - BKG)

Protein conc. NG/100 Microl	Curve I % Bound	Curve II % Bound	Curve III % Bound	Mean curve % Bound
4000.01	-7.1283	-23.6252	-29.7352	-20.1629
2000	-30.9572	-11.4053	21.5886	-6.92464
1000	-3.46232	-15.6823	-14.4603	-11.2016
500	22.8106	37.4745	12.4236	24.2363
250	46.0285	27.0876	60.0815	44.3992
125	64.3585	58.8595	70.4684	64.5621
62.5	79.6334	71.0795	81.4664	77.3931
31.25	103.462	82.6884	96.7413	94.2974
15.625	107.128	80.2444	99.7963	95.723
7.8125	101.018	71.6904	101.018	91.2424
3.90625	87.5764	97.9634	122.403	102.648
1.95313	110.183	95.5194	112.016	105.906
.976563	112.627	123.625	102.851	113.035

Total mean : 20842.7 counts

BLK mean : 377.333 counts

BKG mean : 213.667 counts

Curve formulae :

Curve I : Y = -17.3149 X + 132.621 R = .925334 ***

Curve II : Y = -16.6901 X + 122.518 R = .95339 ***

Curve III : Y = -17.1744 X + 135.378 R = .916417 ***

Mean : Y = -17.0598 X + 130.172 R = .953594 ***

Analysis of covariance of pancreas supernatant T. measurements.

The considerable variation between the pancreas T. levels of the experimental mice prompted the consideration of whether the level of T. measured was dependent on some other measurable factor. It appeared that the levels of polymerised and depolymerised pancreas T. bore little relationship to the sex or size of the mouse, its weight or litter origin. The only parameter which was readily available for each mouse was the weight of the removed pancreas before homogenisation. It was considered that although the tissue supernatants were corrected for this factor by extracting the T. in a constant ratio of tissue weight to volume of buffer, that somehow the initial weight affected the final assayed T. levels.

To investigate whether there was a relationship between the net weight of the removed pancreas and its T. content the pancreas weight for each mouse was compared with its mean polymerised and depolymerised T. content. This was done for the pancreas experiments 'PANC : 04 to 18' as a group and for the time course experiments 'PANC : 19 to 22' individually, in each case regardless of experimental treatments. The actual comparison was carried out by obtaining the correlation coefficient for the relationship between the pancreas weights and mean pancreas T. levels. Where a significant correlation coefficient was found, the experiments were reanalysed using the technique of analysis of covariance.

Analysis of covariance is a means whereby a measure of each item can be obtained before the experiment starts, which is closely related to the final measurement, and can be used to adjust the final measurements, thereby reducing the error variation. This makesuse of the regression of the final measurement (y) on the initial measurement (x) and decides

if the variation of the individual treatment means from the common regression line is greater than the variation of the individual values around the line after the effect of treatment has been removed. The actual analysis is carried out in a similar manner to regression analysis finding the S.S. for x, S.S. for y and the S.P. of x and y in the case of each source of variation. The two variables x and y were tabulated in their treatment groups and the corresponding treatment and replicate totals found for both the x and y variables. These values were then used in the same way as in a normal analysis of variance to find the S.S. for both the x and for the y variables. In addition the S.P. of x and y were also found, for example the total variation S.P. was found from :

$$S.P._{xy} = \sum xy - \frac{(\sum x)(\sum y)}{n}$$

where x and y were the individual variable and n was the total number of pairs of variables. For treatment variation the S.P. was found from :

$$S.P._{xy} = \frac{\sum T_x T_y}{r} - \frac{(\sum x)(\sum y)}{n}$$

where T_x and T_y were the treatment totals and r was the number of replicates making up each total.

Initially a test was made whether a linear relationship existed between the two variables by calculating the significance of b in the model $y = a + b(x - \bar{x})$. First the error S.S. for y due to regression was found from :

$$\frac{(\text{Error S.P.}_{xy})^2}{\text{Error S.S.}_x}$$, and this was used to find the S.S.

of the deviations from the regression :

$$\text{Error S.S.}_y - \frac{(\text{Error S.P.}_{xy})^2}{\text{Error S.S}_x} .$$

Since the S.S. of the regression has 1 D.F. this had the error D.F. less

this one and thus its M.S. could be found. This enabled a Student's t-test to be made on the significant of b using values from the error line of the analysis of covariance table :

$$b = \frac{S.P._{xy}}{S.S._{x}} \quad \text{with}$$

$$S.E._{b} = \sqrt{\frac{\text{Residual M.S.}}{S.S._{x}}} \quad \text{so that}$$

$$t = \frac{b}{S.E._{b}} .$$

Assuming this value of t was significant, so that b must be greater than zero (which it should have been if the initial correlation coefficient was correct), then the next step was to carry out an F-test of the treatment effects. To do this the S.S. for the residual variation when treatment effects were included were found together with S.S. for the residual effects when the treatment effects were excluded. The difference between these was the S.S. for the treatment effect after regression had been allowed for. The method involved creating another row in the covariance table containing S.S.x , $S.S._{y}$ and $S.P._{xy}$ for error and treatment.

Calculation of correlation coefficients for mouse pancreas weights against T. content.

1. Table A9 : Experiments 'PANC : 04' to 'PANC : 18' combined

Wet weight of pancreas before homogenisation :

Mouse No.	Pancreas weight (g)	Mouse No.	Pancreas weight (g)
P1	.175	S1	.095
P2	.147	S2	.085
P3	.056	S3	.052
P4	.145	S4	.089
P5	.127	S5	.085
P6	.143	S6	.098
P7	.170	S7	.203
P8	.193	S8	.202
P9	.264	S9	.199
P10	.125	S10	.146
P11	.153	S11	.217
P12	.142	S12	.136
P13	.099	S13	.116
P14	.092	S14	.098
P15	.159	S15	.125
P16	.128	S16	.116
P17	.069	S17	.082
P18	.116	S18	.084
P19	.079	S19	.079
P20	.101	S20	.072
P21	.119	S21	.086
P22	.130	S22	.062
P23	.097	S23	.096
P24	.119	S24	.123
P25	.181	S25	.099
P26	.156	S26	.093
P27	.119	S27	.088
P28	.132	S28	.110
P29	.121	S29	.163
P30	.149	S30	.121

Mouse No.	Pancreas weight (g)	Mouse No.	Pancreas weight (g)
P31	.177	S32	.164
P32	.208	S33	.133
P33	.117	S34	.066
P34	.147	S35	.114
P35	.190	S36	.113
P36	.116	S37	.113
P37	.067	S38	.077
P38	.100	S39	.071
P39	.106	S41	.108
P40	.089	S42	.102
P41	.110	S43	.093
P42	.139	S44	.160
P43	.142	S45	.112
P44	.175	S46	.036
P45	.087	S47	.118
P46	.166	S48	.123
P47	.182	S49	.092

The corresponding pancreas T. contents are shown in the results section.

Correlation coefficients for the relationship between pancreas wet weight and T. content :

Depolymerised T. (SN - I) - r = 0.07 with n = 94 ;
t = 0.67 with (n - 2) D.F. - NS.

Polymerised T. (SN - II) - r = 0.24 with n = 94 ;
t = 2.37 with (n - 2) D.F. - * .

2. Table A10 : Experiment 'PANC : 19' :

Wet weight of pancreas before homogenisation :

Time period (mins)	Replicate Mouse No. 1	2	3	4
10	.179	.124	.182	.161
20	.165	.144	.178	.203
30	.142	.134	.137	.138
45	.137	.103	.173	.156
60	.140	.139	.138	.187

The corresponding pancreas T. contents are shown in the results section.

Correlation coefficients for the relationship between pancreas wet weight and T. content :

Depolymerised T. - $r = -0.43$ with $n = 20$;
$t = 2.04$ with $(n - 2)$ D.F. - NS.

Polymerised T. - $r = 0.12$ with $n = 20$;
$t = 0.52$ with $(n - 2)$ D.F. - NS.

3. Table A11 : Experiment 'PANC : 20' :

Wet weight of pancreas before homogenisation :

Time period (mins)	Replicate Mouse No. 1.	2.	3.	4.
0	.210	.105	.143	.123
2.5	.105	.149	.140	.126
5	.154	.100	.115	.175
10	.137	.123	.101	.202
20	.152	.127	.126	.212

The corresponding pancreas T. contents are shown in the results section.

Correlation coefficients for the relationship between pancreas wet weight and T. content :

Depolymerised T. - $r = -0.45$ with $n = 20$;
$t = 2.16$ with $(n - 2)$ D.F. - *

Polymerised T. - $r = 0.31$ with $n = 20$;
$t = 1.36$ with $(n - 2)$ D.F. - NS.

4. Table A12 : Experiment 'PANC : 21' :

Wet weight of pancreas before homogenisation :

Treatment	Time period (mins)	Replicate Mouse No. 1.	2.	3.
	10	.103	.097	.058
	20	.093	.064	.104
Saline	30	.071	.093	.101
injection	45	.148	.121	.057
	60	.087	.093	.148
	10	.094	.105	.077
	20	.103	.118	.062
Pilocarpine	30	.119	.103	.065
injection	45	.110	.068	.094
	60	.092	.108	.080

The corresponding pancreas T. contents are shown in the results section.

Correlation coefficients for the relationship between pancreas wet weight and T. content :

Depolymerised T. - $r = -0.21$ with $n = 30$;
$t = 1.15$ with $(n - 2)$ D.F. - NS

Polymerised T. - $r = 0.04$ with $n = 30$;
$t = 0.22$ with $(n - 2)$ D.F. - NS.

5. Table A13 : Experiment 'PANC : 22' :

Wet weight of pancreas before homogenisation :

Time period (mins)	Replicate Mouse No. 1.	2.	3.
10	.140	.142	.155
20	.152	.143	.128
30	.135	.153	.117
45	.150	.141	.152
60	.187	.177	.120

The corresponding pancreas T. contents are shown in the results section.

Correlation coefficients for the relationship between pancreas wet weight and T. content :

Depolymerised T. - r = 0.64 with n = 15 ;
t = 3.01 with (n - 2) D.F. - **
Polymerised T. - r = 0.35 with n = 15; t= 1.60 - NS

These comparisons show that only in the case of measured polymerised pancreas T. was there a significant correlation between pancreas weight and T. content for the combined experiments 'PANC 04 to 18'. So far as the time course experiments are concerned a significant correlation was found for pancreas weight and depolymerised T. in both experiments 'PANC : 20' and 'PANC : 22'. These correlations were corrected for by reanalysis of the data using the analyses of covariance technique.

Analysis of covariance for experiments with a correlation between pancreas weight and T. content.

1. Table A14 : Experiments 'PANC : 04 to 18' combined; polymerised T :

Source of variation	D.F.	$S.S._x$	$S.S._y$	$S.P._{xy}$
Replicate mice	: 46	4494.68	1867.58	31.93
Treatments	: 1	97.20	2.42	15.33
Error	: 46	4501.82	1618.22	-62.41
Total	: 93	9093.70	3488.22	-15.15
Error and treatments	: 47	4599.03	1620.64	-47.07

Source of variation		$\frac{S.P._{xy}^2}{S.S._x}$	$S.S._y - \frac{S.P._{xy}^2}{S.S._x}$	D.F.	M.S.
Replicate mice	:	-	-	-	-
Treatments	:	0.87	2.80	1	2.80
Error	:	-	1617.36	45	35.94
Total	:	-	-	-	-
Error and treatments	:	0.48	1620.16	46	-

For treatments, adjusted F = 0.08 with $^1/_{45}$ D.F. - NS.

2. Table A15 : Experiment 'PANC : 20' ; depolymerised T :

Source of variation	D.F.	$S.S._x$	$S.S._y$	$S.P._{xy}$
Replicate mice	: 2	7.42×10^{-3}	11.10	-0.09
Time	: 4	1.36×10^{-3}	17.58	-0.05
Error	: 13	0.014	66.48	-0.53
Total	: 19	0.023	95.16	-0.67
Error and time	: 17	0.015	84.06	-0.58

Source of variation	$\frac{S.P._{xy}^2}{S.S._x}$	$S.S._y - \frac{S.P._{xy}^2}{S.S._x}$	D.F.	M.S.
Replicate mice	: -	-	-	-
Time	: -	15.21	4	3.80
Error	: 20.06	46.42	12	3.87
Total	: -	-	-	-
Error and time	: 22.43	61.63	16	-

For time, adjusted F = 0.98 with $^{4}/_{12}$ D.F. - NS.

3. Table A16 : Experiment 'PANC : 22' ; depolymerised T. :

Source of variation	D.F.	$S.S._x$	$S.S._y$	$S.P._{xy}$
Replicate mice	: 2	1.04×10^{-3}	8.69	0.05
Time	: 4	1.15×10^{-3}	72.48	0.11
Error	: 8	2.72×10^{-3}	153.83	0.53
Total	: 14	4.91×10^{-3}	235.00	0.69
Error and time	: 12	3.87×10^{-3}	226.31	0.64

Source of variation	$\frac{S.P._{xy}^2}{S.S._x}$	$S.S._y - \frac{S.P._{xy}^2}{S.S._x}$	D.F.	M.S.
Replicate mice	: -	-	-	-
Time	: -	69.91	4	17.48
Error	: 103.27	50.56	7	7.22
Total	: -	-	-	
Error and time	: 105.84	120.47	11	

For time, adjusted F = 2.42 with $^4/_7$ D.F. - NS.

In the case of each experiment with a significant correlation between pancreas weight and measured T. there was an increase in the treatment F value once the S.S. due to the regression was removed. However, the resultant increased M.S. was still not significant when compared with the new error M.S. calculated from the deviations from the regression. Thus although the pancreas weight probably had an effect on the final measured pancreas T. concentration it was not sufficiently great to influence the effect of treatment. The weight of pancreas probably had the greatest effect during the homogenisation procedure prior to separation of its polymerised and depolymerised T. Pancreas of low weight seemed to incur greater losses of material due to adhesion to the homogeniser mortar than those of greater weight. This was due to the very low buffer dilution factor necessary to provide the correct pancreas weight to volume ratio.

REFERENCES

Allen,R.D.(1975),J.Cell.Biol.,64,497-503.

Allison,A.C.and J.F.Nunn (1968),Lancet II, 1326-1329.

Alm,P.(1971),Acta Physiol.Scand., 83,269-277.

Amos,L.A.(1977), J.Cell Biol.,72,642-654.

Amos,L.A.and T.S.Baker (1979),Nature,279,607.

Anderson,P.J.(1979),J.Biol.Chem.,254,2168-2171.

Andrews,P.M.(1977),Amer.J.Anat.,150,53-62.

Atkins,T.W.,L.C.Best,P.R.Flatt,C.J.Bailey and A.J.Matty (1975), Gen.Pharmac.,6,43-47.

Aufderheide,K.J.(1979), J.Cell.Sci.,39,299-312.

Azhar,S.,and E.Reaven (1980),J.Cell Biol.,87,251a.

Babiss,L.E.,R.B.Lufty,J.A.Weatherbee,R.R.Weihing,U.R.Ray and B.N.Fields (1979),J.Virol.,30,863-882.

Bajer,A.(1968),In Soc.Exp.Biol."Aspects of Cell Mobility", Cambridge University Press,285-310.

Baker,T.A.and L.A.Amos (1978),J.Mol.Biol.,123,89-106.

Banerjee,A.C.and B.Bhattacharyya (1979),F.E.B.S. Lett.,99,333-336.

Banks,P.and R.Till (1975),J.Physiol.,252,283-294.

Bardeli,C.F.(1977),J.Cell Sci.,25,205-232.

Barnes,L.D.and G.M.Roberson (1979),Arch.Biochem.Biophys.,196,522-524.

Bartels,P.G.and J.L.Hilton (1973),Pestic.Biochem.Physiol.,3,462-472.

Baudnin,H.,C.Stock,D.Vincent and J.F.Grenier (1975),J.Cell Biol., 66,165-181.

Becker,J.S.,J.M.Oliver and R.D.Berlin (1975), Nature,254,152-154,

Beebe,D.C.(1979),Science,206,836-837.

Ben-Ze'er,A.,S.R.Farmer and S.Penman (1979),Cell,17,319-326.

Benz,L.,B.Eckstein,E.K.Matthews and J.A.Williams (1972), Br.J.Pharmac.,46,66-77.

Berg,N.B.and R.W.Young (1971),J.Cell Biol.,50,469-483.

Bergen,L.G.,and G.G.Borisy (1980),J.Cell Biol.,84,141-150.

Bergstrom -Porter,B.and E.Shelton (1979),Anat.Rec.,195,375-386.

Berridge M.J.(1975), In "Advances in cyclic nucleotide research" V6 (ed.P.Greengard and G.A.Robison), pp 1-98,Raven Press;N.Y.

Bershadsky,A.D.,V.I.Gelfand,T.M.Svitkina and I.S.Tint (1978), Cell Biol.Int.Rep.,2,425-432.

Bhisey,A.N.and J.J.Freed (1971),J.Cell Biol.,50,557-561.

Bibring,T.and J.Baxandall (1971),J.Cell Biol.,48,324-339.

Bibring,T.,J.Baxandall,S.Denslow and B.Walker (1976),J.Cell Biol., 69,301-312.

Bird,M.M.and A.R.Lieberman (1976),Cell Tiss.Res.,169,41-47.

Black,M.M.and J.L.Raymond (1980),J.Cell Biol.,86,616-623.

Blobel,G.and B.Dobberstein (1975a),J.Cell Biol.,67,835-851.

Blobel,G.and B.Dobberstein (1975b),J.Cell Biol.,67,852-862.

Blobel,G.and D.D.Sabatini (1970), N.Cell Biol.,45,130-145.

Bloodgood R.A.and K.R.Miller (1974), J.Cell Biol.,62,660-671.

Bonfils,S.,P.Fromageot and G.Rosselin (1977),(ed) "Hormonal receptors in digestive tract physiology." North Holland:Amsterdam.

Borgers,M.and M.De Brabander (1975).(ed.) "Microtubules and microtubule inhibitors." North Holland:Amsterdam,Elsevier:N.Y.

Borgers,M.and S.DeNollin (1975),J.Parasitol.,61,110-122.

Borisy,G.G.(1972),Anal.Biochem.,50,373-385.

Boxer,L.A.,B.Vanderbilt,S.Bonsib,R.Jersild,H-H.Ya ng and R.L.Baehner (1979), J.Cell Physiol.,100,119-126.

Boxer,L.A.,M.Yoder,S.Bonsid,M.Schmidt,P.Ho,R.Jersild and R.L.Baehner (1979), J.Lab.Clin.Med.,93,506-514.

Brim ijo in,S.,J.Olsen and R.Rosenson (1979), J.Physiol.,287,303-314.

Brinkley,B.R.,G.M.Fuller and D.P.Highfield (1975),In "MT.and MT.inhibitors." (M.Borgers and M.De Brabander) Elsevier:N.Y.

Brinkley,B.R.and P.N.Rao (1973), J.Cell Biol.,58,96-106.

Brown,D.L. and G.B.Bouck (1974), J.Cell Biol.,61,514-536.

Bryan,J.(1976), J.Cell Biol.,71,749-767.

Bryan,J., G.A.Cutler and M.Hayashi (1978),Nature,272,81-82.

Bulinski,J.C. and G.G.Borisy (1979),Prac.Nat.Acad.Sci.U.S.,76,293-297.

Bulinski,J.C.and G.G.Borisy (1980a),J.Cell Biol.,87,792-801.

Bulinski,J.C.and G.G.Borisy (1980b),J.Cell Biol.,87,802-808.

Burchill,B.R.et al (1978),J.Cell Biol.,76,439-447.

Burns,R.(1978),Nature,273,709-710.

Burton,P.R.and H.L.Fernandez (1973),J.Cell Sci.,12,567.

Burton,P.R.and R.H.Himes (1978),J.Cell Biol.,77,120-133.

Burton,P.R.,R.E.Hinckley and G.B.Pierson (1975), J.Cell Biol.,65,227-233.

Butcher,F.R.and R.H.Goldman (1972),Biochem.Biophys.Res.Commun.48,23-27.

Byers,M.R.,B.R.Fink,R.D.Kennedy,M.E.Middaugh and A.E.Hendrickson (1973), J.Neurobiol.,4,125-144.

Campbell,P.N.and G.Blobel (1976),F.E.B.S. Lett.,72,215-226.

Caro,L.G.and G.E.Palade (1964),J.Cell Biol.,20,473-495.

Caron,J.M.and R.D.Berlin (1979),J.Cell Biol.,81,665-671.

Caron,J.M.and R.D.Berlin (1980),J.Cell Biol.,87,255a.

Case,R.M.(1978),Biol.Revs.,53,211-354.

Castle,A.G.and N.Crawford (1978a),Thromb.Haemost.,39,386-403.

Castle,A.G.and N.Crawford (1978b),Int.J.Biochem.,9,439

Ceccarelli,B.,F.Clemente and J.Meldolesi (1975),J.Physiol.,245,617-638.

Center,D.M.,S.E.Wasserman and K.F.Austen (1978),Cell Immunol.,39,325-335.

Chaldakov,G.N.,S.Nikolov and V.Vancov (1977),Acta.Morpho.,25,167-176.

Chalfie,M.and J.N.Thomson (1979),J.CellBiol.,82,278-289.

Chambant-Guerin,A-M.,P.Muller and B.Lossignol (1978),J.Biol.Chem., 253,3870-3876.

Chan,K.Y.and A.H.Bunt (1978),J.Neurocytol.,7,137-144.

Chertow,B.S.,G.A.Williams,G.R.Baker,R.D.Surbaugh and G.K.Hargis (1975a),Exp.Cell Res.,93,388-394.

Chertow,B.S.,R.J.Bu hmann and W.J.Henderson (1975b),B.Lab.Invest., 32,190-200.

Chiraseveenuprapund ,P.and I.N.Rosenberg (1974),Endocrin.,94,1086-1093.

Chu,L.L.H.,R.C.MacGregor and D.V.Cohn (1977),J.Ecll Biol.,72,1-10.

Claude,A.(1970),J.Cell Biol.,47,745-766.

*Cochran,W.G.and G.M.Cox (1957),"Experimental Designs", J.Wiley and Sons Inc.

Collis,P.S.and D.P.Weeks (1978),Science,202,440.

Connolly,J.A.,V.I.Kalnins,D.W.Cleveland and M.W.Kirschner (1975), J.Cell Biol.,76,781-786.

Corces,V.G.,J.Solas,M.L.Solas and J.Avila (1978),Eur.J.Biochem., 86,473-480.

Coughlin,B.A.,H.D.White and D.L.Purich (1980),Biochem.Biophys.Res. Commun.,92,89-94.

Cronly-Dillon,J.and G.W.Perry (1979),J.Physiol.,293,469-484.

Curry,A.and R.D.Butler (1976),J.Ultrastrue.Res.,56,164-176.

Dahl,J.L.and V.J.Weibel (1979),Biochem.Biophys.Res.Commun.,86,822-828.

Daleo,G.R.,M.M.Piras and R.Piras (1977),Arch.Biochem.Biophys., 180,288-297.

Dales,S.(1963),Proc.Nat.Acad.Sci.U.S.,50,268-275.

Dales,S.(1975),Ann.N.Y.Acad.Sci.,253,440-444.

Dales,S.and Y.Chardonnet (1973),Virology,56,465-483.

David-Pfeuty,T., H.P.Erickson and D.Pantaloni (1977),Proc.Nat. Acad.Sci.U.S.,74,5372-5376.

Davidse,L.C.(1973),Pestic.Biochem.Physiol.,3,317-325.

Davidse,L.C.(1975),In "MT and MT inhibitors" (Borgers M.and M.De Brabander - eds.), North Holland,483-495.

Davidse,L.C.and W.Flech (1977),J.Cell Biol.,72,173-193.

Davis,B.J.(1964),"Disc.electrophoresis" Ann.N.Y.Acad.Sci.,121,404-427.

Davis,B.J.and N.R.Lazarus (1976),J.Physiol.,256,709-729.

De Brabander,M.,F.Aerts,G.Geuens,R.van Ginckel,R.Van de Veire and H.van Belle (1978),Chemico-biol.Interact.,23,45-64.

*- for Cleveland et al see end of references.

De Brabander,M.,J.De Mey,M.Johnian and S.Geuens (1977), J.Cell Sci.,28,283-302.

De Brabander,R.Van de Veire,F.Aerts,G.Geuens,M.Borgers,L.Deplenter and J.De Cree (1977),In'Microtubule and Microtubule Inhibitors' (Borgers,M. and M.De Brabander - eds.), North Holland,509-521.

Deitch,A.D. and S.G.Sawicki (1979),Exp.Cell Res.,118,1-14.

Delacourte,A.,M.Plancet,K.Han,H.H ildebrand and G.Biserte (1977), F.E.B.S.Lett.,77,41-46.

Dentler,W.L.,S.Granett and J.L.Rosenbaum (1975),J.Cell Biol.,65,237-241.

Delahurty,T.J. and J.M.Johnston (1976),J.Lipid Res.,17,112-116.

Detrich,H.W. and R.C.Williams (1978),Biochem.,17,3900-3907.

Devis,G.,E.Van Obbergh^{e}n,G.Somers,F.Malaisse-Lagae,L.Orci and W.J. Malaisse (1974),Diabetologia,10,53-59.

Dieg^{g}elman,R.F., and B.Peterkofsky (1972),Proc.Nat.Acad.Sci.U.S.,69, 892-896.

Donoso,J.A.,K.M.Haskins and R.H.Himes (1979),Cancer Res.,39,1604-1610.

Douglas,W.W. (1974),Biochem.Soc.Symp.,39,1-28.

Dousa,T.P.,Y.S.F.Hui and L.D.Barnes (1978),J.Lab.Clin.Med.,92,252-261.

Drury R.A.B. and H.M.Wallington (1980),'Carletons Histological Technique' 5th edn.,Oxford University Press.

Dustin,P. (1947),Nature,159,794.

Dustin,P. (1949),Exp.Cell Res.Suppl.,1,153-155.

Dustin,P. (1978),'MICROTUBULES',Springer-Verlag:N.Y.

Eckert,B.S. and J.A.Snyder (1978),Proc.Nat.Acad.Sci.U.S.,75,334-338.

Edstrom,A.,H.-A.Hansson,H.Larsson and M.Wallin (1978),Cell Tissue Res., 162,35-48.

Eichhorn,J.H. and B.Peterkofsky (1979),J.Cell Biol.,82,572-576.

Erickson,H.P. (1976),In 'Cell motility'(Goldman,R.,T.Pollard and J. Rosenbaum-eds.),Cold Spring Harbour,1069-1080.

Erickson,H.P. and W.A.Voter (1976),Proc.Nat.Acad.Sci.U.S.,73,2813-2817.

Ericson,L.E. and I.Lundquist (1975),Diabetologia,11,467-473.

Ermak,T.H. and S.S.Rothman (1978),J.Ultrastrut.Res.,64,98-113.

Fairbanks,G.,T.L.Steck and D.F.H.Wallach (1971),Biochem.,10,2606-2617.

Farr,R.M.,V.Lucknow and G.Sunderharadas (1979),Exp.Cell Res.,121, 428-432.

Farrell,K.W.,J.A.Kassis and L.Wilson (1979),Biochem.,18,2642-2647.

Fisher,R.A. and F.Yates (1963),'Statistical Tables',Oliver and Boyd,6th edn.

Forgue,S.T. and J.L.Dahl (1979),J.Neurochem.,32,1015-1026.

Formby,B. and K.Capito (1977),Diabetologia,13,377-442.

Francon,J.,A.Fellous,A.M.Lennon and J.Nunez (1978),Eur.J.Biochem., 85,43-54.

Frankel,F.R.,R.W.Tucker,J.Bruce and R.Stenberg (1978),J.Cell Biol., 79,401-408.

Freed,J.J. and M.M.Lebowitz (1970),J.Cell Biol.,45,334-354.

Friedman,P.A. and E.G.Platzer (1978),Biochim.Biophys.Acta.,544, 605-614.

Fuchs,R. and M.Peterlik (1979),F.E.B.S.Lett.,100,357-359.

Fujii,T. and R.Tanaka (1979),Life Sci.,24,1683-1690.

Fujiwara,K. and T.D.Pollard (1978),J.Cell Biol.,77,182-195.

Gabianni,G.,F.Malaisse-Lagae,B.Blondel and L.Orci (1974),Endocrinology, 95,1630-1635.

Geahlen,R.L. and B.E.Haley (1977),Proc.Nat.Acad.Sci.U.S.,74,4375-4377.

Gemmel,R.T. and B.D.Stacy (1977),J.Reprod.Fert.,49,115-117.

Geuze,J.J. and M.F.Kramer (1974),Cell Tiss.Res.,156,1-20.

Giesel,M.,H.Fasold and W.Haase (1978),F.E.B.S.Lett.,92,45-48.

Gillespie,E. and L.M.Lichtenstein (1972),J.Clin.Invest.,51,2941-2947.

Glees,P. and P.E.Spoerri (1977a),J.Neurocytol.,6,353-354.

Glees,P. and P.E.Spoerri (1977b),Anals.Anat.,25,159-168.

Godula,J. (1979),J.Ultrastruct.Res.,68,209-219.

Goldman,I.D. (1977),Biochim.Biophys.Acta.,467,185-191.

Goldstein,M.A. and M.L.Entman (1979),J.Cell Biol.,80,183-195.

Gonatas,N.K.,G.Margolis and L.Kilham (1971),Lab.Invest.,24,101-109.

Gozes,I and U.Z.Littauer (1979),F.E.B.S.Lett.,99,86-90.

Grainger,F. and J.C.Sloper (1976),Cell Tiss.Res.,169,405-444.

Gratzl,M. and D.Schwab (1976),Cytobiologie,13,199-210.

Gray,E.G. and L.E.Westrum (1976),Cell Tiss.Res.,168,445-453.

Gray,E.G. and L.E.Westrum (1979),Cell Tiss.Res.,199,281-288.

Greenwood,F.C.,W.M.Hunter and J.S.Glover (1963),Biochem.,89,114.

Gregory,D.A.,Z.R.Valicevii,M.F.Pugh and L.Swell (1978),Gastroenterology, 74,93-100.

Grenier,G.,J.Van Sande,C.Willems,P.Neve and J.E.Dumont (1975),Biochemie, 57,337-342.

Griffin,L. and T.Pollard (1978a),Biophys.J.,24,23a.

Griffin,L. and T.Pollard (1978b),J.Cell Biol.,78,958-965.

Grill,V. and E.Cerasi (1977),Biochem.Biophys.Acta.,500,385-394.

Hadjian,A.J.,C.Guidicelli and E.M.Chambaz (1977),F.E.B.S.Lett.,77,233 -238.

Hall,A.K. and J.Robinson (1978),J.Endocrinol.,79,42-43.

Haschke,R.H.,M.Byers,B.R.Fink (1974),J.Neurochem.,22,837-844.

Hayashi,M. (1979),J.Biochem.(Tokyo),85,691-698.

Hayat,M.A. (1970),'Principles and Techniques of Electron Microscopy',V1, Van Nostrand Reinhold Co.

Heath,I.B. and M.C.Heath (1978),Cytobiologie,16,393-411.

Hegyenex,M.H.,M.Simon and S.J.Singer (1978),Proc.Nat.Acad.Sci.U.S. 75,3863-3866.

Herzog,W. and K.Weber (1977),Proc.Nat.Acad.Sci.U.S.,74,1860-1864.

Heuser,J.E. and M.W.Kirschner (1980),J.Cell Biol.,86,212-234.

Hiller,G. and K.Weber (1978),Cell,14,795-804.

Himes,R.H.,P.R.Burton,R.W.Kersey and G.B.Pierson (1976),Proc.Nat.Acad. Sci.U.S.,73,4397-4399.

Himes,R.H. and L.L.Houston (1976),J.Supramol.Struct.,5,55-64.

Hinz,H.-J.,M.J.Gorbunoff,B.Price and S.N.Timasheff (1979),Biochem., 18,3084-3088.

Hitchen,E.T. and R.D.Butler (1973),Z.Zellforsch.,144,37-57.

Hitchen,E.T. and R.D.Butler (1974),J.Ultrastruct.Res.,46,279-295.

Hoffstein,S. and G.Weissmann (1978),J.Cell Biol.,78,769-781.

Holmes,K.V. and P.W.Choppin (1968),J.Cell Biol.,39,526-543.

Holze,S.andJ.Beckmann (1977),Diabetologia,13,377-442.

Howell,S.L. (1974),In 'Cytopharmacology of Secretion'(Ceccarelli,B., J.Meldolesi and F.Clementi - eds.),Raven Press:N.Y.,319-327.

Howell,S.L. and M.Tyhurst (1978a),Cell Tiss.Res.,190,163-172.

Howell,S.L. and M.Tyhurst (1978b),Diabetologia,15,213-283.

Huang,B. and D.R.Pitelka (1973),J.Cell Biol.,57,704-728.

Hyams,J.S. and H.Stebbings (1979),Cell Tiss.Res.,196,103-116.

Ireland,C.M.,K.Gull,W.E.Gutteridge and C.I.Pogson (1979),Biochem. Pharmacol.,28,2680-2681.

Iyenger,R.,K.G.Lepper and D.S.Mailman (1976),J.Supra.Strut.,5,521-530.

Izant,J.G. and J.R.McIntosh (1977),J.Cell Biol.,75,263a.

Jackson,W.T. (1969),J.Cell Sci.,5,745-755.

Jacobs,M. (1975),Ann.N.Y.Acad.Sci.,253,562-572.

Jamieson,J.D. and G.E.Palade (1967a),J.Cell Biol.,34,577-596.

Jamieson,J.D. and G.E.Palade (1967b),J.Cell Biol.,34,597-615.

Jamieson,J.D. and G.E.Palade (1971),J.Cell Biol.,50,135-158.

Jarlfors,U. and D.S.Smith (1969),Nature,224,710-711.

Johnson,K.A. and G.G.Borisy (1979),J.Mol.Biol.,133,199-216.

Jones,C.W. (1975),J.Cell Sci.,18,133-156.

Kachadorian,W.A.,S.J.Ellis and J.Muller (1979),Amer.J.Physiol.,236, F14-F20.

Kaliner,M. (1977),J.Clin.Invest.,60,951-959.

Karr,T.L.,A.E.Podrasky and D.L.Purich (1979),Proc.Nat.Acad.Sci.U.S., 76,5475-5479.

Karr,T.L. and D.L.Purich (1979),J.Biol.Chem.,254,10885-10888.

Karr,T.L.,H.D.White and D.L.Purich (1979),J.Biol.Chem.,254,6107-6111.

Karsenti,E.,B.Guilbert,M.Bornens,S.Avrameas,R.Whalen and D.Pantaloni (1978),Histochem.Cytochem.,26,934-947.

Kasbeker,D.S. (1978),Acta.Physiol.Scand.,103,253-266.

Keith,C.H.,J.R.Feramisco and M.Shelanski (1981),J.Cell Biol.,88,234-240.

Kemper,B.,J.F.Habener,A.Rich and J.T.Potts (1975),Endocrinology,96, 903-912.

Kennedy,M.S. and P.A.Insel (1979),Mol.Pharmacol.,16,215-223.

Kenney,D.M. and F.C.Chao (1978),J.Cell Physiol.,96,43-52.

Kern,H.F. (1975),Cell Tiss.Res.,164,261-269.

Khar,A.,J.Kunert-Radek and M.Jutisz (1979),F.E.B.S.Lett.,104,410-414.

Kim,H.,L.I.Binder and J.L.Rosenbaum (1979),J.Cell Biol.,80,266-276.

Kim,K.S. and J.P.Fulton (1975),Virology,64,560-565.

Kirschner,M.W. (1980),J.Cell Biol.,86,330-334.

Kirschner,M.W.,R.C.Williams,M.Weingart and J.C.Gerhart (1974),Proc.Nat. Acad.Sci.U.S.,71,1159-1163.

Kirshner,N.,D.Wallach,Y.Sharoni and M.Schramm (1973),Anal.Biochem.,52, 589-594.

Klein,I.,M.Willingham and I.Pastan (1978),Exp.Cell Res.,114,229-238.

Kobayashi,Y and H.Mohri (1977),J.Mol.Biol.,116,613-618.

Koelle,G.(1970),In'The Pharmacological Basis of Therapeutics'(Goodman, L.S. and A.Gilman - eds.),Macmillan,472-475.

Koenig,H.(1974),In 'Cytopharmacology of Secretion'(Ceccarelli,B.,J. Meldolesi and F.Clementi),Raven Press:N.Y.,273-301.

Kramer,M.F. and Geuse,J.J.(1974),In 'Cytopharmacology of Secretion' (Ceccarelli,B.,J.Meldolesi and F.Clementi - eds.),Raven Press: N.Y.,87-97.

Kramer,M.F. and C.Poort (1968),Z.Zellforsch.mikrosk.Anat.,86,475-486.

Kumagai,H. and E,Nishida (1979),J.Biochem.(Tokyo),85,1267-1274.

Kupchan,S.M.,Y.Komoda,W.A.Court,G.J.Thomas,R.M.Smith,A.karin,C.J.Gilmore, R.C.Haltiwanger and R.F.Bryan (1972),J.Am.Chem.Soc.,94,1354-1356.

Kuznetsov,S.A.,V.I.Rodinov,V.I.Gelfand and V.A.Rosenblat (1978),F.E.B.S. Lett.,95,339-342.

Kuriyama,R. (1977),J.Biochem.(Tokyo),81,1115-1126.

Lacy,P.E. (1970),Diabetes,19,895-905.

Lacy,P.E.,S.L.Howell,D.A.Young and C.J.Fink (1968),Nature,219,1177-1179.

Lacy,P.E.,M.M.Walker and C.J.Fink (1972),Diabetes,21,987-998.

LaFountain,J.R. (1972),Exp.Cell Res.,71,325-328.

Lane,N.J. and J.E.Treherne (1970),J.Cell Sci.,7,217-231.

Langford,G.M. (1978),Exp.Cell Res.,111,139-152.

Langford,G.M. (1980),J.Cell Biol.,87,521-526.

Langford,G.M. and S.Inoué (1979),J.Cell Biol.,80,521-538.

Larsson,H.,M.Wallin and A.Edström (1979),J.Neurochem.,32,155-162

Lee,J.C.,N.Tweedy and S.N.Timasheff (1978),Biochem.,17,2738-2790.

LeGuern,C.,P.Pradelles,F.Dray,C.Jeantet and F.Gras (1977),F.E.B.S. Lett.,84,97-100.

LeMarch nd,Y.,C.Pratzelt,F.Assimacopoulos-Jeannet,E.G.Loten and B.Jeanrenaud (1974),J.Clin.Invest.,53,1512-1517.

LeMarchand,Y.,A.Singh,F.Assimacopoulos-Jeannet,L.Orci,C.Rouiller and B.Jeanrenaud (1973),J.Biol.Chem.,248,6862-6870.

LeMarchand,Y.,A.Singh,C.Patzelt,L.Orci and B.Jeanrenaud (1975),In 'MT. and MT. inhibitors'(Borgers,M. and M.DeBrabander-eds.),Elsevier:N.Y. 153-164.

Lockwood,A.H. (1978),Cell,13,613-628.

Lockwood,A.H. (1979),Proc.Nat.Acad.Sci.U.S.,76,1184-1188.

Loike,J.D.,C.F.Brewer,H.Sternlicht,W.J.Gensler and S.B.Horowitz (1978), Cancer Res.,38,2688-2693.

Lowry,O.H.,N.J.Rosenbrough,A.L.Farr and R.J.Randall (1951),J.Biol. Chem.,193,265-275.

Luftig,R.B. and R.R.Weihing (1975),J.Virol.,16,696-706.

MacGregor,H.C. and H.Stebbings (1970),J.Cell Sci.,6,431-449.

MacKinney,A.A.,R.S.Vyas and K.Powers (1978),J.Pharmacol.Exp.Ther., 204,195-202.

MacKinney,A.A.,R.S.Vyas and D.Walker (1978),J.Pharmacol.Exp.Ther., 204,189-194.

MacKinnon,E.A.,P.J.Abraham and A.Svatek (1973),Z.Zellforsch.,136, 447-460.

Makita,T. and S.Kwaki (1978),Arch.Histo.Jap.,41,167-176.

Makita,T.,M.Morimoto and S.Kiwaki (1974),Histochem.J.,6,185-198.

Malaisse,W.J. (1973),Diabetologia,9,167-173.

Malaisse,W.J.,V.Leclercq-Meyer,E.VanObberghen,G.Somers,G.Devis, M.Ravazzola,F.Malaisse-Lagae and L.Orci (1975a),In 'MT. and MT. inhibitors'(Borgers,M and M.DeBrabander-eds.),Elsevier:N.Y.,143-152.

Malaisse,W.J.,F.Malaisse-Lagae,E.VanObberghen,G.Somers,G.Devis, M.Ravazzola and L.Orci (1975b),Ann.N.Y.Acad.Sci.,253,630-652.

Malaisse,W.J.,F.Malaisse-Lagae,M.O.Walker and P.E.Lacy (1971),Diabetes, 20,257-265.

Malaisse-Lagae,F.,M.Amherdt,F.Ravazzola,A.Sener,J.C.Hutton,L.Orci and W.J.Malaisse (1979),J.Clin.Invest.,63,1284-1296.

Malaisse-Lagae,F.,M.H. reider,W.J.Malaisse and P.E.Lacy (1971),J.Cell Biol.,49,530-535.

Malaisse-Lagae,F.,M.Ravazzola,M.Amherdt,A.Gutzeit,W.Stauffacher, W.J.Malaisse and L.Orci (1975),Diabetologia,11,71-76.

Malawista,S.E. (1968),Arthritis Rheum.,11,191-197.

Malawista,S.E. (1975),Ann.N.Y.Acad.Sci.,253,738-749.

Malawista,S.E.,J.M.Oliver and S.A.Rudolph (1978),J.Cell Biol., 77,881-886.

Mandelkow,E.-M. and E.Mandelkow (1979),J.Mol.Biol.,129,135-148.

Marcum,J.M. and G.G.Borisy (1978a),J.Biol.Chem.,253,2825-2833.

Marcum,J.M. and G.G.Borisy (1978b),J.Biol.Chem.,253,2852-2857.

Marcum,J.M.,J.R.Dedman,B.R.Brinkley and A.R.Means (1978),Proc. Nat.Acad.Sci.U.S.,75,3771-3775.

Marchant,H.J. (1978),Exp.Cell Res.,115,25-30.

Mareel,M. and M.J.DeBrabander (1978),Oncology,35,5-7.

Mareel,M. and M.J.DeBrabander (1978),J.Nat.Cancer Inst.,61,787-792.

Margulis,L. (1973),Int.Rev.Cytol.,34,333-361.

Marotta,C.A.,P.Strocchi and J.M.Gilbert (1978),J.Neurochem.,30,1431-1440.

Matsumoto,G.,T.Kobayashi and H.Sakai (1979),J.Biochem.(Tokyo),86, 1155-1158.

Matsumura,F. and M.Hayashi (1976),Biochem.Biophys.Acta.,453,162-175.

Matthews,E. . (1970),In'Calcium and cellular function'(Cuthbert,A.W.-ed.), Macmillan,163-182.

McCarthy,P.L.,J.E.Shaw and H.G.Remold (1979),Cell.Immun.,46,409-415.

McDaniel,M.L.,C.G.Bry,R.W.Homer,C.J.Fink,D.Ban and P.E.lacy (1980), Metabolism,29,762-766.

McGuire,J.,P.Quinn,S.Knutton (1974),J.Cell Biol.,63,217a.

McIntosh,J.R. (1974),J.Cell Biol.,61,166-187.

Means,A.R. and J.R.Dedman (1980),Nature,285,73-77.

Melchior,V.,C.J.Hollingshead and M.E.Cahoon (1980),J.Cell Biol.,86,881-884.

Meldolesi,J.,J.D.Jamieson and G.E.Palade (1971a),J.Cell Biol.,49,109-129.

Meldolesi,J.,J.D.Jamieson and G.E.Palade (1971b),J.Cell Biol.,49,130-149.

Meldolesi,J.,J.D.Jamieson and G.E.Palade (1971c),J.Cell Biol.,49,150-158.

Mellon,M. and L.I.Rebhun (1976),In 'Cell Motility'(Goldman,R.,T.Pollard, and J.Rosenbaum-eds.),Cold Spring Harbor,1149-1163.

Mir,L.,M.-L.Oustrin,P.Lecointe and M.Wright (1978),F.E.B.S.Lett.,88, 259-263.

Mohri,H. (1976),Biochim.Biophys.Acta.,456,85-127.

Mollenhauer,H.H. (1974),J.Cell Sci.,15,89-97.

Monaco,G.,P.Calissano and D.Mercanti (1977),Brain Res.,129,265-274.

Montague,W.,S.L.Howell and I.C.Green (1976),Horm.Metab.Res.,8, 166-169.

Morris,N.R. and C.E.Oakley (1979),J.Gen.Microbiol.,114,449-454.

Moskalewski,S.,J.Thyberg and U.Friberg (1976),J.Ultrastrut.Res.,54, 304-317.

Murphy,D.B. and G.G.Borisy (1974),J.Cell Biol.,63,236a.

Murphy,D.B. and R.R.Hiebsch (1979),Anal.Biochem.,96,225-235.

Murphy,D.B.,K.A.Johnson and G.G.Borisy (1977),J.Mol.Biol.,117, 33-52.

Murphy,D.B. and L.G.Tilney (1974),J.Cell Biol.,61,757-779.

Murphy,D.B.,R.B.Vallee and G.G.Borisy (1977),Biochem.,16,2598-2605.

Nagle,B.W.,K.H.Doenges and J.Bryan (1977),Cell,12,573-586.

Nakai,Y. and R.Ushiyama (1978),Can.J.Bot.,56,1206-1211.

Nath,K.,J.W.Shay and A.P.Bollen (1978),Proc.Nat.Acad.Sci. U.S., 75,319-323.

Neame,K.D. and C.A.Homewood (1974),'Introduction to Liquid Scintillation Counting',Butterworths.

Nelles,L.P. and J.R.Bamburg (1979),J.Neurochem.,32,477-490.

Neutra,M. and C.P.Leblond (1966a),J.Cell Biol.,30,119-136.

Neutra,M. and C.P.Leblond (1966b),J.Cell Biol.,30,137-150.

Nevalainen,T.J. (1970),Acta.Path.Microbiol.Scand.Suppl.,210,1-74.

Nevalainen,T.J. (1975a),Virchow.Archiv.B,18,119-127.

Nevalainen,T.J. (1975b),Res.Exp.Med.,165,163-168.

Nève,P. and S.H.Woolman (1971),Anat.Rec.,171,81-98.

Nishida,E.,H.Kumagai,I.Ohtsuki and H.Sakai (1979),J.Biochem.(Tokyo), 85,1257-1266.

Oftebro,R.,Ø.Grimmer,T.B.Øyen and S.G.Laland (1972),Biochem.Pharmacol., 21,2451-2456.

Olah,L.V. and L.Hanzely (1973),Cytologia,38,55-72.

Oliver,J.M.,R.D.Berlin,R.L.Baehner and L.A.Boxer (1977),Brit.J.Haematol., 37,311-322.

Oliver,J.M.,J.J.Krawiec and R.D.Berlin (1978),Exp.Cell Res.,116, 229-237.

Oliver,J.M.,S.P.Spielberg,C.B.Pearson and J.D.Schulman (1978),J.Immunol., 120,1181-1186.

Oliver,J.M.,T.E.Ukena and R.D.Berlin (1974),Proc.Nat.Acad.Sci.U.S., 71,394-398.

Orci,L.,K.H.Gabbay and W.J.Malaisse (1972),Science,175,1128-1130.

Orci,L.,F.Malaisse-Lagae and A.Perrelat (1977),Diabetologia,13,377-442.

Osborn,M. and K.Weber (1976),Exp.Cell Res.,103,331-340.

Osborn,M. and K.Weber (1977),Cell,12,561-571.

Osborn,M.,R.E.Webster and K.Weber (1978),J.Cell Biol.,77,R27-R34.

Ostlund,R.E. (1977),Diabetes,26,245-254.

Ostlund,R.E.,J.T.Leung and S.V.Hajek (1979),Anal.Biochem.,96,155-164.

Ostlund,R.E.,J.T.Leung and S.V.Hajek (1980),J.Cell Biol.,85,386-391.

Ostlund,R.E.,B.Pfleger and G.Schonfeld (1979),J.Clin.Invest., 63,75-84.

Palade,G.E. (1975),Science,189,347-358.

Palade,G.E. and R.R.Bruns (1968),J.Cell Biol.,37,633-649.

Patzelt,C.,A.Singh,Y.LeMarchand and B.Jeanrenaud (1975),In'MT. and MT. inhibitors'(Borgers,M. and M.DeBrabender-eds.),Elsevier:N.Y., 165-176.

Paulson,J.C. and W.O.McClure (1974),Brain Res.,73,333-337.

Pekas,J.C. (1971),Am.J.Physiol.,220,799-803.

Pencek,P.F. and R.F.Loizzi (1980),J.Cell Biol.,87,249a.

Penningroth,S.M.,D.W.Cleveland and M.W.Kirschner (1976),In'Cell Motility' (Goldman,R.,T.Pollard and J.Rosenbaum-eds.),Cold Spring Harbor, 1233-1257.

Penningroth,S.M.and M.W.Kirschner (1978),Biochem.,17,734-740.

Pepper,D.A. and B.R.Brinkley (1979),J.Cell Biol.,82,585.

Pernice,B. (1889),Sicilia Med.,1,265-279.

Phaire-Washington,L.,S.C.Silverstein and E.Wang (1980),J.Cell Biol., 86,641-655.

Pickett-Heaps,J.D.(1975),Ann.N.Y.Acad.Sci.,253,352-361.

Pierson,G.B.,P.Burton and R.H.Himes (1978),J.Cell Biol.,76,223-228.

Pierson,G.B.,P.R.Burton and R.H.Himes (1979),J.Cell Sci.,39,89-100.

Pipeleers,D.G.,M.A.Pipeleers-Marichal and D.M.Kipnis (1976),Science, 191,88-90.

Pipeleers,D.G.,M.A.Pipeleers-Marichal and D.M.Kipnis (1977),J.Cell Biol.,74,351-357.

Pipeleers,D.G.,M.A.Pipeleers-Marichal,P.Sherline and D.M.Kipnis (1977), J.Cell Biol.,74,341-350.

Pollard,J.H. (1977),'A Handbook of Numerical Statistical Techniques', Cambridge University Press.

Portier.M.-M.,M.Milet,D.H.Hayes (1979),Eur.J.Biochem.,97,161-182.

Poste,G. and A.C.Allison (1973),Biochim.Biophys.Acta.,300,421-465.

Prescott,A.R. and D.Starling (1980),Cell Biol.Int.Rep.,4,815.

Prus,K. and A.Mattison (1979),Histochem.,61,281-290.

Puck,T.T. (1977),Proc.Nat.Acad.Sci.U.S.,74,4491-4495.

Radley,J.M. (1974),J.Cell Sci.,16,309-332.

Raine,C.S.,B.Ghetti and M.L.Shelanski (1971),Brain Res.,34,389-393.

Ranney,D.F. and J.H.Pincus (1976),Cell.Sh.Surf.Arch.,17,287-295.

Rao,A.G.A.,D.L.Hare and J.R.Cann (1978),Biochem.,17,4735-4738.

Ray,P. and C.A.Strott (1978),J.Cell Biol.,79,282a.

Reaven,E.P. (1975),J.Clin.Invest.,56,49-55.

Reaven,E.P. (1977),J.Cell Biol.,75,731-742.

Reaven,E.P. and S.Azhar (1981),J.Cell Biol.,89,300-308.

Reaven,E.P. and G.M.Reaven (1977),J.Cell Biol.,75,559-572.

Reaven,E.P. and G.M.Reaven (1978),J.Cell Biol.,77,735-742.

Reaven,E.P. and G.M.Reaven (1980),J.Cell Biol.,84,28-39.

Rebhun,L.I. (1972),Int.Rev.Cytol.,32,93-137.

Ridgman,W.J. (1975),'Experimentation in Biology',Blackie.

Roberts,K. and J.S.Hyams-eds. (1979),'Microtubules',Academic Press.

Rodriguez,J.A. and G.G.Borisy (1979),Science,206,463-464.

Rodinov,V.I.,V.I.Gel'fand and V.A.Rozenblat (1976),Biokhimiya,41, 2068-2074.

Roth,L.E.,D.J.Pihalaja and Y.Shigenaka (1970),J.Ultrastruct.Res.,30, 7-37.

Roth,L.E. and Y.Shigenaka (1970),J.Ultrastruct.Res.,31,356-374.

Rothman,S.S. (1975),Science,190,747-753.

Rudd,C.E.,K.A.Rogers,D.L.Brown and J.G.Kaplan (1979),Can.J.Biochem., 57,673-683.

Rudolph,R. and M.Woodward (1978),Anat.Rec.,191,169-183.

Runge,M.S.,H.W.Detrich and R.C.Williams (1979),Biochem.,18,1689-1697.

Runge,M.S.,P.B.Hewgley,D.Puett and R.C.Williams (1979),Proc.Nat. Acad.Sci.U.S.,76,2561-2565.

Samson,F.,J.A.Donoso,I.Hellerbettinger,D.Watson and R.H.Himes (1979), J.Pharm.Exp.Thera.,208,411-417.

Sandoval,I.V. and P.Cuatrecasas (1976),Biochem.Biophys.Res.Comm., 68,169-177.

Sandoval,I.V.,J.L.Jameson,J.Niedel,E.MacDonald and P.Cuatrecasas (1978),Proc.Nat.Acad.Sci.U.S.,75,3178-3182.

Sattilaro,R.F.,W.L.Dentler and E.L.LeCluyse (1981),J.Cell Biol.,90, 467-473.

Sattilaro,R.F.,E.L.LeCluyse and W.L.Dentler (1980),J.Cell Biol.,87, 250a.

Schiff,P.B.,J.Fant and S.B.Horowitz (1979),Nature,277,665-667.

Schiff,P.B.,A.S.Kende and S.B.Horowitz (1978),Biochem.Biophys.Res. Commun.,85,737-746.

Schliwa,M. (1978),J.Cell Biol.,76,605-614.

Schliwa,M. and J.Bereiter-Hahn (1974),Cell Tiss.Res.,151,423-432.

Schliwa,M. and J.VanBlerkom (1981),J.Cell Biol.,90,222-235.

Schmitt,F.O. (1968),Proc.Nat.Acad.Sci.U.S.,60,1092-1101.

Schnaitman,T.,L.I.Rebhun and S.M.Kupchan (1975),J.Cell Biol., 67,388a.

Schultz,M. and P.F.Curran (1970),Phys.Rev.,50,637-718.

Selkoe,D.J. (1979),Brain Res.,172,382-386.

Sentein,P. (1970),Chromosoma,32,97-134.

Sentein,P. (1971),Bull.Ass.Anat.,56,712-720.

Sentein,P. (1975),Exp.Cell Res.,95,233-246.

Sentein,P. and Y.Ales (1974),Chromosoma,45,215-244.

Seybold,J.,W.Bieger and H.F.Kern (1975),Virch.Archiv.A.,368,309-327.

Shay,J.W. and J.W.Fuseler (1979),Nature,278,178-180.

Shelanski,M.L.,F.Gaskin and C.R.Cantor (1973),Proc.Nat.Acad.Sci.U.S., 70,765-768.

Sherline,P.,C.K.Bodwin and D.M.Kipnis (1974),Anal.Biochem.,62,400-407.

Sherline,P.,Y.Lee and L.S.Jacobs (1977),J.Cell Biol.,72,380-389.

Sheterline,P. and J.G.Schofi d (1975),F.E.B.S.Lett.,56,297-302.

Sheterline,P.,J.G.Schofield and F.Mira-Moser (1977), Exp.Cell Res.,104,127-134.

Shigenaka,Y. (1976),Annot.Zool.Jpn.,49,164-176.

Shigenaka,Y.,L.E.Roth and D.J.Pihlaja (1971),J.Cell Sci.,8,127-152.

Shino,M. and E.G.Rennels (1975),Proc.Soc.Exp.Biol.Med.,149,380-383.

Shirahama,T. and A.S.Cohen (1974),Am.J.Pathol.,76,501-520.

Shohat,B. (1973),Z.Krebforsch.,80,97-102.

Siekevitz,P. and G.E.Palade (1958a),J.Biochem.Biophys.Cytol.,4, 203-218.

Siekevitz,P. and G.E.Palade (1958b),J.Biochem.Biophys.Cytol.,4, 309-318.

Siekevitz,P. and G.E.Palade (1958c),J.Biochem.Biophys.Cytol.,4, 557-566.

Sleigh,M.A. (1974) -ed.'Cilia and Flagella',Academic Press:N.Y.

Sloboda,R.D.,W.L.Dentler,R.A.Bloodgood,B.R.Telzer,S.Granett and J.L.Rosenbaum (1976),In'Cell Motility'(Goldman R.,T.Pollard and J.L.Rosenbaum-eds.),Cold Spring Harbor,1171-1212.

Sloboda,R.D.,W.L.Dentler and J.L.Rosenbaum (1976),Biochem.15, 4497-4505.

Sloboda,R.D. and J.L.Rosenbaum (1977),J.Cell Biol.,75,286a.

Sloboda,R.D. and J.L.Rosenbaum (1979),Biochem.,18,48-54.

Slot,J.W.,J.J.Geuze and C.Poort (1974),Cell Tiss.Res.,155,135-154.

Smith,D.S.,U.Järlfors and R.Beranek (1970),J.Cell Biol.,46,199-219.

Smith,D.S.,U.Järlfors and B.F.Cameron (1975),Ann.N.Y.Acad.Sci.,253, 472-506.

Smith,D.S.,U.Järlfors and M.L.Cayer (1977),J.Cell Sci.,27,255.

Snedecor,G.W. and W.G.Cochran (1967),'Statistical Methods',6th edn., Iowa State University Press.

Snyder,J.A. and J.R.MacIntosh (1976),Ann.Rev.Biochem.,45,699-720.

Soifer,D. (1975)-ed.,Ann.N.Y.Acad.Sci.,253.

Solomon,F.,M.Magendantz and A.Salzman (1979),Cell,18,431-438.

SpiegelmanB.M.,S.M.Penningroth and M.W.Kirschner (1977),Cell,12,587-600.

Spilberg,I.,B.Mandell and S.Hoffstein (1979),J.Lab.Clin.Med.,94, 361-369.

Spurr,A.R. (1969),J.Ultrastruct.Res.,26,31-43.

Starling,D. (1976),J.Cell Sci.,20,91-100.

Stearns,M.E. and D.L.Brown (1979),F.E.B.S.Lett.,101,15-20.

Stebbings,H. and C.E.Bennett (1975),In'MT. and MT. inhibitors',(Borgers, M. and M.DeBrabander-eds.),Elsevier:N.Y.

Sternlicht,H. and I.Ringel (1979),J.Biol.Chem.,254,10540-10550.

Stock,C.,J.F.Launay and J.F.Gren er (1977),Bochem.Biophys.Res.Commun.,76, 217-223.

Strott,C.A. and P.Ray (1977),Biochim.Biophys.Acta.,495,119-128.

Summers,K. and M.W.Kirschner (1979),J.Cell Biol.,83,205-217.

Sumner,A.T. and B.E.H.Sumner (1969),'A Laboratory Manual of Micro-technique and Histochemistry',Blackwell.

Sutherland,J.W.H. and J.M.Sturtevant (1976),Proc.Nat.Acad.Sci.U.S., 73,3565-3569.

Tamm,L.K.,R.H.Crepeau and S.J.Edelstein (1979),J.Mol.Biol.,130, 473-492.

Tan,L.P.,M.L.Ng and V.R.Kumar Das (1978),J.Neurochem.,31,1035-1042.

Taylor,A.,M.Mamelak,H.Golbetz and R.Maffly (1978),J.Memb.Biol., 40,213-236.

Temple,R.,J.A.Williams,J.F.Wilber and J.Wolff (1972),Biochem. Biophys.Res.Commun.,46,1454-1461.

Terry,B.J. and D.L.Purich (1979),J.Biol.Chem.,254,9469-9476.

Thompson,W.C.,G.G.Deanin and M.W.Gordon (1979),Proc.Nat.Acad.Sci.U.S., 76,1318-1322.

Thorpe,R.,A.Delacourte,M.Ayers,C.Bullock and B.H.Anderton (1979), Biochem.J.,181,275-284.

Thrasher,J.D. (1973),In'Drugs and the Cell Cycle'(Zimmerman,A.M., G.M.Padilla and I.L.Cameron-eds.),Academic Press,25-48.

Thyberg,J.,J.E.Axelsson and A.Hinek (1977),Brain Res.,137,323-332.

Tilney,L.G.,J.Bryan,D.J.Bush,K.Fujiwara,M.S.Moosekev,D.B.Murphy and D.H.Snyder (1973),J.Cell Biol.,59,267-275.

Tinoco,I. (1957),Arch.Biochem.Biophys.,68,367-372.

Trifaro,J.M.,B.Collier,A.Lastoweka and D.Stern (1972),Molec.Pharmacol., 8,264-267.

Tucker,J.B. (1974),J.Cell Biol.,62,424-437.

Tucker,J.B. (1978),J.Cell Sci.,29,213-232.

Tucker,J.B. and J.B.Mackie (1975),Tiss.Cell,7,601-612.

Valenti,C.E.,R.J.Lombardo,C.Liberˆun and D.P.Cardinali (1980), Experentia,36,1012-1014.

Valenti,C.,M.I.Vacas and D.P.Cardinali (1979),Experentia,35,120-122.

Vallee,R.B. and G.G.Borisy (1977),J.Biol.Chem.,252,377-382.

Vallee,R.B. and G.G.Borisy (1978),J.Biol.Chem.,253,2834-2845.

Vandermeers-Piret,M.C.,J.Camus,J.Rothé,A.Vandermeers and J.Christophe (1971),Am.J.Physiol.,220,1037-1045.

VanObberghen,E.,P.DeMeyts and J.Roth (1976),J.Biol.Chem.,251,6844-6851.

VanObberghen,E.,G.Somers,G.Devis,M.Ravazzola,F.Malaisse-Lagae,L.Orci and W.J.Malaisse (1974),Endocrin.,95,1518-1528.

VanObberghen,E.,G.Somers,G.Devis,M.Ravazzola,F.Malaisse-Lagae,L.Orci and W.J.Malaisse (1975),Diabetes,24,892-901.

VanObberghen,E.,G.Somers,G.Devis,G.D.Vaughan,F.Malaisse-Lagae,L.Orci and W.J.Malaisse (1973),J.Clin.Invest.,52,1041-1051.

Vater,W.,H.Muller and E.Unger (1978),Biochem.Biophys.Res.Commun., 84,721-726.

VanVenrooij,W.J.,C.Poort,M.F.Kramer and M.T.Jansen (1972),Eur.J.Biochem., 30,427-433.

Völkl,A.,W.Bieger and H.F.Kern (1976),Cell Tiss.Res.,175,227-243.

Wang,E.,R.K.Cross and P.W.Choppin (1979),J.Cell Biol.,83,320-337.

Wang,R.W.-J.,L.I.Rebhun and S.M.Kupchan (1976),J.Cell Biol., 70,335a.

Warchol,J.B.,D.C.Herbert,M.Glenn-Williams and E.G.Rennels (1975), Cell Tiss.Res.,159,205-212.

Watari,N. and N.Baba(1968),J.Electron Microsc.,17,327-341.

Weatherbee,J.A.,R.B.Luftig and R.R.Weihing (1978),J.Cell Biol., 78,47-57.

Weber,K. (1975),In'MT. and MT. inhibitors'(Borgers.M. and M.DeBrabander -eds.),Elsevier:N.Y.,313-326.

Webster,R.E.,D.Henderson,M.Osborn and K.Weber (1978),Proc.Nat.Acad.Sci. U.S.,75,5511-5515.

Wehland,J.,W.Herzog and K.Weber (1977),J.Mol.Biol.,111,329-342.

Weingarten,M.D.,A.H.Lockwood,H.Shu-Yung and M.W.Kirschner (1975),Proc. Nat.Acad.Sci.U.S.,72,1858-1862.

Weisenberg,R.C. (1972),Science,177,1104-1105.

Weisenberg,R.,G.G.Borisy and E.Taylor (1968),Biochem.,7,4466-4479.

Weisenberg,R.C.,W.T.Deery and P.J.Dickinson (1976),Biochem.,15,4248-4254.

Westrum,L.E. and E.G.Gray (1976),Brain Res.,105,547-550.

White,J.,A.Yeats and G.Skipworth (1974),'Tables for Statisticians', Stanley Thornes.

Wiche,G.,L.S.Honig and R.D.Cole (1979),J.Cell Biol.,80,553-563.

Williams,J.A. (1977),Cell Tiss.Res.,179,453.

Williams,J.A. and M.Lee (1976),J.Cell Biol.,71,795-806.

Williams,J.A. and J. olff (1970),Proc.Nat.Acad.Sci.U.S.,67,1901-1908.

Witman,D.W.Cleveland,M.D.Weingarten and M.W.Kirschner (1976),Proc. Nat.Acad.Sci.U.S.,73,4070-4074.

Wolin,S.L. and R.S.Kucherlapati (1979),J.Cell Biol.,82,76-85.

Zavala,F.,D.Guenard and P.Potier (1978),Experentia,34,1497-1498.

Zeeberg,B. and M.Caplow (1978),J.Biol.Chem.,253,1984-1990.

Zeeberg,B. and M.Caplow (1979),Biochem.,18,3880-3886.

Zenner,H.P. and T.Pfeuffer (1976),Eur.J.Biochem.,71,177-184.

Zingsheim,H.P.,W.Herzog and K.Weber (1979),Eur.J.Cell Biol.,19, 175-183.

Zor,U.,B.Strulovici and H.R.Linder (1978),Biochem.Biophys.Res.Commun., 80,983-992.

* Cleveland,D.W.,S.Y.Hwo and M.W.Kirschner (1977),J.Mol.Biol., 116,207-227.

Cleveland,D.W.,S.Y.Hwo and M.W.Kirschner (1977),J.Mol.Biol., 116,227-248.