assembly and disassembly and the tubulin-MAP interactions will appear to be stoichiometric. Since no assumptions were made concerning specificity, it is evident that, while constancy of composition is consistent with specificity of interaction, it is not a sufficient condition and the question on whether any of the MAPs (be they HMW or tau) are specific or not must be regarded as still open. A similar conclusion has been reached recently by Williams, Jr. [26] who examined by elegant two-dimensional electrophoresis experiments the question of which MAPs can qualify as potential candidates for specific liganding to tubulin.

The nature of the participation of the MAPs in the microtubule system has been questioned also by observations that a variety of polycationic macromolecules, such as histones, ribonuclease, DEAE dextran and poly-L-lysine, can mimic their effect in microtubule reconstitution, [27] to the extent of affecting C_r in similar manner to MAPs [28]. On the other hand, a number of reports have presented circumstantial evidence favoring the proposition that the MAPs are microtubule-related. First, electron micrographs of microtubules reconstituted in the presence of these proteins display decorations similar to those observed on natural microtubules [22,23]. Secondly, immunofluorescence experiments have been interpreted as indicating that some of the MAPs are located in cells in networks similar to those formed by microtubules [29,30]. Yet, none of these observations can be regarded as more than preliminary and suggestive, so that, at present, it seems most prudent to regard as unresolved the question of the participation in microtubule assembly *in vivo*, as well as *in vitro*, of the non-tubulin proteins (MAPs), such as HMW or tau.

Summary

The present day status of *in vitro* microtubule assembly may be summarized as follows. The minimal requirements are: pure tubulin, GTP, probably Mg^{2+} and the absence of Ca^{2+}. Thermodynamically, assembly fits the model of a nucleated co-operative polymerization. The growth step is entropy driven and involves the release of water molecules. The reaction requires GTP, which is probably hydrolyzed during assembly. The process can be enhanced by non-specific thermodynamic boosters, such as concentrated glycerol, and by a variety of polycations which stabilize the assembled structure by binding to it. It is not clear at present what macromolecular factors control microtubule assembly *in vivo* or which, if any, of the proteins frequently co-crystallized with tubulin during its isolation have any specific relation to the microtubule system.

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References

1 Lee, J. C., Frigon, R. P. and Timasheff, S. N. (1973) *J. Biol. Chem.* 248, 7253–7262
2 Frigon, R. P. and Timasheff, S. N. (1975) *Biochemistry* 14, 4559–4566
3 Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) *Biochemistry* 7, 4466–4479
4 Weisenberg, R. C. (1972) *Science* 177, 1104–1105
5 Borisy, G. G. and Olmsted, J. B. (1972) *Science* 177, 1196–1197
6 Weisenberg, R. C. and Timasheff, S. N. (1970) *Biochemistry* 9, 4110–4116
7 Shelanski, M. L., Gaskin, F. and Cantor, C. (1973) *Proc. Nat. Acad. Sci. U.S.A.* 70, 765–768
8 Gaskin, F., Cantor, C. R. and Shelanski, M. L. (1974) *J. Mol. Biol.* 89, 737–756
9 Oosawa, F. and Kasai, M. (1971) in *Biological Macromolecules* Timasheff, S. N. and Fasman, G. D. eds), Volume 5, pp. 261–322, Dekker, N. Y.
10 Kirschner, M. W., Williams, R. C., Weingarten, M. and Gerhart, J. C. (1974) *Proc. Nat. Acad. Sci. U.S.A.* 71, 1158–1163
11 Erickson, H. P. (1974) *J. Supramol. Struct.* 2, 393–411
12 Lee, J. C. and Timasheff, S. N. (1975) *Biochemistry* 14, 5183–5187
13 Lee, J. C. and Timasheff, S. N. (1977) *Biochemistry* 16, 1754–1764
14 Wyman, J. (1964) *Adv. Protein Chem.* 19, 224–285
15 Timasheff, S. N., Gorbunoff, M. J. and Hinz, H.-J. (1978) *Fed. Proc.* 37, 1790
16 Salmon, E. D. (1975) *Science* 189, 884–886
17 Na, C. and Timasheff, S. N. (1978) *Fed. Proc.* 37, 1791
18 Weisenberg, R. C., Deery, W. J. and Dickinson, P. J. (1976) *Biochemistry* 15, 4248–4254
19 David-Pfeuty, T., Erickson, H. P. and Pantaloni, D. (1977) *Proc. Nat. Acad. Sci. U.S.A.* 74, 5372
20 Penningroth, S. M. and Kirschner, M. W. (1977) *J. Mol. Bio* . 115, 643–673
21 Olmsted, J. B. and Borisy, G. G. (1975) *Biochemistry* 14, 2996–3005
22 Murphy, D. B. and Borisy, G. G. (1975) *Proc. Nat. Acad. Sci. U.S.A.* 72, 2696–2700
23 Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) *Biochemistry* 15, 4497–4505
24 Cleveland, D. W., How, S.-Y. and Kirschner, M. W. (1977) *J. Mol. Biol.* 116, 207–225
25 Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B. and Johnson, K. A. (1975) *Ann. N.Y. Acad. Sci.* 253, 107–132
26 Berkowitz, S. A., Katagiri, J., Binder, H. K. and Williams, Jr., R. C. (1977) *Biochemistry* 16, 5610–5617
27 Erickson, H. P. and Voter, W. A. (1976) *Proc. Nat. Acad. Sci. U.S.A.* 73, 2813–2817
28 Lee, J. C., Tweedy, N. and Timasheff, S. N. (1978) Biochemistry, 17, 2783–2790
29 Sherline, P. and Schiavone, K. (1977) *Science* 198, 1038–1040
30 Connolly, J. A., Kalnins, V. I., Cleveland, D. W. and Kirschner, M. W. (1977) *Proc. Nat. Acad. Sci. U.S.A.* 74, 2437–2440

The biosynthetic pathway of the asparagine-linked oligosaccharides of glycoproteins

R. J. Staneloni and L. F. Leloir

A dolichyl diphosphate oligosaccharide containing glucose, mannose and N-*acetylglucosamine is the precursor of the sugar moiety of some glycoproteins. Studies of glycoproteins produced by virus-infected cells, using endo-*N-*acetylglycosaminidase for the liberation of the sugar moiety, have shown that the glucose-containing oligosaccharide is transferred to protein, then the glucose and some mannose residues are removed and acetlyglucosamine, galactose and sialic acid are added.*

Sugar addition to protein leading to the formation of glycoproteins occurs either directly from the sugar nucleotides or indirectly through lipid intermediates. The latter pathway was reviewed in *TIBS* [1] (see also [2,3]).

Recent work has shown that the asparagine-linked oligosaccharides are first built up on a lipid, then transferred to polypeptide and modified by removal or addition of monosaccharide residues. This work has been based mainly on the finding of a glucose-containing lipid-oligosaccharide, on the discovery of enzymes, the endo-*N*-acetylglucosaminidases, which release some oligosaccharides from glycoproteins and on the use of virus-infected cells.

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Asparagine-linked oligosaccharides

The structures of the oligosaccharides, the biosynthesis of which is considered in

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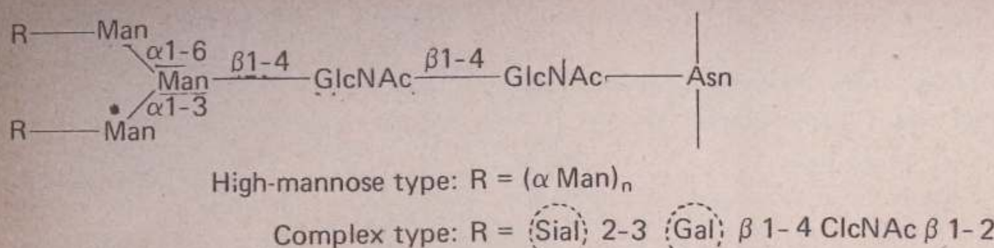

Fig. 1. Structures of the asparagine-linked oligosaccharides. The residues within the dotted circles may be absent. See the text for the explanation of the asterisk (*).

this review, are shown in Fig. 1. Two types of oligosaccharides have been found: the high-mannose and the complex type. Both have a common pentasaccharide structure and differ in the R-side chains. In the high-mannose type the side chains have a variable number of mannose residues and different types of ramification. In some cases, as in the ovalbumin oligosaccharides, there may be, in addition, some acetylglucosamine residues in the side chains. In the complex type the side chains have galactose, *N*-acetylglucosamine and sialic acid, but some may be incomplete. In some cases, as in the α acid-glycoprotein of serum and in the vesicular stomatitis virus glycoproteins, there are three R-chains instead of the two shown in Fig. 1. Thus there is a considerable variety of oligosaccharides but with a common pentasaccharide core.

The G-oligosaccharide

About 10 years ago it was found that a liver enzyme would form dolichyl diphosphate glucose (Fig. 2) by transfer of glucose from UDPglucose to dolichyl phosphate [4]. The compound was then found to react with an endogenous acceptor to yield a substance insoluble in most organic solvents but soluble in chloroform–methanol–water (1:1:0.3) [5]. Mild acid treatment led to the liberation of a substance which appeared to be dolichyl phosphate and of another compound which had the properties of an oligosaccharide [6]. Treatment of the methyl glycoside of the latter with alkali led to the appearance of two positive charges that were interpreted to arise by deacetylation of two hexosamine residues [7]. On paper chromatography with butanol–pyridine–water (4:3:4) or with nitromethane–*n*-propanol–water (2:5:3) it was found to migrate with the R_F value of malto-oligosaccharides of 15–16 or 11–12 units respectively [8]. The original work has been perfected [9–11], and now the oligosaccharide is considered to have the composition: mannose$_9$*N*-acetylglucosamine$_2$ glucose$_2$ [12]. Its structure is presumably similar to a high mannose type of oligosaccharide, with the additional glucose residues. The glucose-containing oligosaccharide combined to dolichyl diphosphate was called glucosylated endogeneous acceptor in the initial studies and more recently has been referred to as G-oligosaccharide [8].

There is evidence that the biosynthetic pathway of dolichyldiphosphate G-oligosaccharide is as shown in Fig. 3. The different steps in which *N*-acetylglucosamine, mannose and glucose are added to dolichyl phosphate have been studied separately with microsomal enzymes of various types of cells [1–3]. The reaction which follows, that is the transfer of the G-oligosaccharide to protein, also seems to be of universal occurrence, at least in animal tissues. However, for some time dolichyl diphosphate G-oligosaccharide was considered to be an oddity because no glycoprotein carrying such a saccharide had been described.

Endo *N*-acetylglucosaminidase

The development of a procedure for removing the amino acid or polypeptide has been a difficulty in the study of asparagine-linked oligosaccharides. Removal of the oligosaccharide is obtained by heating the glycoprotein for about 6 h in 2 N hydroxide plus sodium borohydride [13]. Another procedure is to use an endo *N*-acetylglucosaminidase. These enzymes act by hydrolysing the bond between the two *N*-acetylglucosamine residues of the core (Fig. 1). One of the enzymes was detected by Tarentino and Maley [14] in *Streptomyces griseus* extracts. This enzyme was called H because it acts on the high-mannose type of oligosaccharides containing five or more mannoses but not on the complex type. The di-*N*,*N*′-acetylchitobiose residue is split, irrespective of whether the first *N*-acetylglucosamine is free, reduced or joined to lipid, to asparagine or to an asparagine residue in a protein [15].

Another endo *N*-acetylglucosaminidase (endo D) was detected in *Diplococcus pneumoniae* [16]. It only acts on oligosaccharides which have no substituent on the mannose residue marked with an asterisk (*) in Fig. 1.

Other enzymes were found in *Clostridium perfringens*. They were referred to as C_I and C_{II} and had specificities similar to endo D and H respectively [17].

Studies with virus-infected cells

Animals cells infected with certain viruses are an excellent material for the study of glycoprotein synthesis. Sindbis and vesicular stomatitis virus (VSV) are of the RNA type and have a coat containing glycoproteins. The oligosaccharide of the VSV glycoprotein is of the complex type and in Sindbis there are two glycoproteins each having a high-mannose and a complex oligosaccharide. On infection the protein synthesis of the host cell is suppressed and replaced by that of virus protein. The biosynthesis of the oligosaccharide moiety is carried out by the host cells' glycosyl transferases [18,19]. However, different viruses acting in the same cells may lead to the formation of different oligosaccharides [20]. This is an indication that the polypeptides contain information which determines at which point a high-mannose or a complex oligosaccharide is to be added to it.

Studies with virus-infected cells have been carried out by three groups of workers and most interesting results have been obtained. Robbins *et al.* [21] gave pulses of [^{3}H]mannose (or [^{35}S]methionine) to embryonic chick cells infected with VSV and examined the proteins by polyacrylamide gel electrophoresis, with or without previous treatment with endo H. All of the protein-linked oligosaccharide formed initially was released by endo H but during a chase period the glycoprotein became increasingly resistant to the enzyme. This is as if the oligosaccharide formed initially was the high-mannose type and progressively became the complex type. In

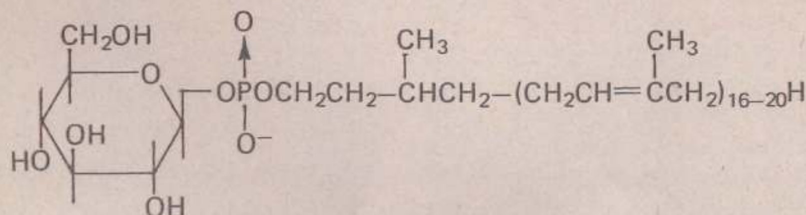

Fig. 2. Dolichyl phosphate glucose.

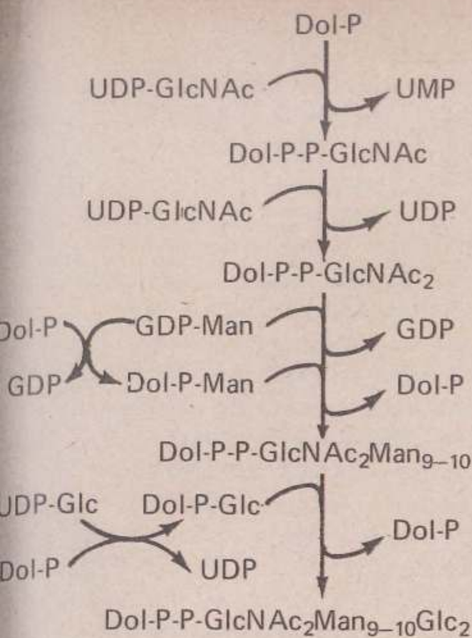

Fig. 3. The biosynthesis of oligosaccharides via the dolichyl derivatives.

other experiments the G-oligosaccharide was separated from the dolichyl phosphate moiety with endo H; the product was then compared by gel filtration with the oligosaccharides released from glycoproteins. After a short pulse (2.5 min) of [^{3}H]-mannose the oligosaccharide released from the glycoproteins was nearly of the same size as the G-oligosaccharide, but as time passed the size decreased progressively. Similar results were obtained with Sindbis virus-infected chick-embryo fibroblasts.

Tabas *et al.* carried out similar experiments with Chinese hamster ovary cells infected with VSV [12]. Their results led them to the conclusion that the lipid-linked G-oligosaccharide is the immediate precursor of the protein-linked oligosaccharide. After transfer to protein, monosaccharide residues are removed, leaving

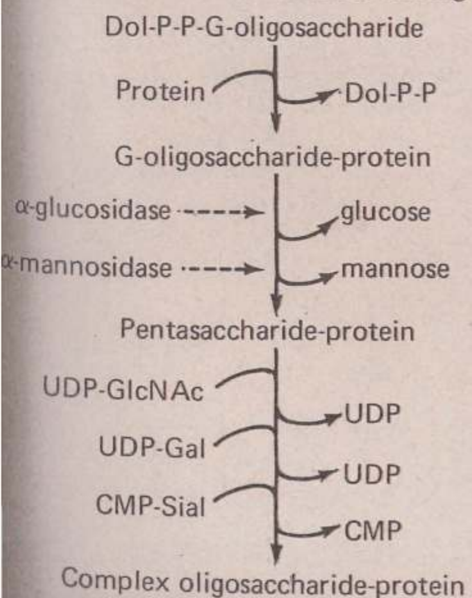

Fig. 4. The formation of complex oligosaccharides

the core pentasaccharide (Man_3 $GlcNAc_2$), which grows again by addition of acetylglucosamine, galactose and sialic acid, thus giving rise to a complex oligosaccharide.

Similar results were obtained by Hunt *et al.* [22]. They found that the initially added oligosaccharide had 7–9 mannoses whereas only three were present in the final product, which also contained branched structures terminated in sialic acid.

In order to find out if the removal of hexose residues from the oligosaccharides also occurred in normal cells and for secreted glycoproteins, experiments were carried out with mouse plasmocitoma cells which secrete immunoglobulin G [12]. It was observed that the intracellular immunoglubulin had an oligosaccharide of the high-mannose type of the approximate composition $hexose_7$ $GlcNAc_2$. In the secreted immunoglobulin the oligosaccharide was different. It contained galactose-*N*-acetylglucosamine external chains and was not acted upon by endo C_{II} (similar to endo H), that is, as if the high-mannose oligosaccharide were the precursor of the complex.

Experiments with tissue slices are consistent with the occurrence of a trimming process. Thyroid slices were incubated with [^{14}C]glucose and the oligosaccharides were removed from the glycoproteins by treatment with alkali and borohydride. These oligosaccharides were smaller than the G-oligosaccharide by 3–5 hexose residues and contained small amounts of glucose [8].

The evidence in favour of the trimming process seems to be very strong. This pathway is schematized in Fig. 4. As to the mechanism of formation of the high-mannose oligosaccharides, the evidence is not so clear. The enzymes that transfer the oligosaccharide from dolichyl diphosphate to proteins do not seem to be very specific. This transfer has been detected *in vitro*, not only with the G-oligosaccharide but also with *N*-acetylglucosamine, di-*N,N'*-acetylchitobiose and the latter with several mannose residues. However the transfer was found to be more efficient for G-oligosaccharide [23]. The high-mannose saccharides could arise either by direct transfer from the corresponding dolichyl derivative or from the G-oligosaccharide followed by trimming.

The enzymes involved in the trimming process are being studied. The glucose residues of the G-oligosaccharide are hardly affected by the commonly available glucosidases but are removed by a microsomal enzyme [24]. Studies on its subcellular distribution showed that while the glucosidase is found in microsomes, an α-mannosidase is found mainly in the Golgi fraction. This agrees with the idea that the G-oligosaccharide is transferred to nascent peptides, and that the glucose is removed soon after. The removal of mannose and the addition of other residues would occur later in the Golgi membranes. The hydrolysing enzymes involved presumably have a delicate specificity in order to leave the three mannoses of the core of the complex oligosaccharides or those which have the required linkage and number in the high-mannose compounds.

References

1 Parodi, A. J. and Leloir, L. F. (1976) *Trends Biochem. Sci.* 1, 58–59
2 Behrens, N. H. (1974) in *Biology and Chemistry of Eucaryotic Cell Surfaces* (Lee, E. Y. C. and Smith, E. E., eds), Vol. 7, pp 159–178, Academic Press, New York
3 Waechter, C. J. and Lennarz, W. J. (1976) *Annu. Rev. Biochem.* 45, 95–112
4 Behrens, N. H. and Leloir, L. F. (1970) *Proc. Nat. Acad. Sci. U.S.A.* 66, 153–159
5 Behrens, N. H., Parodi, A. J. and Leloir, L. F. (1971) *Proc. Nat. Acad. Sci. U.S.A.* 68, 2857–2860
6 Parodi, A. J., Behrens, N. H., Leloir, L. F. and Dankert, M. (1972) *Biochim. Biophys. Acta* 270, 529–536
7 Parodi, A. J., Staneloni, R. J., Cantarella, A. I., Leloir, L. F., Behrens, N. H., Carminatti, H. and Levy, J. A. (1973) *Carbohyd. Res.* 26, 393–400
8 Staneloni, R. J. and Leloir, L. F. (1978) *Proc. Nat. Acad. Sci. U.S.A.* 75, 1162–1166
9 Spiro, M. J., Spiro, R. G. and Bhoyroo, V. D. (1976) *J. Biol. Chem.* 251, 6400–6408
10 Spiro, R. G., Spiro, M. J. and Bhoyroo, V. D. (1976) *J. Biol. Chem.* 251, 6409–6419
11 Spiro, M. J., Spiro, R. G. and Bhoyroo, V. D. (1976) *J. Biol. Chem.* 251, 6420–6425
12 Tabas, I., Schlesinger, S. and Kornfeld, S. (1978) *J. Biol. Chem.* 253, 716–722
13 Chen, W. H., Lennarz, W. J., Tarentino, A. L. and Maley, F. (1975) *J. Biol. Chem.* 250, 7006–7013
14 Tarentino, A. L. and Maley, F. (1974) *J. Biol. Chem.* 249, 811–817
15 Arakawa, M. and Muramatsu, T. (1974) *J. Biochem.* 76, 307–317
16 Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y. and Kobata, A. (1975) *J. Biol. Chem.* 250, 8569–8575
17 Ito, S., Muramatsu, T. and Kobata, A. (1975) *Arch. Biochem. Biophys.* 171, 78–86
18 Burge, B. W. and Huang, A. S. (1970) *J. Virol.* 6, 176–182
19 Schlesinger, S., Gottlieb, C., Feil, P., Gelb, N. and Kornfeld, S. (1976) *J. Virol.* 17, 239–246
20 Sefton, B. W. (1976) *J. Virol.* 17, 85–93
21 Robbins, P. W., Hubbard, S. C., Turco, S. J. and Wirth, D. F. (1977) *Cell* 12, 893–900
22 Hunt, L. A., Etchison, J. R. and Summers, D. F. (1978) *Proc. Nat. Acad. Sci. U.S.A.* 75, 754–758
23 Turco, S. J., Stetson, B. and Robbins, P. W. (1977) *Proc. Nat. Acad. Sci. U.S.A.* 74, 4411–4414
24 Ugalde, R. A., Staneloni, R. J. and Leloir, L. F. (1978) *FEBS Lett.* (in press)