CYCLIC AMP, SIALYLATION AND B₁₆ MELANOMA

by

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SUMMARY

Glycosyltransferase and glycosidase activities were measured in suspensions of B_{16} melanoma cells from both sparse and confluent cultures. Evidence confirming the validity of the glycosyltransferase assay system was evaluated, and metal ion requirements were determined. It was found that in B_{16} melanoma cells from sparse cultures neuraminidase was totally inhibited by the addition of the sialyltransferase substrate, CMP-N-acetylneuraminate, but the neuraminidase from confluent cultures was only partially inhibited.

When B_{16} melanoma cells were grown with 1 mM cyclic AMP and 1 mM theophylline, or with related compounds, changes in growth and morphology were observed, and alterations in glycosyltransferase and glycosidase activities were found. In particular sialyltransferase activity was increased. It was found that under these conditions neuraminidase in B_{16} melanoma cells from confluent cultures was totally inhibited by the addition of CMP-N-acetylneuraminate, but neuraminidase from sparse cultures was only partially inhibited. Glycoproteins removed from the B_{16} melanoma cells by extraction with Triton X-100 were found to be sialylated in a manner consistent with these findings.

The mechanism by which CMP-N-acetylneuraminate inhibited neuraminidase was investigated in a model system using influenza virus neuraminidase and B_{16} melanoma cells from confluent cultures. As in the case of the endogenous
neuraminidase the inhibition of exogenous neuraminidase was greater if B₁₆ melanoma cells had been grown with 1 mM cyclic AMP and 1 mM theophylline. The inhibition of exogenous neuraminidase was totally reversed on incubation of the supernatant obtained, after removal of the B₁₆ melanoma cells, with the neuraminidase assay substrate. Inhibition was associated with hydrolysis of CMP-N-acetyl-neuraminate and the production of free N-acetylneuraminate. With a view to further work a hypothesis based on these findings has been proposed.
# ABBREVIATIONS

With the exception of the abbreviations shown below all abbreviations used in this thesis are standard abbreviations listed in the Biochemical Journal, Vol. 193, pp. 1-27, 1981.

<table>
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<th>Abbreviation</th>
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<tr>
<td>cyclic AMP</td>
<td>adenosine-3',5'-cyclic-monophosphate</td>
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<tr>
<td>GGT</td>
<td>γ-glutamyltranspeptidase</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid</td>
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<tr>
<td>h.p.l.c.</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>I.U.</td>
<td>international units</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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CHAPTER ONE

INTRODUCTION

The aim of this work was to investigate effects of adenosine-3',5'-cyclic-monophosphate (cyclic AMP) on the biosynthesis of membrane glycoproteins of B₁₆ mouse melanoma cells, which provide a convenient system for the study of melanoma.

Some effects of cyclic AMP on membrane glycoproteins were demonstrated by earlier work in this laboratory (Goswell et al., 1977) undertaken as an investigation of the biochemistry of B₁₆ melanoma (Deveridge and Whittaker, 1974). Initially, this early work (Deveridge and Whittaker, 1974; Goswell et al., 1977) involved a preliminary and limited study of the effects of exogenous cyclic AMP (used in conjunction with the phosphodiesterase inhibitor theophylline, known to potentiate the intracellular concentration of cyclic AMP; Robinson et al., 1971) on the morphology of B₁₆ melanoma cells in tissue culture. This was based on the conjectured role of cyclic AMP in cell proliferation, growth and development as suggested by phenomenological studies on a variety of cell lines (Pastan and Johnson, 1974; Ryan and Heidrick, 1974; Willingham, 1976) dealing with morphological changes, density dependent inhibition, cell mobility, cell adhesion, and agglutinability by lectins. Because morphological effects were found in the above experiments in B₁₆ melanoma cells (Deveridge and Whittaker, 1974; Goswell et al., 1977) attention was transferred to the effects of exogenous cyclic AMP on the biosynthesis and structure of membrane glycoproteins. The latter were of interest because of the apparent
biochemical correlation between alterations in glycoprotein biosynthesis and the malignant characteristics of cells. Alterations in glycoprotein structure were known to be associated with transformation (Wu et al., 1969; Glick, 1979; Santer and Glick, 1980) and glycoproteins were known to play an important role in the regulation of growth, differentiation and the social behaviour of cells (Warren et al., 1978). However, the straightforward approach of comparing biochemical parameters of B16 melanoma cells with the corresponding parameters of their normal counterparts is not possible because it is not feasible to grow pure cultures of skin melanocytes. The finding that treatment of B16 melanoma cells with cyclic AMP and theophylline had effects on membrane glycoproteins, as mentioned above (Goswell et al., 1977) suggested a further study of the regulatory functions of cyclic AMP on glycoprotein biosynthesis and structure in these cells. Such a study would contribute to the understanding of the biochemical role of glycoproteins and might help to elucidate their role in malignancy.

The use of cyclic AMP in the above experiments was originally based on the concept that some transformed cells in vitro had reduced intracellular concentrations of cyclic AMP (Johnson et al., 1971; Otten et al., 1971). It seemed likely that a lower level of cyclic AMP may be a consequence of the primary transforming event, and thus responsible for alterations in the levels or activities of the enzymes involved in the synthesis or degradation of membrane glycoproteins involved in the regulation of cell behaviour. This hypothesis, widely held at the time when the project for this
thesis was conceived, suggested that such changes would be prevented or reversed by exogenous cyclic AMP or its derivatives, or with agents such as theophylline, all of which increase intracellular cyclic AMP levels.

When it eventually became apparent that the effects of transformation on growth and morphology of cells were not mediated by cyclic AMP in any consistent way (Pastan and Willingham, 1978) and that the effects of cyclic AMP on cell proliferation may be stimulatory or inhibitory depending on the stage of the cell cycle or on cell type (Boynton and Whitfield, 1983) it was clear that the above hypothesis, as a generalisation, had to be abandoned. Nevertheless, changes in glycoprotein biosynthesis which can be correlated with changes in cyclic AMP levels are important for an understanding of cell surface phenomena, whatever the role of cyclic AMP. Whether, in a particular case, cyclic AMP acts as a positive or as a negative regulator of cell proliferation, or of some other functional characteristic, then the changes in glycoprotein biosynthesis must be appropriate for this role. Thus while the theoretical context, in which the effects of cyclic AMP were studied, changed and evolved as the field developed, the investigation of changes in the oligosaccharide chains of membrane glycoproteins during biosynthesis, or subsequently, remained the primary aim. Analysis of the precise structure of the oligosaccharide chains of particular glycoproteins was not attempted. Instead, attention was specifically directed to those enzymes which might be responsible for the formation of abnormal glycoproteins, namely, to the glycosyltransferases and glycosidases involved.
in the biosynthesis or alteration of the oligosaccharide chains of glycoproteins.

The work described in this thesis has been focused on the effects on glycosyltransferases and glycosidases when cyclic AMP and theophylline were added to the culture medium in which the B16 melanoma cells were grown. L. Sharmeen (1985) working in this laboratory has measured the intracellular levels of cyclic AMP in B16 melanoma cells and has shown that growing the cells in the presence of exogenous cyclic AMP, when combined with theophylline, brings about an increase in the intracellular level of cyclic AMP. The mechanism by which theophylline participates in this effect is unknown, but theophylline is known to alter cell physiology by a variety of mechanisms; not only does it potentiate the intracellular concentration of cyclic AMP by inhibiting phosphodiesterase (Robinson et al., 1971) but it also has a role in the translocation of intracellular calcium (Peach, 1972). It also potentiates inhibitors of prostaglandin synthesis (Vinegar et al., 1976; Horrobin et al., 1977) and reduces catecholamine uptake and metabolism (Kalsner et al., 1975). Theophylline is also known to block adenosine receptors for adenylyl cyclase (Green and Stanberry, 1977; Wolff and Cook, 1977). Nevertheless the consistent finding by Sharmeen (1985) that an appropriate combination of cyclic AMP and theophylline when added to the growth medium of B16 melanoma cells brought about a doubling of the intracellular cyclic AMP concentration was considered to justify the use of this combination in the investigation of the effects of intracellular cyclic AMP on glycosyltransferases and glyco-
sidases. The possibility that theophylline may affect these enzymes by some other mechanism was recognised and accepted.

The following sections deal, by way of introduction, with the structure and biosynthesis of glycoproteins and glycosphingolipids, and their relationship to transformation and malignancy; with the relationship of cyclic AMP levels, both exogenous and endogenous, to transformation and malignancy, and to changes in the structure and levels of particular glycoproteins and glycolipids; with alterations in glycosyltransferase and glycosidase activities in various systems; and with B₁₆ melanoma cell surface biochemistry.

**GLYCOPROTEINS**

The following monosaccharides are the major components of oligosaccharide side chains of glycoproteins of cell membranes (Schacter, 1978): L-fucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and the sialic acids (Corfield and Schauer, 1982a). D-Xylose, first reported in trace amounts in glycoproteins (Jeanloz, 1968), occurs principally in glycosaminoglycans (Baker et al., 1972; Stoolmiller et al., 1972).

Glycoproteins have been classified into three major groups (Kornfeld and Kornfeld, 1976; Zinn et al., 1978) with linkages as follows: those with an O-glycosidic linkage between the hydroxyl groups of serine or threonine and N-acetylgalactosamine (Ser(Thr)-GalNAc-type); those with an O-glycosidic linkage between the hydroxyl group of hydroxylysine and galactose (Gal-Hyl-type); and those with an N-glycosidic linkage between the amide nitrogen of asparagine
and N-acetylglucosamine (Asn-GlcNAc-type). The latter may be further subdivided into two sub-groups (Parodi and Leloir, 1979): those with simple (high mannose) chains containing N-acetylglucosamine and mannose, and those with complex chains containing the disaccharide Gal-β-1,4-GlcNAc (lactosamine) and in addition sialic acid and fucose. Oligosaccharide chains linked to asparagine have a pentasaccharide containing two separate α-mannosyl residues attached to one β-mannosyl residue, which is attached in turn to the disaccharide N,N'-diacetylchitobiose. The C-1 position of the terminal reducing sugar residue of each glycoprotein sub-group is covalently linked to a functional group on the appropriate amino acid in the polypeptide chain. Chains may be branched; fucose and sialic acid are found only at non-reducing ends of the oligosaccharide chains. In addition to the three major groups of glycoproteins, with the linkages described above, the O-glycosidic linkage between the hydroxyl group of serine and xylose (Ser-Xyl-type) which occurs in glycosaminoglycans (Roden, 1980) may be regarded as constituting a fourth group.

**GLYCOPROTEIN BIOSYNTHESIS**

Glycoprotein oligosaccharide chains are known to be synthesized either from sugar nucleotides or from sugar derivatives of polyrenols (Schacter, 1978).

The biosynthesis of Ser(Thr)-GalNAc-type glycoproteins involves the addition of sugars, supplied as nucleotide-sugars (derived as shown in Fig. 1.1). This synthesis probably begins during peptide assembly on membrane bound
FIG. 1.1 Biosynthetic scheme for the nucleotide-sugars
(after Schacter, 1978)
FIG. 1.2 Biosynthesis of ovine submaxillary mucin (OSM) and porcine submaxillary mucin (PSM). The interrupted arrows designate enzymic reactions which have not yet been fully established by *in vitro* experiments (after Schacter, 1978)
ribosomes; post-ribosomal glycosylation is then continued in the endoplasmic reticulum after release of the peptide from the ribosome, and is finally completed in the Golgi apparatus. Monosaccharides are incorporated one by one in a stepwise assembly process. No evidence exists for pre-assembly of oligosaccharides as a polyprenol bound intermediate but this possibility has not been completely ruled out. Fig. 1.2 summarises the biosynthetic pathway for the major oligosaccharides of ovine (OSM) and porcine (PSM) submaxillary mucins and indicates that five different glycosyltransferases are involved. There is a branch point in the biosynthetic pathway after the first N-acetylglactosamine is incorporated. If galactose is incorporated before sialic acid the pathway proceeds towards longer oligosaccharide chains; if sialic acid is incorporated before galactose, galactose cannot be incorporated and assembly stops at the disaccharide stage. The key enzyme in this control process is the galactosyltransferase. This enzyme is strongly bound to membrane, is activated by Triton X-100 and has an absolute requirement for Mn$^{2+}$.

The biosynthesis of the Gal-Hyl-type linkage, which occurs in collagen, involves the post-translational hydroxylation of lysyl residues to form hydroxylysyl residues. After hydroxylation certain lysine units are glycosylated by the sequential action of two enzymes, galactosyltransferase and then glucosyltransferase. The glycosylation and hydroxylation apparently occur in concert in the endoplasmic reticulum and cease when the chains fold into the triple helix (Butler, 1978).
FIG. 1.3 Structure of a dolichol-linked precursor oligosaccharide (a) and representative asparagine-linked oligosaccharides from glycoproteins of high mannose (b) and complex (c) forms (after Hubbard & Ivatt, 1981).
The biosynthesis of Asn-GlcNAc-type glycoproteins (Hubbard and Ivatt, 1981) involves the preassembly of the oligosaccharide core as a polyprenol bound intermediate (Fig. 1.3a). After transfer of the oligosaccharide to asparagine it is then processed and extended. The extent of the processing determines whether a high mannose (Fig. 1.3b) or a complex chain (Fig. 1.3c) is formed. The polyprenol acceptor on which the core oligosaccharide is assembled is dolichol diphosphate. With the exception of the last four mannosyl residues and the three glucosyl residues, the sugars are supplied as nucleotide-sugars. The last four mannosyl residues added are those attached to the mannosyl residue which is linked α-1,6 to the innermost β-1,3(4)-linked mannosyl residue (Fig. 1.3a) and these are supplied from mannosylphosphoryldolichol. The three glucosyl residues are supplied from glucosylphosphoryldolichol. The transfer of the oligosaccharide from the dolichol diphosphate to the asparagine residue of the protein occurs before the nascent peptide is released from the ribosome. During passage of the glycoprotein through the rough endoplasmic reticulum the three glucose residues are removed. The terminal glucose residue is removed by glucosidase I and the other two residues are removed by glucosidase II-III (probably a single enzyme). Most of the processing takes place in the Golgi apparatus. Recent work has shown that the Golgi apparatus has several specialised compartments (Dunphy et al., 1981; Goldberg and Kornfeld, 1983; Dunphy et al., 1985). Transport between compartments involves budding of vesicles from cisternae and diffusion of vesicles, followed by attachment and fusion of
the vesicles with the appropriate cisterna (Rothman et al., 1984). The first (cis) compartment is capable of phosphorylating mannose residues. The second (medial) compartment contains mannosidase I (Tabas and Kornfeld, 1979) which removes the four $\alpha$-(1,2)-mannosyl residues, and two other enzymes: N-acetylglucoasminyltransferase I which attaches an N-acetylglucosamine residue to the innermost $\alpha$-(1,3)-mannosyl residue (Schacter and Roseman, 1980), and mannosidase II which removes the two remaining non-core mannose residues. The third (trans) compartment contains N-acetylglucoasminyltransferase II and galactosyltransferase, which extend the oligosaccharide chain. The oligosaccharide is also the substrate for a fucosyltransferase but the order in which these last three enzymes work is not known. If the mannosidases, for some reason, do not operate, oligosaccharide chains of the high mannose type result. The triantennary structure shown in Fig. 1.3c is a typical end product in which terminal sialic acid residues have been added by sialyltransferase, also in the third (trans) compartment. A great variety of alternate structures are possible; for example, hybrid oligosaccharides occur which combine in a single structure features usually associated exclusively with high mannose or complex type oligosaccharides (Tai et al., 1977; Yamashita et al., 1978). All of the hybrid oligosaccharides studied contain an N-acetylglucosaminyl residue linked $\beta$-(1,4) to the $\beta$-linked innermost mannose residue. Possibly the order in which individual enzymes act can determine the final structure of the oligosaccharide. Beyer et al. (1979) showed that, for example, sialylation and fucosylation are often alternative oligosaccharide chain termination
reactions; i.e. terminal branches containing both fucose and sialic acid cannot be produced. However the substrate specificities of these and other glycosyltransferases do not completely account for the regulation of glycan synthesis. Since different glycoproteins produced by the same cells carry distinct families of complex oligosaccharides, other factors, such as the structure of the protein being processed, must also direct complex glycan assembly. Glycoproteins which are destined for the plasma membrane have sequences of hydrophobic amino acids which keep them bound in the membrane of the endoplasmic reticulum (Struck and Lennarz, 1980). It appears that they remain incorporated in the membrane of the vesicles which transport them first to the Golgi apparatus and then to the cell surface membrane (Rothman and Fine, 1980).

The biosynthesis of the serine-xylose-type linkage, which initiates the formation of the protein-carbohydrate linkage triplet (xylose-galactose-galactose) of connective tissue polysaccharides, involves the transfer of xylose to serine hydroxyl groups in the core proteins (Roden, 1980). It is not known whether xylose transfer occurs before the core protein has been completed on the polysomes, but the xylosyltransferase is said to be totally located in the cytosol (Guillaumond and Louisot, 1975; Serres-Guillaumond and Louisot, 1976). Galactosyltransferases I and II, each with an appropriately different specificity, then act in sequence and a specific glucuronyltransferase then transfers glucuronic acid to the second galactose residue. In the case of chondroitin sulphate alternating transfer of N-acetylgalactosamine and glucuronic acid to the non-reducing terminus of the growing
chain then follows, this polymerisation being brought about by two further enzymes, an N-acetylgalactosaminyltransferase and a second glucuronyltransferase.

GLYCOSPHINGOLIPIDS

The monosaccharide components of glycosphingolipids are the same as those listed above as the major components of oligosaccharide chains of glycoproteins of cell membranes, except that D-mannose and D-xylose are not found. Glycosphingolipids are derivatives of ceramide and consist of: the ceramide monosaccharides, or cerebrosides, containing either glucose or galactose, together with their sulphate derivatives (cerebroside sulphatides); ceramide oligosaccharides, containing as many as ten sugar residues, but no sialic acid; and gangliosides, which are ceramide oligosaccharides containing as many as three sialic acid residues (Hakomori, 1981).

GLYCOSPHINGOLIPID BIOSYNTHESIS

Glycosphingolipids are synthesised from nucleotide-sugars. The cerebrosides are synthesised by the transfer of either glucose or galactose from the corresponding nucleotide-sugar to the C-1 hydroxyl group of a ceramide. The ceramide oligosaccharides are synthesised by the sequential addition of sugar residues from the corresponding nucleotide-sugars to a cerebroside by a series of glycosyltransferases. Thus the biosynthesis is analogous to the biosynthesis of the O-linked oligosaccharides in glycoproteins. Gangliosides are synthesised similarly except that a sialic acid residue is
added at appropriate sites by sialyltransferase. Since each glycosyltransferase appears to have quite specific requirements for pH optima, nucleotide-sugar concentration, and both divalent cations and cofactors, it would be improbable that the optimum conditions for the synthesis of a given glycosphingolipid would prevail at all times. The multiglycosyltransferase system would thus predict considerable heterogeneity or incompleteness among glycosphingolipids (Dawson, 1978). Nevertheless it may well be that individual glycosphingolipids each have specific biological functions. Almost all glycosyltransferases are membrane bound and are associated in particular with the Golgi apparatus; the rough endoplasmic reticulum also contains a full complement of these glycolipid glycosyltransferases for synthesising gangliosides but the plasma membrane contains negligible ganglioside glycosyltransferase activities (Schacter et al., 1970; Keenan et al., 1974).

GLYCOPROTEINS AND GLYCOLIPIDS IN TRANSFORMATION AND MALIGNANCY

Relatively few studies of the effects of malignant transformation on membrane glycoproteins have been carried out. Warren et al. (1972) isolated glycopeptides from virus transformed baby hamster kidney (BHK) cells by gel filtration and found that transformed cells contained more early-eluting asparagine-linked glycopeptides, containing both fucose and sialic acid, than did non-transformed cells. This material has been shown to be similarly increased in a wide variety of cells (Warren et al., 1978). The early eluting glycopeptides from transformed cells were different from those from control
cells even though the proteins from which they came were the same molecular weight. Tuszyński et al. (1977) concluded that these results were a consequence of many changes in the carbohydrate components of many glycoproteins. Glick and Buck (1973) showed that the amounts of such high molecular weight glycopeptides were greater than those in untransformed cells. In similar studies Blithe et al. (1980) concluded that as a consequence of transformation there had been a shift in the biosynthetic pattern towards those glycoproteins containing large sugar chains. Van Beek et al. (1973) found that glycopeptides of this kind had a higher sialic acid content after transformation.

Warren et al. (1972) demonstrated an alteration in sialyltransferase activity in transformed cells. It was found that there was three times more sialyltransferase activity in the transformed cells than in the control non-transformed cells but this increase in activity appeared specific for transformed cell glycopeptide acceptor (Atkinson and Hakimi, 1980).

Morré et al. (1978) found that in rats bearing transplantable hepatoma serum levels of lipid-bound sialic acid were elevated 2.5 times. Similar results were obtained with sera from mice bearing transplantable mammary carcinomas and from human cancer patients. These workers also found that ganglioside patterns showed a progressive simplification in sequence from hyperplastic nodules through well differentiated hepatomas to poorly differentiated hepatomas. These findings provide new emphasis for gangliosides in both cancer detection and as regulatory signals for growth and multiplication of cells.
Takasaki et al. (1980) showed that polyoma-transformed BHK cells had more oligosaccharides, with highly branched outer chains, than did normal BHK cells. This increased branching was due to an increase in N-acetyl-glucosamination, which permitted the formation of abnormal oligosaccharide chains with five or six branches.

Kobata and Yamashita (1984) have characterised the structures of the sugar moieties of γ-glutamyltranspeptidase (GGT). This is the first time that the structure of the oligosaccharide chains of a glycoprotein enzyme has been determined. Earlier work by others had shown the physicochemical characteristics of GGT change according to the physiological state of cells, especially in malignant transformation (Taniguchi, 1974; Novogrodsky et al., 1976; Jaken and Mason, 1978; Tsuchida et al., 1979; Yamamoto et al., 1981). Fiala et al. (1972) found that GGT activity is tremendously elevated in hepatoma induced in rats by feeding 3'-methyl-4-dimethylaminoazobenzene. Since the elevation had been observed in the preneoplastic nodule of liver (Fiala and Fiala, 1973), the enzyme was expected to be a good marker for the diagnosis of hepatoma. GGTs produced in azo dye-induced rat hepatoma (Taniguchi, 1974), rat malignant mammary tissue (Jaken and Mason, 1978), and human renal carcinoma (Hada et al., 1978) had more acidic isoelectric points than those of GGTs in the respective normal tissues. Since the differences mostly disappear after sialidase treatment (Hada et al., 1978), the transformational changes of GGTs would appear to involve mainly the carbohydrate moieties of the enzyme molecules. Comparative studies of oligosaccharides
FIG. 1.4 Structure of sugar chains of γ-glutamyltranspeptidases purified from AH-66 cells and from normal rat liver (after Kobata and Yamashita, 1984)
obtained from the enzymes purified from rat AH-66 hepatoma and from normal rat liver revealed that more than 40% of the sugar chains of the hepatoma enzyme contain bisecting N-acetylglucosamine residues (Fig. 1.4) which are not found in those of the liver enzyme. Other structural differences among the sugar chains of the two enzymes have become evident from the work of Kobata and Yamashita (1984). Although heterogeneity was observed in the number of sialic acid and fucose residues per molecule, the structures of acidic sugar chains of liver GGT indicated that they are complete bi-, tri-, and tetra-antennary complex-type sugar chains. On the other hand, many of the complex-type sugar chains of the hepatoma GGT have incomplete outer chains. While bisecting N-acetylglucosamine residues were found in the sugar chains of GGT purified from rat, bovine and mouse kidney, they were not found in the sugar chains of the mouse and rat liver GGT. Therefore it appears that malignant transformation induces an enzyme which is suppressed in the normal liver tissue. Another important fact is that the total number of asparagine-linked sugar chains in one molecule of the AH-66 GGT was approximately four times higher than that in one molecule of the rat liver GGT. Since the enzyme from various tumour and normal tissues showed the same amino acid composition (Taniguchi et al., 1983) and the same immunogenicity, the increase in the number of sugar chains per molecule of the hepatoma GGT would appear to be induced by some change during biosynthesis of the GGT molecule. Possibly alterations in the relative rates of translation and glycosylation, and the degree of postranslational folding of the polypeptide chain (Pless and Lennarz, 1977)
when glycosylation takes place, may account for the difference in the number of sugar chains found in the iso-enzyme form of GGT. Kobata and Yamashita (1984) have also shown that a human hepatoma GGT has sialylated complex-type sugar chains with bisecting N-acetylg glucosamine residues and can be discriminated from the human liver GGT, which lacks the bisecting sugar chain, by its behaviour on agarose columns containing bound Phaseolus vulgaris lectin (Cummings and Kornfeld, 1982).

Other work from the laboratory of Kobata (Miguuchi et al., 1983) has shown that human chorionic gonadotrophin (hCG) from the urine of patients with choriocarcinoma contained abnormal sugar chains. More than 97% of the sugar chains of choriocarcinoma hCG were free from sialic acid, while the sugar chains of normal hCG were mostly sialylated. Choriocarcinoma hCG also contains unusual biantennary complex-type sugar chains in addition to regular tri-, bi-, and mon-antennary sugar chains. These sugar chains have two outer chains linked at the C-2 and C-4 positions of the same α-mannosyl residue of the tri-mannosyl core. There are also twice as many fucosylated chains as in normal hCG.

TRANSFORMATION, MALIGNANCY AND CYCLIC AMP

A recent review by Boynton and Whitfield (1983) has considered two conflicting views about the role of cyclic AMP in transformation and placed them in some perspective. Some of the earliest reports in this field suggested that cyclic AMP was a positive regulator of cell proliferation (Haynes et al., 1960; Selye et al., 1961; MacManus and Whitfield,
1969; Malamund, 1969; Sutherland, 1971). Other early reports (Burk, 1968; Ryan and Heidrick, 1968) indicated that cyclic AMP was a negative regulator, and in the course of the next decade further reports (Pastan et al., 1971; Sheppard, 1972; Teel and Hall, 1973; Cooper and Smith, 1973; Pastan and Johnson, 1974; Ryan and Heidrick, 1974; Berridge, 1975; Chlapowski et al., 1975; Pastan et al., 1975; Friedman, 1976; Friedman et al., 1976; Barker and Isles, 1977; Schonhofer and Peters, 1977) led to the overwhelming acceptance of the notion that cyclic AMP was a principal negative regulator of cell proliferation. Some may have thought of it as the primary regulator. Any evidence against this view was regarded by most investigators as merely an exception to the rule. As Boynton and Whitfield (1983) further point out "The reason for the acceptance of the negative view was a stream of convincing reports that cyclic AMP derivatives and cyclic AMP-elevating agents inhibited the growth of pure cultures of conventional established cell lines, and that the cyclic AMP levels in some cells rose as they became quiescent in confluent or serum-starved cultures and sharply dropped when they were stimulated to resume proliferating".

In challenging the hypothesis that cyclic AMP is a negative regulator Boynton and Whitfield (1983) describe examples of cyclic AMP (Mengato et al., 1979; Taylor-Papadimitriou et al., 1980; Tsang et al., 1980; Mares et al., 1981), derivatives of cyclic AMP (Klein and Loizzi, 1977; Nomura et al., 1978; Pentland et al., 1981; adenylate cyclase stimulators (Klee et al., 1980; Boynton and Whitfield, 1981; Whitfield et al., 1982) and cyclic nucleotide phosho-
diesterase inhibitors (Rechler et al., 1977; Lehnert, 1979) directly stimulating cell proliferation. They give evidence that the $G_1$ and $G_2$ programme of cell cycle events in a wide variety of cells each includes one transient cyclic AMP surge (Zeilig et al., 1976; Boynton and Whitfield, 1979; Whitfield et al., 1980). They conclude that a regulator such as cyclic AMP will have an entirely different effect when generated by the cell at the properly scheduled point or points in the cell cycle than it will when experimentally forced to appear in abnormal amounts at one or more inappropriate points in the cell cycle. This, they say, is because not only the profile of surface receptors, but also the profile of functioning enzyme systems comprising the target processes, changes as the cells progress through a cycle.

The above comments have dealt with the relationship between cyclic AMP and cell proliferation. In addition many workers have attempted to correlate levels of cyclic AMP with other cellular characteristics. However, it is often difficult to decide the extent to which changes in other cell properties are consequences of changes in cell proliferation rates, as distinct from those properties which are linked more directly to transformation. For example, several reports have correlated low intracellular cyclic AMP levels with the transformed cell phenotype (rapid growth, loss of contact inhibition, transformed-type cell morphology and orientation, and lectin agglutinability), and high cyclic AMP levels with a more normal cell phenotype (slow growth, contact inhibition, normal cell morphology and orientation, and non-agglutinability by lectins) (Otten et al., 1971; Carchman et al.,
Similarly several groups have reported that the addition of cyclic AMP, dibutyryl cyclic AMP or theophylline to transformed cell lines resulted in altered cellular morphology and a decreased tendency for cells to pile up in culture (Johnson and Pastan, 1971; Sheppard, 1971; Johnson et al., 1971; Willingham and Pastan, 1975a and b). Johnson and Pastan (1972) observed that transformed fibroblasts treated with dibutyryl cyclic AMP quickly became more adherent to the substratum and their motility decreased. Transformed fibroblasts that had previously grown in multilayers in which the cells were randomly orientated were converted to a monolayer of elongated cells growing parallel to each other. The cells appeared more normal. Curtis et al. (1979) demonstrated that cyclic AMP significantly inhibited tumour formation in mice when it was injected at the same time as a promoter of tumor formation. Cho-chung (1980) hypothesised that nuclear translocation of a cyclic AMP-receptor ternary complex in the cytoplasm may be the key event leading to tumor regression in vivo.

In extending these results to the study of transformation and malignancy caution needs to be exercised. If the lack of regulation in malignant cells was merely a consequence of abnormal cyclic AMP levels the situation would be relatively straightforward. If on the other hand the regulatory mechanism that constrains the proliferation of normal cells is missing or defective as a consequence of the failure of operation of some other mechanism in neoplastic cells, then the effects of cyclic-AMP on proliferation of neoplastic cells may bear no relation to the role of cyclic nucleotide in the
regulation of the proliferation of normal cells. Hunt and Martin (1980) considered that cyclic nucleotides are not universal determinants of cell growth rate in cultured cells but concede that this does not rule out the possibility of such a role in individual types of tumors. They concluded that changes in the rate of formation of cellular products in tumors may be due to derangement in cyclic nucleotide metabolism and that while it was not possible to generalise about the role of cyclic nucleotides in tumors, a comparative study in differential functions in normal and neoplastic cells in relation to cyclic nucleotide metabolism is of value.

**EFFECT OF EXTRACELLULAR CYCLIC AMP ON INTRACELLULAR CYCLIC AMP LEVELS**

There is a good deal of evidence which suggests that exogenous cyclic AMP does not enter cells but exerts its effects by being converted to adenosine, which stimulates surface membrane adenylate cyclase, thus raising the concentration of endogenous cyclic AMP (Sattin and Rall, 1970; Kaukel et al., 1972; Hilz and Kaukel, 1973; Clark and Gross, 1974; Granner et al., 1975; Snyder and Seegmiller, 1976; Green and Stanberry, 1977; Green, 1979). One exception to this is S-91 Cloudman and melanoma adenylate cyclase which is inhibited by adenosine; this inhibition is enhanced by Mn$^{2+}$ (Bregman et al., 1980; Wolf et al., 1981).

In this laboratory Sharmeen (1985) has found that the mean intracellular cyclic AMP level in B$\text{$_16$}$ melanoma cells grown to confluence in tissue culture for 72 h was 16.4 ± 5.0 (S.E.M.) pmol/10$^6$ cells, and that treatment for 48 h with
1 mM cyclic AMP and 1 mM theophylline added 24 h after seeding consistently led to a doubling of the intracellular cyclic AMP level (Student's 't' test $P < 0.001$, $n = 30$). Treatment with 1 mM cyclic AMP alone or 1 mM theophylline alone did not consistently result in a significant increase in the intracellular cyclic AMP level. She showed further that the cells degraded cyclic AMP in the growth medium to inosine. Although AMP was found to be an intermediate in this process no free adenosine was found in the medium; however, cells readily removed exogenous adenosine and at the same time free inosine appeared in the medium. When cells were incubated with inosine the intracellular level of cyclic AMP increased. Adenosine did not have this effect, even though it is rapidly converted to inosine, suggesting either that adenosine interferes with the stimulatory effect of inosine, or that adenosine may bind to inhibitory receptors and that inosine may interfere with this effect.

Although in the above experiments theophylline was originally used because of its capacity to inhibit phosphodiesterase (Butcher and Sutherland, 1962; Turle and Kipnis, 1967) leading to an increase in extracellular cyclic AMP levels, there are a number of alternative mechanisms for the action of theophylline and these have already been referred to (see p. 4). For example, it is possible that theophylline functions here by blocking the effect of extracellular adenosine on adenosine receptors (Blume et al., 1973).

Another approach to the study of the effects of intracellular cyclic AMP is to use analogues of cyclic AMP which are able to enter the cell directly. Dibutyryl cyclic AMP
appears to have a high resistance to extracellular and intracellular phosphodiesterases, permeates more readily into cells and becomes deacylated to the monobutyryl cyclic AMP, which has an affinity for protein kinases comparable to that of cyclic AMP (Kaukel et al., 1972; Hilz and Kaukel, 1973). 8-Bromo cyclic AMP produces effects similar to those of dibutyryl cyclic AMP and is often used to eliminate possible effects of the butyrate produced when dibutyryl cyclic AMP is used (Nielson and Puck, 1980; Prasad, 1980; Littlefield et al., 1982; D'Anna et al., 1983; Martel et al., 1983; Suthanthiran et al., 1983; Wintersberger et al., 1983). While analogues such as the above mimic cyclic AMP they may have other effects, attributable to the groups attached to the cyclic AMP, which complicate the interpretation of results.

SECRETION OF CYCLIC AMP

The energy-dependent egress of cyclic AMP from intact cells was first found by Davoren and Sutherland (1963). Sharmeen (1985) showed that when B_{16} melanoma cells were incubated in serum free medium at 37°C for 2 h the intracellular level of cyclic AMP did not change significantly but the extracellular level of cyclic AMP rose from zero to approximately 60 nM. When the experiment was carried out in the presence of 30 μM prostaglandin A1 the intracellular level of cyclic AMP increased; the extracellular level of cyclic AMP increased initially but subsequently decreased so that after 2 h of incubation the extracellular concentration of cyclic AMP was only 35% of that in the control. Prosta-
glandin A1 is known to be an inhibitor of cyclic AMP extrusion
(Rindler et al., 1978) and the above results suggest that the release of cyclic AMP by the control cells was due to energy-linked secretion and was not merely a consequence of leakiness of the cells. Cyclic AMP egress does not appear to be a major mechanism for disposing of intracellular cyclic AMP, but the quantity of cyclic AMP released by this mechanism from the cells may well have some extracellular function in vivo, possibly via degradation to adenosine (Barber and Butcher, 1983).

**CYCLIC AMP, GLYCOPROTEINS AND GLYCOLIPIDS**

Sakiyama and Robbins (1973) concluded that the glycosphingolipid pattern of transformed NIL cells remained unchanged when the cells were grown with dibutyryl cyclic AMP. Yogeeswaran et al. (1972) who studied the glycosphingolipids of normal and virally transformed 3T3 mouse fibroblasts reached similar conclusions. On the other hand, using a surface labelling technique, Gahmberg and Hakamori (1973) demonstrated clearly that the surface profile of transformed cells changed towards that of normal cells when their growth behaviour was reverted by the addition of exogenous cyclic AMP; i.e. the label in the high molecular weight galactoproteins greatly increased and the label in ceramide oligosaccharide (globoside) could be enhanced to a normal level. Mansoor Baig and Roberts (1973) noted significant changes in the composition of glycopeptides in Chinese hamster ovary (CHO) cells when these were grown in the presence of the more lipid soluble dibutyryl cyclic AMP. Truding et al. (1974) observed *de novo* synthesis of a glycoprotein (105,000 $M_r$), which was correlated with morphological differentiation, and
found that the synthesis of this glycoprotein could also be induced by added dibutyryl cyclic AMP. Van Veen et al. (1976) observed that when CHO cells were grown in the presence of dibutyryl cyclic AMP there was a decrease in the size of surface fucopeptides. Imada et al. (1980) demonstrated that added cyclic AMP markedly induced the surface expression of an integral membrane glycoprotein of 135,000 $M_r$ in CHO cells; a phosphodiesterase inhibitor potentiated this induction. The induction was more efficient in dense cultures than in sparse cultures, and Imada and Imada (1982) proposed that the interaction of surface components during cell-cell contact played a role in the expression of the surface glycoproteins.

Muramatsu and Muramatsu (1982) showed that the addition of dibutyryl cyclic AMP to embryonal carcinoma cells, which had been induced to differentiate by retinoic acid, led to a decrease in the synthesis of large fucopeptides whose core structure is composed of galactose and N-acetylglucosamine. Parish et al. (1978) found that cyclic AMP induced the synthesis of a new plasma membrane glycoprotein in the course of inducing differentiation of Dictyostelium discoideum. Williams et al. (1980), using a plasmid which contained a eukaryotic gene from Dictyostelium discoideum, demonstrated that cyclic AMP rapidly and specifically reduced the transcription of the eukaryotic gene; the gene product is a developmentally regulated lectin which is thought to play a role in cellular adhesion.
ALTERATIONS IN GLYCOSYLTRANSFERASE AND GLYCOSIDASE ACTIVITIES

All glycosyltransferases have a number of characteristics in common: they catalyze the transfer of a monosaccharide from a nucleotide-sugar to an acceptor molecule which may be from any of the groups of complex carbohydrates; addition is to the nonreducing end of the growing oligosaccharide chain; a membrane location is suggested for the biosynthesis of complex carbohydrates; and high enzyme specificity is shown (Corfield and Schauer, 1982b). Although there is an extensive literature on the glycosyltransferases (reviewed by Shur and Roth, 1975; Schacter and Roseman, 1980; Beyer et al., 1981), reports of changes in their activity, as they are affected by alterations in cyclic AMP levels or by transformation, malignancy or infection in a particular tissue, are not so numerous, and will be discussed further. Changes in glycosidase activities similarly affected will also be considered.

Cyclic AMP-related changes

There is some evidence for a possible role of cyclic AMP in receptor-mediated regulation of glycosyltransferase activities (McLawhon et al., 1981). These workers showed that exposure of a mouse neuroblastoma cell line to opiates produced a naloxone-reversible inhibition of cyclic AMP synthesis and prevented, in a concentration-dependent manner, the formation of both ganglioside $G_{M2}$ (GalNAc-[NeuNAc]-Gal-Glc-ceramide) from $G_{M3}$ (NeuNAc-Gal-Glc-ceramide) and ganglioside $G_{M1}$ (Gal-GalNAc-[NeuNAc]-Gal-Glc-ceramide) from $G_{M2}$ in cell-free extracts. In contrast, the receptor-mediated
elevation of intracellular cyclic AMP levels by agents such as prostaglandin E (in the presence of isobutylmethylxanthine), or the addition of dibutyryl cyclic AMP markedly stimulated the activities of UDP-N-acetylgalactosamine ganglioside $G_{M3}$ N-acetylgalactosaminyltransferase and UDP-galactose, ganglioside $G_{M2}$ galactosyltransferase. An overall increase in the synthesis of more complex gangliosides was also observed in a hybrid cell line following elevation of cyclic AMP levels by treatment with serotonin and pargyline. The data presented support the hypothesis that cyclic AMP may have a role in the regulation of sialoglycosphingolipid biosynthesis.

Sudo and Onodera (1975) found that galactosyltransferase and sialyltransferase in the plasma membrane of SV 40-transformed mouse cells were inhibited by the addition of dibutyryl cyclic AMP, while those of normal cells did not respond to this compound. The differential effects of added dibutyryl cyclic AMP on the membrane-bound glycosyltransferases were observed both in isolated plasma membranes and in intact cell membranes. They suggested that some of the morphological restorations of normal characteristics during reverse transformation are partly due to the direct effect of this compound on the cell membrane. By contrast they found that microsomal sialyltransferase activity was unaffected by the addition of dibutyryl cyclic AMP but microsomal galactosyltransferase activity was stimulated in both normal and transformed cells.

Other changes

A number of reports indicate that there is increased sialyltransferase activity in association with malignancy or
transformation. Bosman (1972b) found elevated levels of sialyltransferase activity and surface glycoprotein acceptor, as well as higher amounts of incomplete glycoprotein, in virally transformed mouse fibroblasts, as compared to the corresponding normal fibroblasts. Bosman and Hall (1974) detected elevated levels of sialyltransferase, neuraminidase, galactosyltransferase and β-galactosidase in malignant tumors of the human colon and breast. Aoi and Yokota (1978) showed that the level of sialyltransferase activity in the microsomes of human embryo kidney cells was increased when cells were infected with oncogenic adenovirus, and there was a positive correlation between the sialyltransferase activity and tumor-genicity. Steimer and Despont (1980) demonstrated that chronic lymphatic leukemia cells had higher levels of sialyltransferase and sialic acid content than did other lymphoid cells and considered that these findings may be responsible for the fact that these cells live longer than normal cells. Satt et al. (1981) found that leukemic thymus homogenates contained two-fold higher activities of the enzyme lactosylceramide sialyltransferase and CMP-N-acetylneuraminate: ganglioside $G_{M1}$ sialyltransferase, compared to homogenates of non-leukemic cells. Sometimes a particular sialyltransferase was increased while others were decreased. Takahashi et al. (1982) showed that Golgi fractions from rat hepatoma had much lower levels of glycoprotein sialyltransferase and of the CMP-N-acetylneuraminate:asialo-ganglioside $G_{M1}$ sialyltransferase, but had an increased activity of CMP-N-acetylneuraminate: asialo-ganglioside $G_{M3}$ sialyltransferase. Their results suggested that the hepatoma Golgi fraction had a high activity for the formation of di- and triglycosylceramides, for which
the liver Golgi fractions showed negligible activity. In other cases serum sialyltransferase levels were increased. Ip (1980) observed that elevation of serum sialyltransferase levels were closely related to the degree of cellular proliferation in growth of the mammary gland. Liu et al. (1979) showed that sialyltransferase was released in large amounts by two hepatoma cell lines but only in minimal amounts in a cell line derived from normal liver. Serum sialic acid levels and sialyltransferase levels have been investigated as monitors of tumor burden in malignant melanoma patients (Ryan and Fennelly, 1981) but according to other authors sialic acid levels more closely reflect tumor burden than does sialyltransferase activity (Silver et al., 1979; Herrmann and Gielen, 1979).

Other workers have found either no change, or a decrease, in sialyltransferase activity in the systems studied. Myers-Robfogel et al. (1981) showed that transformed chick embryo fibroblasts had higher neuraminidase activity and less total sialic acid content than did the corresponding untransformed cells; they found no alteration in the activity of the enzyme synthesizing or degrading the substrate for sialyltransferase (CMP-N-acetyllneuraminate). In a previous study they found no change in sialyltransferase activity in this system (Spataro et al., 1975). On some occasions the substrate binding properties of the enzyme may vary, as Olofsson et al. (1980) found; in both green monkey kidney cells and baby hamster kidney cells, infected with herpes simplex virus, the apparent $K_M$ of microsomal-bound sialyltransferase increased while the opposite was true for the galactosyltransferase. Spataro et al. (1978) considered that the decrease in cell surface sialic acid found in senescent human diploid fibro-
blasts might be due either to a sialyltransferase deficiency or to a decrease in the substrate, CMP-N-acetylneuraminate. Chatterjee (1979) noted that sialyltransferase activity was significantly lower in spontaneously metastasizing rat mammary tumors than in non-metastasizing tumors, while the levels of N-acetylglucosaminyltransferase were comparable in the two groups. Ingraham and Alhadeff (1978a) found that metastatic tumors in human liver appeared to have significantly reduced sialyltransferase activity and more bound sialic acid than did uninvolved adjacent tissue, and considered that end product inhibition may be occurring in the metastatic sites. Low sialyltransferase activity was not due to removal of the substrate, CMP-N-acetylneuraminate, by CMP-N-acetylneuraminate hydrolase. Alhadeff and Holzinger (1982a) showed that soluble sialoglycoconjugates from most human liver metastatic tumors gave gel filtration profiles different from those of non-cancerous human livers.

Sometimes alterations in glycosyltransferases found are of a qualitative rather than a quantitative nature. Podolsky and Weiser (1979) and Podolsky et al. (1978) found a cancer associated galactosyltransferase in pooled effusions, from patients with various cancers, which appears to be structurally and kinetically distinct from the normal enzyme. Podolsky et al. (1978b) and Podolsky and Isselbacher (1980) have also demonstrated that a galactosyltransferase glycopeptide acceptor prepared from human malignant effusions produces selective inhibition of transformed cell growth in animal and tissue culture systems. The cancer-associated galactosyltransferase acceptor functions as an acceptor for
the cancer-associated galactosyltransferase. Roth and White (1972) provided evidence to suggest that sparse 3T3 mouse fibroblasts incorporated more galactose than did confluent 3T3 fibroblasts and attributed this to the presence of greater amounts of galactosyl acceptors. Most studies indicate an increase in galactosyltransferase activity in association with malignancy or transformation. Chatterjee and Kim (1977) showed that galactosyltransferase activity was higher in spontaneously metastasizing as compared to nonmetastasizing rat mammary tumors. LaMont et al. (1977) found that there was a marked increase in specific activity of surface galactosyltransferase in serum-stimulated, as compared to resting, fibroblasts. Dividing but not resting fibroblasts released galactosyltransferase, but not sialyl- or fucosyltransferase, in soluble form into the tissue culture medium. The release of galactosyltransferase was greater from virally transformed than from nontransformed fibroblasts. Similar effects were obtained in lipid studies. In NIL-2 hamster fibroblasts the activities of two galactosyltransferases catalysing the formation of di- and triglycosylceramides increased considerably as culture density increased, although maximal activities were found before appreciable cell contact occurred; there was no evidence that the transformed cells produced a dialysable soluble inhibitor of transferase activities (Chandrabose et al., 1976). Lactosylceramide α-galactosyltransferase increased two- to three-fold in baby hamster kidney cells (BHK) and NIL hamster fibroblasts when the growth of these cells were contact inhibited. The activity of this enzyme in BHK and NIL cells was 10–50% of the activity of the growing normal cells and was not influenced by cell population density. On the other hand
β-galactosyltransferase did not increase as cell population density increased and was not affected by transformation. The activity of α-galactosyltransferase was higher in polyoma transformed cells (Kijimoto and Hakamori, 1971).

Much less evidence exists for variation in the activities of other glycosyltransferases or glycosidases in association with malignancy or transformation. Gorka (1977) found that N-acetylg glucosaminyltransferase, in spleen cells of mice injected with mouse mammary tumor virus, was enhanced, although this increase in activity was not due to synthesis of new enzyme. Chatterjee and Kim (1978) demonstrated a positive correlation between the capacity of rat mammary tumors to metastasize spontaneously and the level of a fucosyltransferase which transferred fucose to an acceptor with a terminal β-N-acetylg glucosaminide residue. Bauer et al. (1977) showed that fucosyltransferase activity in rat hepatoma was significantly elevated whereas sialyltransferase activity was greatly decreased. α-L-Fucosidase in the tumor was seven times higher than in host liver. The activity of neuraminidase in the tumor was lowered by 50% or remained unchanged when compared to the activity in host liver. Bauer et al. (1978) found that the determination of serum fucosyltransferase activity could facilitate the diagnosis of neoplasia and the success of surgery, chemotherapy or radiotherapy in the case of carcinoma of the colon.

In summary, it can be seen that no simple picture emerges indicating that there is a consistent relationship between the levels of glycosyltransferases and glycosidases which characterises transformed and malignant cells, in
contradistinction to normal cells. Nevertheless it does appear that in the majority of systems glycosyltransferases tend to be increased in activity in transformed and malignant cells. In situations where they are decreased in activity the presence of inhibitors and loss of enzyme from the cells need to be considered. Perhaps the fact that there is an alteration in the normal enzyme pattern is more significant than whether there is an increase or decrease in glycosyltransferase or glycosidase activity.

**B16 MELANOMA AND CYCLIC AMP**

Kreider *et al.* (1973) found that when a melanotic clone of B16 melanoma cells was treated with various concentrations of cyclic AMP, dibutyryl cyclic AMP or theophylline, cellular replication was retarded, melanogenesis was enhanced and morphological alterations such as cellular hypertrophy and exaggerated dendrite formation were observed. Kreider *et al.* (1975) studied a melanotic clone of B16 melanoma *in vivo* and *in vitro* and found that 12 h after adding 1 mM theophylline to growing cultures the number of cells incorporating $[^3H]$-thymidine and the rate of uptake of $[^3H]$thymidine into DNA were significantly reduced. After 24 h incubation with 1 mM theophylline cells contained approximately 80 pmol of cyclic AMP per mg of protein whereas control cells contained approximately 70 pmol of cyclic AMP per mg of protein (Student's 't' test $P < 0.01$, $n = 7$). After seven days the number of cells in the control cultures had increased twentyfour-fold, whereas theophylline-treated cells increased only six-fold. Compared to the controls the theophylline-treated cells contained ten times the melanin and an elevated cyclic AMP content. They
found also that theophylline treatment of hosts bearing B<sub>16</sub> melanoma tumors failed to reduce the tumor growth rate. Kolb and Manfield (1980) reported that B<sub>16</sub> melanoma cells grown for 48 h with 1 mM theophylline had cyclic AMP levels (specific values were not given) which were routinely four times greater than the levels in untreated B<sub>16</sub> melanoma cells.

Sheppard et al. (1984) found that the accumulation of cyclic AMP induced by melanocyte stimulating hormone or by forskolin showed a strong positive correlation with the ability of B<sub>16</sub> melanoma clones to form pulmonary tumor colonies. Highly metastatic tumor cell clones showed greater than a 30-fold increase in cellular cyclic AMP when exposed to melanocyte stimulating hormone or forskolin. By contrast clones with limited metastatic abilities responded to the same agonist with only a 2 to 3-fold increase in intracellular cyclic AMP. On the other hand, Agarwal and Parks (1983) showed that intraperitoneally administered forskolin reduced B<sub>16</sub> melanoma colonisation in the lungs of mice by more than 70%.

Sezzi et al. (1984) reported that flunarizine, a drug which binds to calmodulin, increases the intracellular concentration of cyclic AMP in B<sub>16</sub> melanoma cells and inhibits both the growth rate and decreases the survival fraction of the cells in vitro. However Walker et al. (1984) showed that about 50% of the total cyclic AMP phosphodiesterase activity in B<sub>16</sub> melanoma cells was calmodulin-activated. The response to cyclic AMP is also affected by retinoic acid. Rogelj et al. (1984) showed that retinoic acid increased the amount of a cyclic AMP binding protein (probably protein kinase I) in
B₁₆ melanoma cells.

Giotta et al. (1978) found that 0.5 mM GTP caused in vitro growth inhibition of B₁₆ melanoma cells after two days and also induced morphological and biochemical differentiation. Although the mechanism of these effects is unknown they considered that it was possible that GTP traversed the cell plasma membrane. They found that the cyclic AMP concentration of cells treated for four days averaged 1.5 pmol/mg of cell protein whereas the cyclic AMP concentration of control cells averaged 1.2 pmol/mg of cell protein, but nevertheless they thought it possible that GTP might have acted through cyclic AMP by causing transient increases in cyclic AMP levels.

Giotta et al. (1980) also found that 5-bromodeoxyuridine caused B₁₆Fl mouse melanoma cells to develop a flattened morphology and simultaneously adhere tenaciously to the substratum on which they were growing. The cellular concentration of cyclic AMP remained unchanged but there was a striking increase in the number of organised microtubules and microfilaments, although the amount of tubulin in the cells had not increased. They concluded that cyclic AMP and 5-bromodeoxyuridine may operate in this respect by similar but independent mechanisms. In view of the results of Yamada et al. (1976), who showed that the major cell surface glycoprotein of chick embryo fibroblasts caused cell flattening and increased adhesion to the substratum without elevating cellular cyclic AMP concentrations, Giotta and co-workers considered that the organisation of the cytoskeletal components could be directed from the cell surface. Both cyclic AMP and 5-bromodeoxyuridine have been shown to enhance synthesis of cell
surface-associated and secreted sialoglycoproteins (Goggins et al., 1972; Kreider et al., 1973; Sheik Fareed et al., 1978) and acidic mucopolysaccharides (Satch et al., 1975).

**B₁₆ MELANOLOGY, GLYCOCONJUGATES AND METASTASES**

Recently a number of laboratories have studied the biochemical differences between various B₁₆ melanoma cell lines which differed in their capacity to metastasize. Rieber and Rieber (1981) found that B₁₆ melanoma cells with lower metastatic potential have greater surface protease and/or glycosidase activity, which in turn leads to a greater turnover of the surface components required for cell-cell interaction and subsequent cellular survival during the invasive process. Rieber and Castillo (1984) have studied metastatic variants in B₁₆ melanoma produced by treatment with bromodeoxyuridine. Variants with low colonising ability have a hydrophilic variety of two particular glycoproteins (Mᵣ 140,000 and 110,000) whereas cells with increased lung colonising ability have proteins which are similar but hydrophobic and more susceptible to protease treatment. Reiber et al. (1984) have reported alterations in other surface glycoproteins involved in tumor cell detachment from extracellular matrix which also seem to be involved in metastatic behaviour, and McCarthy and Furcht (1984) have studied adhesive matrix glycoproteins which encourage B₁₆ melanoma cell migration.

Kramer et al. (1982) found that B₁₆ melanoma cells elaborated a glycosidase capable of cleaving glycosaminoglycans. Miner et al. (1982) showed that the exposure of a cell surface component (Mᵣ 90,000) of B₁₆ melanoma cells had a correlation
with high brain colonisation potential. Irimura et al. (1981) found that tunicamycin-induced cell surface glycoprotein changes in B16 F1 melanoma cells appeared to interfere with those tumor cell-host cell interactions that lead to arrest and survival of blood-borne malignant cells. Irimura and Nicholson (1981) concluded that the glycoproteins involved were those responsible for determining the adhesive properties of malignant cells. Schroeder and Gardiner (1984) studied membrane lipid composition in B16 melanoma cells with different metastatic properties and found that the highly metastatic B16 F10 melanoma cells had a lower cholesterol:phospholipid ratio and a lower polyunsaturated fatty acid content than cell variants with a low metastatic potential.

Yogeeswaran and Salk (1981) found that the ability of B16 melanoma and other murine tumor cells to metastasize was positively correlated with the total sialic acid content of the cells in culture, with the degree to which the sialic acid was exposed on the tumor cell surface, and particularly with the degree of sialylation of galactosyl and N-acetylgalactosaminyl residues in cell surface glycoconjugates. However, Yogeeswaran and Tao (1980) found previously that not all clones of B16 melanoma cells with reduced malignancy have a low surface sialic acid content. Dobrossy et al. (1981) found that when cells were treated with neuraminidase, highly metastasizing cells released approximately twice as much sialic acid as did cells with low metastatic potential.

Lotan and Raz (1983) found that cells of a B16 melanoma variant with a low metastatic potential failed to aggregate in the presence of asialofetuin in contrast with the parent
B₁₆ Fl melanoma cell line suggesting that the ability to undergo aggregation in the presence of glycoproteins is an important property of malignant cells which may influence anchorage-independent growth and the formation of metastases. Lin et al. (1983) found that several B₁₆ melanoma clones resistant to wheat-germ agglutinin toxicity had, compared to non-resistant clones, a decreased content of sialic acid in one glycoconjugate that binds to wheat-germ agglutinin, and had only O-linked oligosaccharides. Other work from the same laboratory has investigated whether acid mucopolysaccharides, especially hyaluronic acid, have a major role in cell adhesion. Satoh et al. (1974) showed that both melanotic and amelanotic clones of B₁₆ mouse melanoma cells produced high molecular weight chondroitin of varying sulphate content, predominantly 4-sulphate, but only insignificant quantities of hyaluronic acid. On the other hand mouse iris melanocyte cultures, which they used as a variety of control cell with respect to melanoma cells, did not produce chondroitin, and hyaluronic acid accounted for approximately half of their heteropolysaccharide. Similarly human foetal uveal melanocyte glycosaminoglycans were found to consist of chondroitin 4-sulphate (42%), heparin sulphate (25%), chondroitin 6-sulphate (16%), and hyaluronic acid (17%); whereas HM7 human melanoma cultures produced no chondroitin 6-sulphate, increased quantities of heparin sulphate, and less hyaluronic acid (Bhavanandn, 1981). The HM7 human melanoma cells by contrast contained O-glycosidically linked and N-glycosidically linked oligosaccharides which contained sialic acid, and these oligosaccharides were markedly reduced in foetal uveal melanocyte cells (Bhavanandn et al., 1981; Umemoto et al., 1981).
B16 MELANOMA, GLYCOSYLTRANSFERASES AND GLYCOSIDASES

Bosmann et al. (1973) examined cells from both sparse and confluent cultures of each of two cell lines of B16 melanoma cells, one with high metastatic potential and one with low metastatic potential. They measured the activity levels of enzymes incorporating glycosyl groups, supplied as the corresponding nucleotide-sugars, into either endogenous or exogenous acceptors. They found that 0.1% Triton X-100 homogenates of cells from sparse cultures of the line with high metastatic potential incorporated more glucosyl, sialyl, galactosyl and N-acetylglucosaminyl groups than did similar homogenates of cells with low metastatic potential. When confluent cells were used the increases were similar but smaller, except that there were no increases in glucosyl or galactosyl transfer when exogenous acceptor was omitted. When similar studies were carried out with cell suspensions instead of homogenates, and in the absence of exogenous acceptor, there were once again higher levels (two- to three-fold) of glycosyl transfer in the cells with a high metastatic potential; the groups transferred included, in addition to the four listed above, fucosyl and mannosyl groups. These workers also measured glycosidase activities in 0.1% Triton X-100 homogenates of cells from sparse B16 melanoma cultures, and found that those from a cell line with a high metastatic potential had more than twice the levels of β-D-galactosidase, β-L-fucosidase, N-acetyl-β-D-galactosaminidase, and N-acetyl-β-D-glucosaminidase than did those from a cell line with a low metastatic potential. The D-mannosidase activities were, however, the same for both cell lines. In addition they found
that, in sparse B₁₆ melanoma cultures, cells from a line with high metastatic potential had a much higher electrophoretic mobility than did cells from a line with low metastatic potential. Treatment with neuraminidase released 2.5 times as much sialic acid from the former than from the latter. In confluent cultures these differences were not found, cells from both lines resembling those in sparse cultures of the line with low metastatic potential.

Finne et al. (1980) found that N-glycosidic glycopeptides from a poorly metastasizing clone (Jumblatt et al., 1980), selected because it was resistant to agglutination by wheat-germ agglutinin, contained only half the amount of sialic acid than did the parent cells, and there was a concomitant increase in the amount of fucose in these glycopeptides. The lost sialic acid was bound to C-3 of galactose, whereas the increased fucose was found on C-3 of 4-substituted N-acetylglucosamine. Finne et al. (1982) isolated revertant clones from previously characterised wheat-germ agglutinin-resistant clones of B₁₆ melanoma cells by selection for resistance to Lotus tetragonolobus lectin. Comparison of the wheat-germ agglutinin-resistant clones with the parent and revertant clones indicated that this phenotype was correlated with an increased sensitivity to the Lotus lectin, a 60- to 70-fold increase in α-(1,3) fucosyltransferase activity and a decreased sialic acid content of the N-glycosidic chains of glycoproteins. They considered that the presence of α-(1,3) bound fucose on N-acetylglucosamine residues would interfere with the addition of sialic acid by α-(2,3) linkages in the carbohydrate units, and that this change could explain the
resistance to wheat-germ agglutinin and the increased sensitivity to the Lotus lectin. They suggested that a change in a regulatory gene for the fucosyltransferase could be the possible primary cause for the changed phenotype. In further work from the same laboratory Prieels et al. (1983) isolated a fucosyltransferase from wheat-germ agglutinin-resistant cells which is able to transfer fucose to oligosaccharides containing galactose β-(1,4)N-acetylglucosamine and galactose β-(1,4)glucose structures. It is unable to transfer fucose to sialylated glycoproteins.

The significance and consequences of the above changes in glycosyltransferases and glycosidases are for the most part obscure, but it is clear that differences in enzyme activity between various sublines of B₁₆ melanoma exist. Thus the effects of treatment with cyclic AMP or other agents may differ from one subline to another and generalisations concerning these effects can only be made with the appropriate reservations.

AIMS OF THE WORK IN THIS THESIS

The original motivation behind this work was the desire to obtain a better understanding of the biochemistry of melanoma. As discussed in the preceding sections, early work in this laboratory, based on the contention that there was an inverse correlation between intracellular levels of cyclic AMP and malignancy, had demonstrated effects of exogenous cyclic AMP and theophylline on glycoprotein structure in B₁₆ melanoma cells. The initial aim of the work in this thesis was to identify which glycosyltransferases and
glycosidases were altered in activity when cyclic AMP and theophylline were added to the medium in which the B₁₆ melanoma cells were grown. This assumes that the activities, specificities and locations of glycosyltransferases and glycosidases are principal determinants in the structure of the oligosaccharide chain of glycoproteins. This approach would not permit elucidation of the precise structure of sugar chains but it might indicate how normal function and structure of oligosaccharide chains of glycoproteins could be restored. A secondary aim of this work was to investigate whether morphological changes were associated with any such biochemical changes. In view of the lack of precise knowledge about the significance of morphological changes the interpretation of such findings requires considerable caution.

The importance of sialyltransferase and sialylation in malignancy have also been outlined in the introduction. Thus when it was found that treatment of B₁₆ melanoma cells with cyclic AMP and theophylline increased sialyltransferase activity the aims were extended to include an investigation of the nature of this increase and its relationship to neuraminidase activity. It was found that in B₁₆ melanoma cells grown with cyclic AMP and theophylline the B₁₆ melanoma neuraminidase was completely inhibited by the addition of the sialyltransferase substrate CMP-N-acetylneuraminate. Consequently the aims were further extended to include a detailed study of the mechanism of this inhibition. This section, because of the wide ranging biological significance of sialic acid (Schauer, 1985), extending beyond its role in malignancy, constitutes an important part of this thesis.
CHAPTER TWO

MATERIALS

GDP-L-[U-\(^{14}\)C]fucose (170 mCi/mmol), UDP-D-[U-\(^{14}\)C]-glucose (294 mCi/mmol), UDP-D-[U-\(^{14}\)C]galactose (347 mCi/mmol), GDP-D-[U-\(^{14}\)C]mannose (173 mCi/mmol), UDP-N-acetyl-D-[1-\(^{14}\)C]galactosamine (61.5 mCi/mmol), UDP-N-acetyl-D-[U-\(^{14}\)C]-glucosamine (323 mCi/mmol), CMP-N-acetyl-D-[4,5,6,7,8,9-\(^{14}\)C]-neuraminic acid (304 mCi/mmol and 164 mCi/mmol), N-acetyl-D-[4,5,6,7,8,9-\(^{14}\)C]neuraminic acid (250 mCi/mmol), D-[U-\(^{14}\)C]-glucose (298 mCi/mmol), N-acetyl-D-[U-\(^{14}\)C]mannosamine (293 mCi/mmol), L-[U-\(^{14}\)C]asparagine (105 mCi/mmol), D-[1-\(^{14}\)C]ribose (3.09 mCi/mmol), [2-\(^{14}\)C]uridine (56 mCi/mmol), [U-\(^{14}\)C]-cytidine (479 mCi/mmol), N-[\(^{3}\)H]acetyl-D-mannosamine (500 mCi/mmol) and DL-[G-\(^{3}\)H]threonine (1.43 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Bucks., U.K.). UDP-D-[U-\(^{14}\)C]xylose (160 mCi/mmol) and Aquasol scintillation cocktail were purchased from New England Nuclear (Boston, MA, U.S.A.). \([^{32}\text{P}]\)orthophosphoric acid (4.63 Ci/mmol) was obtained from the Australian Atomic Energy Commission (Lucas Heights, N.S.W., Australia). Cyclic AMP, theophylline, 4-aminopyrine, \(p\)-nitrophenyl-\(\alpha\)-D-galactopyranoside, \(p\)-nitrophenyl-\(\beta\)-D-galactopyranoside, \(p\)-nitrophenyl-\(\alpha\)-L-fucoside, \(p\)-nitrophenyl-\(\beta\)-L-fucoside, \(p\)-nitrophenyl-\(\alpha\)-D-mannoside, \(p\)-nitrophenyl-N-acetyl-\(\beta\)-D-glucosaminidase, \(p\)-nitrophenyl-N-acetyl-\(\beta\)-D-galactosaminidase, neuraminidase (Clostridium perfringens, type IX, 39 I.U./mg), N-acetyl-neuraminic acid, lactalbumin hydrolysate, HEPES, cytochrome C (type VI: horse heart), albumin (bovine), albumin (egg), \(\gamma\)-globulin (bovine), and polyethylene glycol were purchased.
from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Neuraminidase (Influenza virus, 1.6 I.U./mg), neuraminidase (Vibrio cholerae, 500 I.U./ml), pronase and forskolin were obtained from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). Foetal calf serum was purchased from the Commonwealth Serum Laboratories (Parkville, Vic., Australia). Minimum Essential Medium (EAGLE) (Cat. No. 410-1500) was purchased from Gibco Laboratories (Grand Island, N.Y., U.S.A.). Crystapen and streptomycin sulphate were obtained from Glaxo-Australia, (Boronia, Vic., Australia), and fungizone from E.R. Squibb and Sons (Princeton, N.J., U.S.A.). Bio-Gel A-0.5 m Agarose (200-400 mesh) and Bio-Gel P-300 (50-150 mesh) were obtained from Bio.Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-50 (Superfine), Sephadex G-25 and Dextran T500 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Trypsin (1:250 'Difco' certified) was purchased from Difco Laboratories (Detroit, MI, U.S.A.). 3-Methoxyphenol was obtained from Fluka AG (Buchs. S.G., Switzerland). 4-Methylumbelliferyl-N-acetyl-α-D-neuraminate ammonium salt tetrahydrate was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Berks., U.K.). 4-Methylumbelliferone was purchased from Boehringer Mannheim GmbH (Mannheim, West Germany). 2-(3-Methoxyphenol)-N-acetyl-α-D-neuraminic acid, originally obtained from Boehringer Mannheim GmbH and purified to remove excess p-unsubstituted phenols, was a gift from M.A. Stewart, Department of Biochemistry, University of Sydney (Sydney, Australia). All other chemicals used were of reagent grade.
CHAPTER THREE

METHODS

ANIMAL AND TUMOUR MAINTENANCE

The B₁₆ amelanotic mouse melanoma cell line (SZ₆) was maintained in vivo in C57 BL/6J mice. The tumour cell line was begun in the Biochemistry Department, University of Sydney, by Deveridge (1973). Initially the tumour was kindly supplied by Dr. E.M. Stephenson, Department of Zoology, University of Sydney. The C57 BL/6J mice were obtained from the Bosch animal house at the University of Sydney.

The tumour cell line was maintained in vivo by the serial subcutaneous transplantation of tumour homogenates from mature tumours into new hosts. A mouse with a mature tumour was killed by cervical dislocation, the tumour excised, washed with a small volume of phosphate buffered saline, placed on a watchglass and finely minced in 2 ml of phosphate buffered saline with the aid of a pair of curved scissors to form an homogenate. The tumour homogenate (0.2 ml) was injected subcutaneously into the left dorsal loin of the new host. The transplanted tumour subsequently underwent localized development and reached maturity 11-12 days after transplantation.

TISSUE CULTURE SOLUTIONS

Tissue Culture Medium:

8.46 g Minimum Essential Medium (EAGLE)
1.71 g sodium bicarbonate
0.36 g lactalbumin hydrolysate
2.38 g HEPES
24 mg crypstatpen
50 mg streptomycin sulphate
2.5 mg fungizone

The above were dissolved in distilled water, and the solution made up to 900 ml. To this 100 ml of foetal calf serum was added and the solution sterilized by Millipore filtration. Immediately before use the pH was adjusted to 7.4 by the dropwise addition of 1 M hydrochloric acid.

**Versene Buffered Saline:**

8.0 g sodium chloride
0.2 g sodium EDTA
0.2 g potassium chloride
1.15 g disodium hydrogen orthophosphate (anhydrous)
0.2 g potassium dihydrogen orthophosphate
0.2 g glucose
0.067 g sodium hydroxide
0.02 g phenol red

The above were dissolved in distilled water and the solution made up to 1 l, adjusted to pH 7.4 with 2.8% sodium bicarbonate (w/v) and sterilized by Millipore filtration.

**Hanks's Buffered Saline:**

8.0 g sodium chloride
0.4 g potassium chloride
0.12 g disodium hydrogen orthophosphate (anhydrous)
0.06 g potassium dihydrogen orthophosphate
1.0 g glucose

The above were dissolved in distilled water and the solution
made up to 1 l, adjusted to pH 7.4 with 2.8% sodium bicarb-
onate (w/v) and sterilized by Millipore filtration.

**Tris-Maleate Buffer:**

The pH of an aqueous solution of 0.05 M Tris
containing 0.25 M sucrose was adjusted to 7.4 at 4°C by the
dropwise addition of 1 M solution of maleic acid.

**Trypsin Solution:**

A solution of 2.5 g of 'Difco' certified 1:250
trypsin in 100 ml of Hanks's buffered saline was sterilized
by Millipore filtration.

**0.25% (v/v) Trypsin-Versene Buffered Saline:**

A 5 ml volume of the trypsin solution was diluted
to 50 ml with versene buffered saline.

**Phosphate Buffered Saline:**

8.0 g sodium chloride
0.2 g potassium chloride
0.2 g potassium dihydrogen orthophosphate
1.146 g disodium hydrogen orthophosphate (anhydrous)
0.01 g phenol red

The above were dissolved in distilled water and the solution
made up to 800 ml. To this were added 100 ml of 9 mM calcium
chloride and 100 ml of 10.5 mM magnesium chloride, and the
solution was sterilized by Millipore filtration.

**TISSUE CULTURE**

Tissue cultures were set up from homogenates prepared as described above. To aid cell dispersion 2 ml of 0.25% (w/v) trypsin-versene buffered saline were added to the homogenate, which was then mixed for 3 min with a pasteur pipette. The trypsin was then inactivated with 4 vol. of tissue culture medium. The suspension was centrifuged at 110 g ($r_{av.} 10 \text{ cm}$) for 3 min at room temperature. The supernatant was aspirated off and the pellet from the homogenate resuspended in 10 ml of tissue culture medium. Aliquots of this suspension were transferred to 168 ml McCartney bottles, and the contents made up to a final volume of 10 ml with tissue culture medium. Bottles were laid flat to allow growth over the largest possible area.

Following overnight growth at 37°C the tissue culture medium was discarded and replaced by fresh tissue culture medium. The medium was again replaced at 48 h intervals until the cells reached a confluent growth pattern, as monitored by an inversion light microscope.

Once confluent growth was attained the culture was passaged as follows in order to increase cell numbers from the parent tumour. Old tissue culture medium was discarded and the culture washed with 10 ml of versene buffered saline. The cells remained adherent to the glass substratum. To encourage cell dislodgment 3 ml of 0.25% (w/v) trypsin-versene buffered saline were added to the bottles and with
the aid of a silicon wedge and judicious shaking the cells were removed from the glass substratum. The trypsin was then inactivated by tissue culture medium (as above) and the cells resuspended in an appropriate volume of tissue culture medium. For seeding into McCartney bottles 1 ml containing approximately $10^6$ cells was used, and 9 ml of tissue culture medium added. For seeding into Falcon flasks (25 cm$^2$/side) 1 ml containing $5 \times 10^5$ cells was used and 4 ml of tissue culture medium added. The newly passaged cells were incubated at 37°C and the medium changed after 24 h.

Unless otherwise indicated cells used had been passaged only once and were harvested 72 h after subculturing. Where indicated, cyclic AMP and theophylline were added 24 h after passaging, each to a final concentration of 1 mM. Labelling with radioisotopes was also carried out as indicated, 24 h after subculturing.

SCANNING ELECTRON MICROSCOPY

$B_{16}$ melanoma cells for morphological observation by scanning electron microscopy (SEM) were grown to subconfluent or confluent densities on small, dry heat-sterilized glass cover slips located in Falcon tissue culture flasks containing 5 ml of tissue culture medium. To half the number of flasks 1 mM cyclic AMP and 1 mM theophylline were added. Cell fixation was a modification of the method described by Arro et al. (1981). The fixative, 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer with 0.1 M sucrose (pH 7.2: total osmolality 510 mOsmol; vehicle osmolality
300 mOsmol), was prewarmed to 37°C and slowly added to the original tissue culture medium (296 mOsmol) until equal volumes of each were present. Care was taken to add the fixative without moving the culture dish and to maintain the temperature at 37°C. The cell culture was left standing undisturbed for 10 min, after which time it was gently rinsed with, and ultimately replaced by, pure fixative, and then left for 1 hour at room temperature before being stored overnight at 4°C. This time scale was found to be most suitable for the preservation of motile structures and did not result in artefacts due to precipitation of protein from tissue culture medium. After initial glutaraldehyde fixation the cells were washed three times in 0.15 M sodium cacodylate-HCl buffer, pH 7.2, and subjected to secondary fixation using 1% (w/v) OsO₄ in 0.15 M sodium cacodylate-HCl, pH 7.2, for 1 h at room temperature. Following OsO₄ fixation the cells were dehydrated in a graded series of ethanol solutions (50, 70, 80, 90, 100% ethanol in water v/v) and dried with CO₂ using the critical point drying method of Anderson (1951). The dried specimens were mounted on aluminium stubs and covered with 10 nm of gold-palladium using a magnetron sputter coating process (custom modified Dynavac coating unit, University of Sydney, Sydney, Australia) calibrated with a filament thickness monitor. This thickness was necessary to ensure adequate secondary electron production. Dried coated specimens were examined immediately in a Jeol JSM 35C scanning electron microscope. Prolonged storage was found to alter the fine surface detail of the specimens.
HISTOLOGICAL PREPARATION OF CELL CULTURES

B16 melanoma cells were grown in Falcon tissue culture flasks and on small, dry heat-sterilized glass cover slips located in the tissue culture flasks for 72 h, for the last 48 h of which cyclic AMP or related derivatives, and/or theophylline, were present in the tissue culture medium (1 mM cyclic AMP and 1 mM theophylline; 1 mM dibutyryl cyclic AMP and 1 mM theophylline; 1 mM 8-bromocyclic AMP and 1 mM theophylline; 1 mM theophylline; or 1 μM adenosine). As used in fixation for scanning electron microscopy, 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose was prewarmed to 37°C and added to the cultures which were then left to stand for 10 minutes at 37°C. This was followed by gentle rinsing with pure fixative and the cultures were then left to stand in fresh fixative for 20 minutes at room temperature. The cells were then washed twice with phosphate buffered saline, and the flasks broken to expose the cells, which were then stained with Mayer's Acid Alum Haemotoxylin (modified 1942) and Phloxine, and monitored until suitable colour developed. The stained slides were mounted using 10% (v/v) phosphate buffered saline in glycerol and photographed using a Zeiss photomicroscope and Kodak Ektachrome 160 Tungsten film.

HARVESTING

Unless otherwise indicated confluent cells were harvested in Hanks's buffered saline at 37°C (pH 7.4). The medium was decanted and the cells were washed with 10 ml of
Hanks's buffered saline. Cells were then allowed to stand for 20 min in fresh Hanks's buffered saline. The bottles were then gently agitated and the cells removed from the substratum with the aid of a silicon wedge. They were then washed twice with Hanks's buffered saline, and counted using a Neubauer haemocytometer. Unless otherwise indicated cells were suspended at a concentration of $5 \times 10^6$ ml. In some earlier experiments cells were harvested at 4°C by a similar procedure in which 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose, replaced Hanks's buffered saline.

**RADIOACTIVE COUNTING**

Radioactivity of aqueous solutions or of scrapings from TLC plates was measured by counting samples in Aquasol or toluene-Triton X-100 (2:1, v/v) scintillant containing 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-di[2-(5-phenyloxazolyl)]benzene using a Nuclear Chicago Isocap 300 liquid scintillation spectrometer, calibrated according to the manufacturer's recommendation before use. Counts per minute (c.p.m.) were corrected for quenching and converted to disintegrations per minute (d.p.m.) from which pmol of labelled substrate incorporated were calculated. Radioactivity of material dried on glass fibre discs or on paper was measured by counting samples in toluene scintillant which was similar to the above except that 10 ml of toluene replaced the 10 ml of toluene-Triton X-100 mixture. It is not possible to prepare a quench correction curve for samples counted on the paper or glass fibre discs since samples are soluble in the quenching agent (water). Therefore a sample from a known
radioactive standard, treated in the same way as the sample being assayed, was used to calculate the counting efficiency. Picomoles of substrate incorporated were calculated from d.p.m. In later experiments the glass fibre discs were dispensed with and the dissolved samples were counted in 10 ml of Aquasol scintillant and the results corrected for quenching using a quench curve. Non-radioactive blanks appropriate for each system were measured and the resulting values subtracted from the counts for each sample.

GLYCOSYLTRANSFERASE ASSAY

The method used was based on the assay for cell suspensions, as described by Bosman (1972b); whereby incorporation of labelled substrate into acid-precipitable material was measured. This method is suitable for assaying enzymes on the outer surface of the plasma membrane (ectoglycosyltransferases) and has been used by numerous workers for this purpose (Patt and Grimes, 1976; Spataro et al., 1979; Pan and Datta, 1980; Dobrossy et al., 1981; Duc Dodon et al., 1984). This method is also suitable for assaying intracellular glycosyltransferase activity in broken cell preparations or in intact cells where some mechanism exists for transport of the nucleotide-sugar into the cell.

Either Hanks's buffered saline, pH 7.4, or a solution of 0.25 M sucrose in 50 mM Tris-maleate buffer, pH 7.4, was used, as indicated in individual protocols, to suspend the cells for incubation and to redissolve the labelled nucleotide-sugar substrates, which had been dried under N₂. Each
incomplete assay mixture contains $5 \times 10^5$ B16 melanoma cells in 0.1 ml in one of the above solutions, and in addition either 10 µl of 0.1 M MnCl$_2$ or 10 µl of water. When cells were harvested with 0.25 M sucrose in 50 mM Tris-maleate buffer, pH 7.4, at the temperature of melting ice, they were preincubated for 15 min before being assayed. This was not necessary when Hanks's buffer (pH 7.4) was used and cells were harvested at 37°C. The reaction was initiated by the addition of 450 pmol of nucleotide-sugar in a volume of 10 µl. After incubation for various time intervals at 37°C, each reaction was terminated by the addition of 0.4 ml of 1% (w/v) phosphotungstic acid in 0.5 M HCl. Each precipitate was collected by centrifugation at 1000 g ($r_{av}$. 13 cm) for 10 min, washed twice with 10% (w/v) trichloroacetic acid and once with ethanol/ether (2:1, w/v), and dried at 80°C. The dried sample was then dissolved in 0.5 ml of 1 M NaOH at 37°C, and 100 µl of this solution was applied to a glass fibre disc (Whatman GF/C, 2.4 cm diameter) and dried at room temperature. Radioactivity was measured in 10 ml of toluene scintillant prepared as described in Radioactive Counting, and the results expressed as pmol of exogenous substrate incorporated. Blanks were prepared in which reaction mixtures containing all components except substrate were boiled for 10 min; after the subsequent addition of substrate they were incubated for 2 h at 37°C.

Preliminary studies indicated that the addition of bovine serum albumin (to increase the bulk of the precipitate and reduce losses during washing) immediately before the addition of phosphotungstic acid, in the above method, made
no difference to the amount of label measured as being incorporated from CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminic acid and GDP-[\(^{14}\text{C}\)]mannose. Such a step was therefore omitted from the procedure.

A limitation of this assay system is that unless adequate exogenous glycosyl-acceptors are included the glycosyltransferase activity measured in this system is not necessarily proportional to the quantity of glycosyltransferase present, as the number of glycosyl-acceptors may be limiting. However, it was the intention of this project to measure the changes in activity in cells in which exogenous acceptors were not available. Changes in activity of sialyltransferase with respect to an exogenous acceptor (asialofetuin) have been measured by Sharmeem (1985) and will be discussed later.

**DISRUPTION OF CELLS FOR SIALYLTRANSFERASE (EC 2.4.99.1)**

**ESTIMATION**

Cells were suspended at a concentration of 5 x 10^6/ml in 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose, and disrupted either by sonic oscillation for 6 sec in a Branson B-12 Sonifier (20 kHz; power output 7 w) or by homogenizing in a 7 ml Dounce homogenizer with 50 strokes of a B pestle. Both procedures were carried out in an ice bath and monitored by light microscopy. The disrupted cells were dispensed in 100 µl aliquots for assay purposes.
GLYCOSIDASE ASSAY USING B16 MELANOMA CELL HOMOGENATE

*p*-Nitrophenyl substrates were used to estimate some glycosidase activities in B16 melanoma cell homogenates using the procedures of Bosman (1972a, 1973). Cells were harvested in Hanks's buffered saline, pH 7.4, as previously described, then suspended at a concentration of $5 \times 10^6$/ml in 0.1% Triton X-100 in Hanks's buffered saline, pH 7.4, and homogenised in a Dounce homogenizer with 50 strokes of a tight fitting A pestle at the temperature of melting ice. To obtain the supernatant as used by Bosman (1972a, 1973), the homogenate mixture was stirred for 16 h at 5°C before being centrifuged at 30,000 $g$ ($r_{av.}$ 11.02 cm) for 1 h at the same temperature. The amount of a given glycosidase in the cell extract was determined by taking 200 µl of cell extract (except for N-acetyl-β-D-glucosaminidase and N-acetyl-β-D-galactosaminidase assays where 40 and 100 µl of homogenate respectively, made up to a total volume of 200 µl with Hanks's buffered saline, pH 7.4, was used), 200 µl of 0.5 M citrate buffer, pH 4.3, and 400 µl of the appropriate 2 mM *p*-nitrophenyl substrate. The mixture was incubated at 37°C for 1 h and the reaction terminated using 400 µl of 1 M Tris hydrochloric acid buffer, pH 8.3 (Asp, N-G., 1981). The resultant colour was read at 420 nM in a Varian (SuperScan or series 634) spectrophotometer (Varian Instrument Group, Walnut Creek, CA, U.S.A.). *p*-Nitrophenol was used as the standard, and an absorbance of 0.8 was equivalent to 55 pmol per ml. Parallel substrate blanks were determined with each estimation.
NEURAMINIDASE (ACYLNEURAMINYL HYDROLASE, EC 3.2.1.18) ASSAY

Neuraminidase was assayed by three methods. The first was a modification of the fluorometric method developed by Poitier et al. (1979) with 4-methylumbelliferyl-α-D-N-acetyl-neuraminate as the substrate. This assay was used both on homogenates and on supernatants from cell suspensions, but the preliminary procedure for each of these was different as set out below. The second assay was a modification of the spectrophotometric method described by Santer et al. (1978) using 2-(3-methoxyphenyl)-N-acetyl-α-D-neuraminate. This assay was used for supernatants from cell suspensions, but discontinued during the project as the substrate became unavailable. Results from the fluorometric and spectrophotometric assays were comparable. The third method was developed in this laboratory and was based on radioactive labelling of cells.

Neuraminidase Assay Using 4-Methylumbelliferyl-α-D-N-acetylneuraminide

(a) Procedure for homogenates

B16 melanoma cells were harvested in Hanks's buffered saline, pH 7.4, as previously described, suspended at a concentration of 10⁷ cells/ml in harvesting buffer, then cooled to the temperature of melting ice and homogenised in a Dounce homogeniser with 50 strokes of a tight fitting A pestle. The homogenate was then sonicated with two bursts, each of six seconds duration, using a Branson B12 Sonifier (20 kHz; power output 7 w) at the same temperature. The sonicate was
centrifuged at 30,000 g (r_{av.} 11.02 cm) for 1 h at 4°C and the supernatant was removed and assayed. Substrate (300 μl of 0.1 mM 4-methylumbelliferyl-α-D-N-acetylenuraminate in 0.2 M sodium phosphate buffer, pH 6.0) was mixed with 300 μl of homogenate supernatant and incubated at 37°C. The incubation was stopped after 1 h with 3 ml of 0.133 M glycine buffer, pH 10.7, containing 0.06 M sodium chloride and 0.083 N sodium carbonate. Free 4-methylumbelliferone was measured in an Aminco SPF 500 ratio spectrofluorometer (American Instrument Co., Silver Springs, MD, U.S.A.) using excitation light at 365 nm and fluorescence emission at 450 nm. As 4-methylumbelliferyl-α-D-N-acetylenuraminate is prone to hydrolysis on incubation (Poitier et al., 1979) reaction mixtures to be used as substrate blanks (from which enzyme and homogenate supernatant were omitted) were prepared and used to set the zero reading on the spectrofluorometer scale. Reaction mixtures to be used as enzyme blanks (from which substrate was omitted) were also prepared and did not give significant fluorescence. Standards were prepared with 4-methylumbelliferone, using the same settings that were used for the assay measurement (Damping, 0.1; Band-pass 4), and the experimental values converted to pmol using the standard curve.

(b) **Procedure for supernatants from cell suspensions**

This method was used principally to assay influenza virus neuraminidase activity, which was determined as in the preceding section, with the cell wash (supernatant obtained after B_{16} melanoma cells had been incubated with influenza virus neuraminidase) replacing the homogenate supernatant. In some experiments incubations were also carried out for
various time intervals where the cells were not removed prior to the addition of substrate but were centrifuged from the suspension before fluorometric measurements were taken. Reaction mixtures to be used as substrate blanks (from which enzyme and cells were omitted) were prepared and used to set the zero reading on the spectrofluorometer scale. Reaction mixtures to be used as enzyme blanks (from which substrate was omitted) were also prepared and did not give significant fluorescence.

Neuraminidase Assay Using 2-(3-methoxyphenyl)-N-acetyl-α-D-neuraminate

Substrate (100 μl of solution containing 1.0 mg of 2-(3-methoxyphenyl)-N-acetyl-α-D-neuraminate per ml of 0.2 M sodium phosphate buffer, pH 6.0) was mixed with 100 μl of cell wash (supernatant obtained after B16 melanoma cells had been incubated with influenza virus neuraminidase) and the incubation was stopped after various times by the addition of 1.3 mM amino-antipyrine in 1 M Tris-hydrochloric acid buffer pH 8.5, containing 1.33% (v/v) ethanol. As reported by Palese et al. (1973) 2-(3-methoxyphenyl)-N-acetyl-α-D-neuraminate is very stable at pH 6.0. A pink colour developed rapidly after the addition of 50 μl of freshly prepared 6 mM potassium ferricyanide. This colour, stable for several hours at room temperature (Asp, N-G., 1971), was read at 400 nm in a Union Carbide Centrifichem (RocheSerius, Sydney, Australia) or in a Varian (SuperScan or series 634) spectrophotometer. Reaction mixtures to be used as enzyme blanks (from which substrate was omitted) and as substrate blanks (from which
enzyme was omitted) were also prepared. These were treated similarly and the resulting values subtracted from the assay results. Standards were prepared with 3-methoxyphenol, and the pmol of product formed were obtained from a standard curve prepared for each series of assays.

**Neuraminidase Assay Utilising Radioactive Labelling of Cells**

B16 melanoma cells were grown in the presence of the sialic acid precursor (Monaco et al., 1975) N-[3H]acetylmannosamine (2 μCi/10 ml medium) for 48 h. The cells were harvested and washed three times and suspensions of 10⁶ cells/0.1 ml of Hanks's buffer were incubated for various times at 37°C. The reaction was stopped by 0.4 ml of 1% phosphotungstic acid in 0.5 ml HCl. The cell precipitates were washed with 10% (w/v) trichloroacetic acid twice, followed by an ethanol:ether (2:1) wash. The cell precipitates were dried at 80°C and then dissolved in 0.5 ml of 1 M NaOH by overnight incubation at 37°C. 200 μl of this was counted for radioactivity incorporation using 10 ml of Aquasol scintillant. The difference in ³H incorporation between 0 time and 2 h was the neuraminidase activity during that time, as the radioactive material released in the supernatant was then identified as sialic acid by thin layer chromatography (Fig. 4.9b).
MODEL SYSTEM FOR STUDYING NEURAMINIDASE INHIBITION: EFFECT OF CMP-N-ACETYLC-[\textsuperscript{14}C]NEURAMINATE ON INFLUENZA VIRUS NEURAMINIDASE INCUBATED WITH \textit{B_{16}} MELANOMA CELLS

\textit{B_{16}} melanoma cells suspended in Hanks's buffered saline, pH 7.4, were incubated with influenza virus neuraminidase (5 I.U. per 10\textsuperscript{7} cells), with or without CMP-N-acetyl-[\textsuperscript{14}C]neuraminate (4.5 nmol/ml). After various time intervals the cell suspension was centrifuged at 8000 g (\textit{r}_{av}. 5 cm) for 2 min and the supernatant (cell wash) was removed and assayed for neuraminidase activity by one of the two alternative methods described in the preceding section. For h.p.l.c. experiments 0.3 ml reaction mixtures were used.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) of sialic acid and sialyl compounds was undertaken on aluminium sheet silica gel 60 plates (Merck, Art. 5553, 20 x 20 cm) which had been activated by heating to 100°C for 20 min. Samples were spotted 2 cm from the bottom of the plate and run in various solvent systems. A satisfactory system for separation was that of Hansen (1975) where isopropanol:acetone:0.1 M lactic acid (2:2:1 v/v/v) solvent was used. After chromatography the plates were air dried, the section containing the N-acetyl-neuraminate marker cut off and stained with an aniline-diphenylamine solution and then heated to 105°C for 15 min to develop the colour. Radioactivity on the silica gel plates was determined by scraping 1 cm sections into scintillation vials containing 10 ml of Triton-toluene scintillant and
counted using a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. Blanks were prepared by scraping a similar amount of gel from a non-radioactive plate into a scintillation vial and counting as above.

GEL FILTRATION CHROMATOGRAPHY

B16 melanoma cell extracts were examined by High Performance Liquid Chromatography (h.p.l.c.), or by liquid chromatography using Sephadex or Agarose. For h.p.l.c., samples (250 µl) were applied to Varian Micro Pak TSK G2000 SW (0.75 cm x 30 cm) or TSK G 3000 SW (0.75 cm x 50 cm) steric exclusion columns, each protected by a 10 cm TSK SW guard column and connected to a Varian (Model 5020) liquid chromatograph (Varian Instrument Group, Walnut Creek, CA, U.S.A.). The columns were eluted with Hank's buffered saline, pH 7.4, at a flow rate of 1 ml per minute and a pressure of 18 atmospheres at 25°C. Elution profiles of absorbance at 280 nm were obtained using a Vari-chrom continuous flow liquid chromatography detector. In addition, 0.5 ml fractions were collected for radioactive analysis where appropriate. To separate glycoproteins extracted by Triton X-100, 2.5 cm x 70 cm columns of Bio-Gel A-0.5 m Agarose (200-400 mesh), 2.5 cm x 70 cm columns of Bio-Gel P-300 Acrylamide (50-150 mesh), and 0.8 cm x 90 cm columns of Sephadex G-50 (superfine) were used. Bio-Gel A-0.5 m and P-300 columns were washed with 3 bed volumes of the eluant buffer (0.05 M Tris-chloride, pH 8.0, containing 1 mM Triton X-100) before use. Likewise the Sephadex G-50 columns were washed before use with 3 bed volumes of 0.1 M Tris-acetate, pH 9.0 containing 0.1% (w/v)
2-mercaptoethanol. For all reported results a recovery of labelled substrate greater than 95% was obtained. During storage of Sephadex or Agarose columns sodium azide was added to the column buffer to give a final concentration of 0.05% (w/v). For storage of h.p.l.c. columns the buffer was replaced with methanol. When required u.v. absorption spectra of eluted fractions were recorded using a Varian SuperScan spectrophotometer and the area under the curve calculated using a Varian CDS 111L recorder.

PREPARATION OF TRITON X-100 EXTRACTS

Cell surface membrane proteins were solubilised with Triton X-100; this technique has been used to solubilise many membrane proteins without loss of biological activity (Bonsall and Hunt, 1971; Clarke, 1975; Helenius and Simons, 1975; Pollet et al., 1981).

Extracts of B16 Melanoma Cells

B16 melanoma cells awaiting extraction were kept at the temperature of melting ice. Buffer was removed after centrifugation at 150 g (r̄, 13 cm) for 3 min. B16 melanoma cells were then resuspended (5 x 10⁶ cells per ml) in ice cold 1 mM Triton X-100 in 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose, and incubated at the temperature of melting ice for 20 min. B16 melanoma cells were then removed by centrifugation at 1000 g (r̄, 13 cm) for 10 min and the supernatant (Triton X-100 extract) was collected. Electron microscopy carried out as a check on this procedure
revealed that the surface membrane was stripped from the cell. The remainder of the cell appeared to be intact but it is inevitable the membrane extract will be contaminated with some intracellular material.

Extracts of B₁₆ Melanoma Cell Surface Membranes

B₁₆ melanoma cell surface membranes isolated by the procedure to be described below were centrifuged at 4000 g (r<sub>av</sub>. 25 cm) for 10 min and resuspended (membranes from 6 x 10<sup>7</sup> cells per ml) in ice cold 1 mM Triton X-100 in 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose. The suspension was incubated at the temperature of melting ice for 20 min, the membranes removed by centrifugation at 4000 g (r<sub>av</sub>. 25 cm) for 10 min and the Triton X-100 extract collected.

PRONASE DIGESTION OF MATERIAL DERIVED FROM TRITON X-100 EXTRACTS OF B₁₆ MELANOMA CELLS

Pooled fractions of eluate were dialysed, lyophilised and resuspended in 1 ml of phosphate buffered saline, pH 7.4, and incubated with 20 μl of a 1% solution of pronase in the same buffer, pH 7.4, added daily for five days, under toluene. At the end of the five day incubation the samples were centrifuged at 1000 g (r<sub>av</sub>. 13 cm) for 15 min and the supernatant collected (Mansoor Baig and Roberts, 1973).
ISOLATION OF CELL SURFACE MEMBRANES

A two phase polymer system, based on the method of Warren and Glick (1969), was used for the isolation of cell surface membranes. The two phases were prepared by mixing the following solutions in a 500 ml separating funnel and allowing them to separate into their two phases at 4°C over 48 h:

100 g  20% (w/w) dextran T500
51.5 g  30% (w/w) polyethylene glycol
166.5 ml  0.22 M phosphate buffer, pH 6.5
40 ml  0.01 M zinc chloride
49.5 ml  distilled water.

The cells for surface membrane isolation were harvested in Hanks's buffered saline as described in Methods and then washed twice in 0.15 M sodium chloride before transferring them to a Dounce homogenising flask. Seven volumes of 1 mM zinc chloride was then added to the homogenising flask and the cell suspension mixed by gentle shaking. The cells were swollen for 10 min at room temperature followed by 10 min at 4°C. A loose-fitting (type B) pestle was used to rupture the cells at 4°C and 90-95% breakage, as monitored by light microscopy, was obtained with 20-30 strokes. The homogenate was then centrifuged at 2500 g (r<sub>av.</sub> 25 cm) for 60 min. The pellet was resuspended in 10 ml of the upper phase of the two phase polymer system and then mixed with 10 ml of the lower phase. This was centrifuged at 3500 g (r<sub>av.</sub> 25 cm) for 15 min. The two phases were then poured into a second centrifuge tube and centrifuged under the same conditions for
15 min. The membranes were removed from the interface of the two phase polymer system with a Pasteur pipette and washed free of polymer system in 50 ml of distilled water at 4000 g (r_{av.} 25 cm) for 15 min. Membranes were resuspended in 0.05 M Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose.

DESCENDING PAPER CHROMATOGRAPHY

The enzymic breakdown of CMP-N-acetyl-[^{14}C]neuraminic acid was monitored by descending paper chromatography (Spix et al., 1979). Incubation mixtures were chromatographed on Whatman 3MM paper using ethyl acetate:pyridine:glacial acetic acid:water (5:5:1:3 v/v/v/v) as solvent. After 20 h chromatography the papers (48 cm long) were dried, cut at 1.5 cm intervals and counted in a toluene scintillant as described under radioactive counting. Non-radioactive markers were simultaneously chromatographed and stained with silver nitrate. Non-enzymic hydrolysis of CMP-N-acetyl-[^{14}C]neuraminic acid was measured in the same way.
CHAPTER FOUR

RESULTS

MORPHOLOGY AND PROLIFERATION OF B16 MELANOMA CELLS: EFFECTS OF CYCLIC NUCLEOTIDES

B16 melanoma cells were grown in tissue culture as described in Methods. Fig. 4.1(a-h) and Fig. 4.2(a-i) show a series of scanning electron micrographs photographed at various magnifications as described in Methods. B16 melanoma cells shown in Fig. 4.1(a-d) and Fig. 4.2(c-i) were control cells, while those shown in Fig. 4.1(e-h) and Fig. 4.2(a and b) were grown with 1 mM cyclic AMP and 1 mM theophylline, a treatment known to bring about a doubling of the intracellular concentration of cyclic AMP as described in Chapter 1.

On seeding into tissue culture bottles B16 melanoma cells quickly adhered to the glass substratum. With further incubation at 37°C their number increased and the culture became confluent after 72 h. The growth of the tissue culture was associated with a diversification of cell size and shape. The majority of cells became elongated, with thin pseudopodial projections which frequently passed over or under adjacent cells (Fig. 4.1a-c). Higher power micrographs show that some of these cells had numerous microvilli on their surface (Fig. 4.2c); on others small pits were visible (Fig. 4.2d); but most had relatively few and relatively short microvilli. Cells assumed a rounded shape when they were about to divide (Fig. 4.2f-i).

Treatment of B16 melanoma cells cultures with 1 mM
FIG. 4.1  COMPARATIVE MORPHOLOGY OF B16 MELANOMA CELLS GROWN WITH AND WITHOUT CYCLIC AMP AND THEOPHYLLINE (SCANNING ELECTRON MICROGRAPHS)

B16 melanoma cells were grown in tissue culture as described in Methods, with and without the addition of 1 mM cyclic AMP and 1 mM theophylline to the growth medium. Cells were fixed, dehydrated, dried at the critical point, coated with gold-palladium and scanned as described in Methods for scanning electron microscopy.

(a-c) Confluent cultures of control B16 melanoma cells showing serial magnifications of the same field.

(d) A control B16 melanoma cell from a sparse culture.

(e-g) Confluent cultures of B16 melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline showing serial magnification of the same field.

(h) A B16 melanoma cell from a sparse culture grown with 1 mM cyclic AMP and 1 mM theophylline.
FIG. 4.2 MORPHOLOGICAL CHARACTERISTICS OF B_{16} MELANOMA CELLS
(SCANNING ELECTRON MICROGRAPHS)

B_{16} melanoma cells were grown and prepared as for Fig. 4.1

(a) B_{16} melanoma cells from a confluent culture
grown with 1 mM cyclic AMP and 1 mM theophylline.

(b) Higher power micrograph of part of the field
shown in (a).

(c and d) High power micrographs of the surface of 2
different control B_{16} melanoma cells.

(e) Confluent culture of control B_{16} melanoma cells.

(f-i) Higher power micrographs of dividing B_{16} melanoma
cells. Each picture is from a different region
of the field shown in (e).
cyclic AMP and 1 mM theophylline resulted in a general overall increase in apparent cell size and a decrease in the number of pseudopodia (Fig. 4.1e-g). The characteristic pleomorphism of malignancy was displayed by both treated and untreated tissue cultures but in treated tissue cultures there was less overlapping of cells. The pattern of microvilli present on the cell surface did not differ noticeably from that present on the untreated cells. Specimen shrinkage is inherent in the preparation of tissue cultures for scanning electron microscopy and this can be clearly seen in Fig. 4.2(a) and at a higher magnification in Fig. 4.2(b). Observation of this preparation artefact, however, permits the conclusion that there are very few microvilli in areas of close contact between cells. When present the microvilli extend into the tissue culture medium.

The B₁₆ melanoma cell shown in Fig. 4.1(d) is typical of cells from a sparse control culture where the majority of cells tend to be elongated or rounded. The cell shown in Fig. 4.1(h) is typical of cells in sparse cultures treated with 1 mM cyclic AMP and 1 mM theophylline, where the majority of cells tend to be flat or stellate. These cells from both control and treated sparse cultures (Fig. 4.1d and Fig. 4.1h respectively) are larger than comparable cells from control and treated confluent cultures (Fig. 4.1c and Fig. 4.1g).

Fig. 4.3 shows six histological preparations, prepared as described in Methods, of control B₁₆ melanoma cells (Fig. 4.3a-c) and of B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.3d-f). In confluent cultures both at lower magnification (Fig. 4.3a and d) and at
FIG. 4.3  COMPARATIVE MORPHOLOGY OF B₁₆ MELANOMA CELLS GROWN WITH AND WITHOUT CYCLIC AMP AND THEOPHYLLINE (HAEMATOXYLIN AND PHLOXINE STAIN)

B₁₆ melanoma cells were grown in tissue culture as described in Methods, with and without the addition of 1 mM cyclic AMP and 1 mM theophylline to the growth medium. Cells were fixed and stained with Haematoxylin and Phloxine as described in Methods for histological preparation of cell cultures.

(a) Confluent culture of control B₁₆ melanoma cells at low magnification.

(b) Higher magnification of the preparation shown in (a).

(c) Control B₁₆ melanoma cells from a sparse culture.

(d) Confluent culture of B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline at low magnification.

(e) Higher magnification of the preparation shown in (d).

(f) B₁₆ melanoma cells from a sparse culture grown with 1 mM cyclic AMP and 1 mM theophylline.
higher magnification (Fig. 4.3b and e), the effects of adding 1 mM cyclic AMP and 1 mM theophylline were essentially similar to those found in scanning electron micrographs shown in Fig. 4.1 and Fig. 4.2; namely, cells treated with 1 mM cyclic AMP and 1 mM theophylline appeared to be less elongated and there was less overlap between adjacent cells. In addition the round cells can be seen to have mitotic figures. In sparse cultures (Fig. 4.3c and Fig. 4.3f), B16 melanoma cells had a tendency to form clusters. The addition of 1 mM cyclic AMP and 1 mM theophylline to the growth medium (Fig. 4.3f) decreased the number and length of cell processes and appeared to increase cell size.

Protein assays (Lowry, 1951) were carried out on B16 melanoma cells grown with and without 1 mM cyclic AMP and 1 mM theophylline and it was found that the total protein in μg per million cells was 272 ± 18.7 (S.E.M., n=20) and 266 ± 20.4 (S.E.M., n=20) respectively; that is, there was no significant difference. For this reason, in subsequent experiments, biochemical parameters were expressed per number of cells rather than per quantity of protein.

Histological preparations of B16 melanoma cells that had been grown with various analogues of cyclic AMP and/or theophylline as listed in Table 4.1 were examined. The effect on the morphology of B16 melanoma cells when 1 mM theophylline was used in combination with 1 mM dibutyryl cyclic AMP or 1 mM 8-bromo cyclic AMP was essentially similar to that obtained with 1 mM cyclic AMP and 1 mM theophylline. Treatment with each of these combinations also produced a 15% decrease in B16 melanoma cell numbers (Table 4.1). The
**TABLE 4.1** Effect of various cyclic AMP derivatives and/or theophylline on cell numbers of B16 melanoma cells

B16 melanoma cells, seeded at a concentration of $2 \times 10^4$ per cm$^2$, were grown as described in Methods for tissue culture except that cells were grown for 48 h in the presence of the compounds listed in the table, which were added 24 h after subculture. Cells were harvested and counted as described in Methods. In each case the number shown is the mean of six counts, each from a different bottle of cells, ± S.E.M. All cells were from a single subculture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/cm$^2$ (x $10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.8 ± 0.1</td>
</tr>
<tr>
<td>1 mM cyclic AMP and 1 mM theophylline</td>
<td>11.9 ± 0.2*</td>
</tr>
<tr>
<td>1 mM dibutyryl cyclic AMP and 1 mM theophylline</td>
<td>11.2 ± 0.1*</td>
</tr>
<tr>
<td>1 mM 8-bromo cyclic AMP and 1 mM theophylline</td>
<td>11.8 ± 0.1*</td>
</tr>
<tr>
<td>1 mM theophylline</td>
<td>10.2 ± 0.2*</td>
</tr>
</tbody>
</table>

*P < 0.01 when compared to control cells (Student's 't' test)
effect of 1 mM theophylline alone was to decrease B16 melanoma cell numbers by 25%, but its effect on cell morphology was less than that of the above-mentioned combinations.

**Mn<sup>2+</sup> REQUIREMENT FOR GLYCOSYLTRANSFERASES**

Before investigating the effects of cyclic AMP and theophylline on the glycosyltransferases in B16 melanoma cells, for the reasons outlined in Chapter 1, it was necessary to investigate the metal ion requirements of these enzymes. Since a number of reports indicated that the galactosyltransferase of bovine milk (Berliner and Wong, 1975; Powell and Brew, 1976; Tsopanakis and Herries, 1978) and the galactosyltransferase and glucosyltransferase involved in collagen biosynthesis (Kivirikko and Myllyla, 1979) had a requirement for Mn<sup>2+</sup>, preliminary experiments to investigate the effect of Mn<sup>2+</sup> on the incorporation of <sup>14</sup>C sugars from nucleotide-[<sup>14</sup>C]sugars were carried out. Myllyla et al. (1979) had noted that the concentration of Mn<sup>2+</sup> used in most reports ranged from 2.5 - 25 mM, so the effect of an approximately similar concentration of Mn<sup>2+</sup> (8.3 mM) was investigated using the glycosyltransferase assay described in Methods. Each of the eight sets of results showing the time course of incorporation of label (Fig. 4.4a-h) was obtained from cells of different sub-cultures.

Mn<sup>2+</sup> appeared to be essential for incorporation of label from UDP-N-acetyl-[<sup>14</sup>C]galactosamine (Fig. 4.4e), UDP-N-acetyl-[<sup>14</sup>C]glucosamine (Fig. 4.4f) and UDP-[<sup>14</sup>C]galactose (Fig. 4.4g). Added Mn<sup>2+</sup> increased the incorporation of label from GDP-[<sup>14</sup>C]fucose (Fig. 4.4c) and UDP-[<sup>14</sup>C]xylose (Fig.
FIG. 4.4 TIME-DEPENDENCE OF THE INCORPORATION OF $^{14}\text{C}$ FROM NUCLEOTIDE-$^{14}\text{C}$ SUGARS BY B16 MELANOMA CELLS WITH OR WITHOUT Mn$^{2+}$

Cells were incubated with the following nucleotide-$^{14}\text{C}$ sugars: (a) CMP-N-acetyl-$^{14}\text{C}$ neuraminic acid; (b) GDP-$^{14}\text{C}$ mannose; (c) GDP-$^{14}\text{C}$ fucose; (d) UDP-$^{14}\text{C}$ xylose; (e) UDP-N-acetyl-$^{14}\text{C}$ galactosamine; (f) UDP-N-acetyl-$^{14}\text{C}$ glucosamine; (g) UDP-$^{14}\text{C}$ galactose; (h) UDP-$^{14}\text{C}$ glucose. The uptake of $^{14}\text{C}$ was measured using the glycosyltransferase assay described in Methods. Each point represents the mean value of two determinations. When duplicate determinations deviate from the mean by more than 5% they are indicated by error bars. All calculations assume that labelled sugars are incorporated as such.

- - - with 8.3 mM Mn$^{2+}$

O--O without added Mn$^{2+}$
4.4d), decreased the incorporation of label from CMP-N-acetyl-[¹⁴C]neuraminic acid (Fig. 4.4a) and from GDP-[¹⁴C]mannose, but had no effect on the incorporation of label from UDP-[¹⁴C]glucose.

A limited study was carried out to compare the effects of another divalent cation Mg²⁺ with the effects of Mn²⁺. 8.3 mM Mg²⁺ added either instead of or in addition to Mn²⁺, had little effect on the incorporation of label from either UDP-[¹⁴C]glucose or GDP-[¹⁴C]fucose. Mg²⁺ was not added in standard assay procedures in subsequent experiments.

**EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON [¹⁴C] INCORPORATION FROM NUCLEOTIDE-[¹⁴C]SUGARS**

As described in Chapter 1, supplementing the growth medium with 1 mM cyclic AMP and 1 mM theophylline for 48 h brings about a doubling of the intracellular cyclic AMP level. It was therefore appropriate to investigate whether the capacity of B₁₆ melanoma cell suspensions (harvested from confluent subcultures) to incorporate label from various nucleotide-[¹⁴C]sugars was altered by this treatment. Each of the eight sets of results shown (Fig. 4.5a-h) was obtained from cells of different subcultures using the glycosyltransferase assay described in Methods; 8.3 mM Mn²⁺ was added to each assay mixture.

In test cells, grown with cyclic AMP and theophylline, the incorporation of label from CMP-N-acetyl-[¹⁴C]neuraminic acid after 2 h (Fig. 4.5a) had increased by 85% relative to that in control cells. By comparison Fig. 4.5b-h show that the
FIG. 4.5 TIME-DEPENDENCE OF THE INCORPORATION OF $\text{^{14}C}$ FROM NUCLEOTIDE-$[\text{^{14}C}]$SUGARS BY B$_{16}$ MELANOMA CELLS GROWN WITH AND WITHOUT CYCLIC AMP AND THEOPHYLLINE

Cells were incubated with the following nucleotide-$[\text{^{14}C}]$sugars: (a) CMP-N-acetyl-$[\text{^{14}C}]$neuraminlate; (b) GDP-$[\text{^{14}C}]$mannose; (c) GDP-$[\text{^{14}C}]$fucose; (d) UDP-$[\text{^{14}C}]$xylose; (e) UDP-N-acetyl-$[\text{^{14}C}]$-galactosamine; (f) UDP-N-acetyl-$[\text{^{14}C}]$glucosamine; (g) UDP-$[\text{^{14}C}]$galactose; (h) UDP-$[\text{^{14}C}]$-glucose. The uptake of $\text{^{14}C}$ was measured using the glycosyltransferase assay described in Methods. Each reaction mixture contained 8.3 mM Mn$^{2+}$. Each point represents the mean value of two determinations. When duplicate determinations deviate from the mean by more than 5% they are indicated by error bars. All calculations assume that labelled sugars are incorporated as such.

□□□ with 1 mM cyclic AMP and 1 mM theophylline

●●● without 1 mM cyclic AMP and 1 mM theophylline
changes in incorporation of $[^{14}C]$ from other nucleotide-$[^{14}C]$-sugars were small. Since changes in sialyltransferase activity have often been associated with transformation and malignancy, as described in Chapter I, further work to define the effect of cyclic AMP on sialyltransferase was indicated.

The above results had some limitations. First, they were obtained in the presence of Mn$^{2+}$ which has been shown to inhibit sialyltransferase (Fig. 4.4a). Second, the effect of Mn$^{2+}$ and those of cyclic AMP and theophylline had not been compared in a single subculture. Therefore the effects of cyclic AMP and theophylline and the effects of Mn$^{2+}$ on the incorporation of $^{14}$C from CMP-N-acetyl-$[^{14}C]$neuraminic acid were tested both separately and combined, in cells from the same subculture, which were divided into two batches.

One batch was grown for 72 h in control medium and the other was grown for 24 h in control medium and then for 48 h in medium supplemented with 1 mM cyclic AMP and 1 mM theophylline. At this time the subcultures had reached confluence. After harvesting, cells from each batch were incubated for 2 h with CMP-N-acetyl-$[^{14}C]$neuraminic acid and without 8.3 mM Mn$^{2+}$ and the incorporation of $^{14}$C was measured using the glycosyltransferase assay. A preliminary study was carried out in the absence of Mn$^{2+}$ to investigate whether increasing the concentration of the substrate, CMP-N-acetyl-$[^{14}C]$neuraminic acid, from 450 pmol to 900 pmol/1.3 ml reaction mixture, increased the incorporation of $^{14}$C during the incubation for 2 h at 37°C, but no significant increase in the $^{14}$C was found (n=5, P > 0.1 Student's 't' test). Therefore the assay was used as before.
The results, shown in Table 4.2, indicate that the effect of Mn\textsuperscript{2+} on the sialyltransferase activity in control cells (24 ± 4% inhibition; a result which confirms the inhibition shown in Fig. 4.4a) does not differ significantly from the effect of Mn\textsuperscript{2+} on sialyltransferase activity in cells grown with cyclic AMP and theophylline (31 ± 5%). The effect of cyclic AMP and theophylline on sialyltransferase activity measured in the absence of Mn\textsuperscript{2+} (73 ± 6% stimulation) did not differ significantly from the effect of cyclic AMP and theophylline measured in the presence of Mn\textsuperscript{2+} (57 ± 13% stimulation, a result which confirms the stimulation shown in Fig. 4.5a). These data not only provide validity for the effects shown in the previous experiments (Fig. 4.4a, Fig. 4.5a) but also indicate that the effects of Mn\textsuperscript{2+} and the effects of cyclic AMP and theophylline on the incorporation of \textsuperscript{14}C from CMP-N-acetyl-\textsuperscript{14}C]neuraminic are independent of each other.

One further relationship remained to be determined: whether the effect of cyclic AMP and the effect of theophylline were independent of each other, or whether they potentiate each other. In Chapter I it has been noted that Sharmeen (1985) has shown that only a combination of exogenous cyclic AMP and theophylline consistently resulted in a significant increase in the intracellular cyclic AMP level. If the mechanism whereby exogenous cyclic AMP and theophylline operate is via an increase in the intracellular cyclic AMP level, then for the above reasons it would not be expected that either exogenous cyclic AMP alone or theophylline alone would consistently result in an effect on sialyltransferase activity. To investigate this, cells from a single sub-
TABLE 4.2  Independence of effects of Mn$^{2+}$ and of cyclic AMP and theophylline on the incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminic into B16 melanoma cells

As described in Methods, incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminic over 2 h was measured using the glycosyltransferase assay, and 8.3 mM MnCl$_2$ was added to incubation mixtures as indicated. When added, 1 mM cyclic AMP and 1 mM theophylline were included in the cell culture medium for 48 h before harvesting. In each case the incorporation shown is the mean of five assays ± S.E.M. and the calculations assume that the labelled sugar is incorporated as such. All cells were from a single subculture.

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$C incorporation (pmol/500,000 cells)</th>
<th>Relative incorporation (%)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37 ± 0.01 = a$^1$</td>
<td></td>
</tr>
<tr>
<td>With Mn$^{2+}$</td>
<td>0.28 ± 0.01 = b$^1$</td>
<td>$b/a = 76 ± 4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$d/c = 69 ± 5$</td>
</tr>
<tr>
<td>With cyclic AMP</td>
<td>0.64 ± 0.01 = c$^1$</td>
<td>$c/a = 173 ± 6$</td>
</tr>
<tr>
<td>and theophylline</td>
<td></td>
<td>$d/b = 157 ± 13$</td>
</tr>
<tr>
<td>With Mn$^{2+}$,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclic AMP and</td>
<td>0.44 ± 0.03 = d$^1$</td>
<td></td>
</tr>
<tr>
<td>theophylline</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$ A one way analysis of variance shows a significant difference between groups at the 1% level.

$^2$ Propagation of precision indices was carried out according to accepted procedures (Margenau and Murphy, 1956)
culture were seeded into bottles and divided into four batches; one batch was grown for 72 h in control medium and each of the other three batches was grown for 24 h in control medium and then for 48 h in medium supplemented respectively with 1 mM cyclic AMP, with 1 mM theophylline, and with 1 mM cyclic AMP and 1 mM theophylline. After harvesting, cells were incubated for 2 h with CMP-N-acetyl-[\(^{14}\)C]neuraminic in the absence of Mn\(^{2+}\) and the incorporation of \(^{14}\)C into cells from each batch was measured using the glycosyltransferase assay as before.

Table 4.3 shows that in the test cells grown with both cyclic AMP and theophylline, the incorporation over 2 h of label from CMP-N-acetyl-[\(^{14}\)C]neuraminic had increased by 126% relative to that in control cells. This differs substantially from the sum of the independent effects of cyclic AMP (4% stimulation) and of theophylline (78% stimulation).

**INCORPORATION OF \(^{14}\)C FROM NUCLEOTIDE-\(^{14}\)C SUGARS BY SPARSE B\(_{16}\) MELANOMA CELLS**

The incorporation of \(^{14}\)C from nucleotide-\(^{14}\)C sugars by B\(_{16}\) melanoma cells grown to confluence in medium supplemented with 1 mM cyclic AMP and 1 mM theophylline has been shown to differ from that of control cells. Therefore an experiment was designed to test whether such incorporation may in some way be dependent on the overall cell density in tissue culture. B\(_{16}\) melanoma cells were grown so that at the time of harvesting there were only \(2 \times 10^6 \pm 10\%\) cells per cm\(^2\); such cells will be referred to as sparse cells.
TABLE 4.3 Interdependence of effects of cyclic AMP and of theophylline on the incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminic into B$_{16}$ melanoma cells

As described in Methods, incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminic acid into B$_{16}$ melanoma cells over 2 h was measured using the glycosyl transferase assay. When added together and separately, 1 mM cyclic AMP and 1 mM theophylline were included in the tissue culture medium for 48 h before harvesting. In each case the incorporation shown is the mean of the five assays ± S.E.M. and the calculations assume that the labelled sugar is incorporated as such. All cells were from a single subculture.

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$C incorporation (pmol/500,000 cells)</th>
<th>Relative incorporation (%) $^{*2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.27 ± 0.01 = a</td>
<td></td>
</tr>
<tr>
<td>With cyclic AMP</td>
<td>0.28 ± 0.01 = b</td>
<td>$\frac{b}{a} = 104 ± 7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\frac{d}{c} = 127 ± 5$</td>
</tr>
<tr>
<td>With theophylline</td>
<td>0.48 ± 0.01 = c$^*$</td>
<td>$\frac{c}{a} = 178 ± 10$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\frac{d}{b} = 218 ± 11$</td>
</tr>
<tr>
<td>With cyclic AMP and</td>
<td>0.61 ± 0.01 = d$^*$</td>
<td>$\frac{d}{a} = 226 ± 12$</td>
</tr>
<tr>
<td>theophylline</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{*1}$ P < 0.01 when compared to control cells (Student's 't' test)

$^{*2}$ Propagation of precision indices was carried out according to accepted procedures (Margenau and Murphy, 1956)
Reduced cell numbers were obtained by decreasing the number of cells used to seed the tissue culture flasks from $2 \times 10^4$ to $4 \times 10^3$ cells per cm$^2$; otherwise the tissue cultures were maintained as described in Methods.

Supplementing the growth medium of sparse cells with 1 mM cyclic AMP and 1 mM theophylline for 48 h was shown to affect the capacity of harvested cells to incorporate label from various nucleotide-[¹⁴C]sugars. Each of the seven sets of results shown in Fig. 4.6 were obtained from cells of the same subculture using the glycosyltransferase assay described in Methods. 8.3 mM Mn$^{2+}$ was included in all assay mixtures except those involving CMP-N-acetyl-[¹⁴C]neuraminate and GDP-[¹⁴C]mannose; Mn$^{2+}$ has previously been shown to inhibit the transfer of label from these nucleotide sugars (Fig. 4.4). For all nucleotide-[¹⁴C]sugars tested, cells grown in 1 mM cyclic AMP and 1 mM theophylline incorporated more $^{14}$C into the phosphotungstate precipitate than did control cells. The increased $^{14}$C uptake from UDP-[¹⁴C]galactose, UDP-N-acetyl-[¹⁴C]galactosamine, GDP-[¹⁴C]fucose and CMP-N-acetyl-[¹⁴C]neuraminate was in each case approximately 100%; from UDP-N-acetyl-[¹⁴C]glucosamine and GDP-[¹⁴C]mannose it was approximately 50%; but from UDP-[¹⁴C]glucose it was less than 50%.

**EFFECT OF EXOGENOUS SUGARS ON $^{14}$C INCORPORATION FROM NUCLEOTIDE-[¹⁴C]SUGARS BY B₁₅ MELANOMA CELLS**

Incorporation of $^{14}$C from UDP-[¹⁴C]glucose could be due to the activity of a glucosyltransferase. It is also possible that cells incubated with UDP-[¹⁴C]glucose might incorporate $^{14}$C via the formation of [¹⁴C]glucose. This was
FIG. 4.6 INCORPORATION OF $^{14}$C FROM NUCLEOTIDE-$[^{14}$C]SUGARS BY SPARSE B16 MELANOMA CELLS

Cells from sparse cultures were harvested as described in Methods for confluent cells, and incubated at 37°C for 2 h with the following nucleotide-$[^{14}$C]sugars: (A) UDP-$[^{14}$C]galactose; (B) UDP-N-acetyl-$[^{14}$C]galactosamine; (C) UDP-N-acetyl-$[^{14}$C]glucosamine; (D) UDP-$[^{14}$C]glucose; (E) GDP-$[^{14}$C]fucose; (F) CMP-N-acetyl-$[^{14}$C]-neuraminic; (G) GDP-$[^{14}$C]mannose. The uptake of $^{14}$C was measured using the glycosyltransferase assay described in Methods. Each incubation mixture with the exception of those containing CMP-N-acetyl-$[^{14}$C]neuraminic and GDP-$[^{14}$C]mannose contained 8.3 mM Mn$^{2+}$. Each bar represents the mean of 5 determinations ± S.E.M. All calculations assume that labelled sugars are incorporated as such.

- with 1 mM cyclic AMP and 1 mM theophylline
- without 1 mM cyclic AMP and 1 mM theophylline (control)

* $P < 0.01$ when compared to control cells (Student's 't' test)
investigated by the addition of unlabelled glucose to incubation mixtures prepared, in duplicate, as in the glycosyltransferase assay described in Methods, except that the final volume of each incubation mixture was increased to 0.13 ml in order to accommodate the addition of unlabelled sugar as necessary. If the processes involved in the uptake and incorporation of glucose were not saturated, then it would follow that the rate of uptake and incorporation of glucose would increase in parallel with the increased concentration of glucose in the medium, and no change in the rate of $^{14}$C incorporation would be observed because the specific activity of any $[^{14}C]$glucose formed would be decreased by an equivalent amount (Hirschberg et al., 1976). If the glucose concentration was increased beyond that necessary to saturate either or both of the processes of uptake and incorporation, then the rate of $^{14}$C incorporation would no longer rise in parallel with the glucose concentration; in fact it would fall in parallel with the decreased specific activity of the $[^{14}C]$glucose.

Fig. 4.7(a) indicates that the incorporation of label from 450 pmol of UDP-$[^{14}C]$glucose/0.13 ml reaction mixture (3.46 μM) is unaffected by the addition of amounts of unlabelled glucose up to 45 nmol/0.13 ml reaction mixture (346 μM).

Linear regression analysis of the data in Fig. 4.7(a) confirms that increasing concentrations of unlabelled glucose in the medium do not decrease the incorporation of $^{14}$C from nucleotide-$[^{14}C]$glucose.
FIG. 4.7 EFFECT OF EXOGENOUS GLUCOSE ON $^{14}$C INCORPORATION FROM UDP-$[^14]$C GLUCOSE BY B<sub>16</sub> MELANOMA CELLS

(a) The uptake of $^{14}$C (O) from UDP-$[^14]$C glucose in the presence of unlabelled glucose (4.5 pmol to 45 nmol) using the glycosyltransferase assay described in Methods and modified as described in the text. Each reaction mixture contains 8.3 mM MnCl<sub>2</sub> and 450 pmol of UDP-$[^14]$C glucose in a volume of 0.13 ml (i.e. 3.46 μM UDP-$[^14]$C glucose). The unlabelled glucose added per reaction mixture ranged from 4.5 pmol to 45 nmol and this is expressed on a logarithmic scale, as the log of the nanomolar concentration of glucose. The final concentration of unlabelled glucose is indicated above each point. The equation of the line of best fit for each of the mean values is $y = 0.736 + 0.002x$.

(b) The uptake of $^{14}$C (●) from $[^14]$C glucose over the same range of concentrations as that of the glycosyltransferase assay modified as described above. Each reaction mixture contained 8.3 mM MnCl<sub>2</sub>. The final concentration of glucose is indicated above each point.

In both (a) and (b) each point represents the mean value of two determinations which did not deviate from the mean by more than 5%. All cells were from the same subculture.
In a separate experiment it was found also that the addition of 25 mM glucose (which is at least 25 times the Km value for glucose uptake in PG19 mouse melanoma, a melanoma originating in a C57BL mouse; White et al., 1981) did not decrease the $^{14}$C incorporation from 450 pmol of UDP-$^{14}$C-glucose. Again, Fig. 4.7(b) shows that very little $^{14}$C was incorporated when the 0.13 ml incubation mixtures contained 450 pmol of [$^{14}$C]glucose (3.46 μM) instead of UDP-$^{14}$C]glucose. It can be seen also that if the quantity of [$^{14}$C]glucose was doubled, i.e. if it was 6.92 μM (indicated by the heavy line X) this would make very little difference to the $^{14}$C incorporation. This is relevant to the results in Fig. 4.7(a) because it shows that, in the reaction mixture containing 3.46 μmol of UDP-$^{14}$C]glucose and 3.46 μmol of unlabelled glucose, very little, if any, of the $^{14}$C incorporation can be attributed to incorporation via [$^{14}$C]glucose, even if all of the UDP-$^{14}$C]glucose has been converted to [$^{14}$C]glucose.

Using the glycosyltransferase assay, also carried out in 0.13 ml reaction mixtures (n=5) it was found, first, that 0.50 ± 0.01 (S.E.M.) pmol of N-acetyl-$^{14}$C]neuraminic/500,000 cells was incorporated in 2 h from 450 pmol of CMP-N-acetyl-$^{14}$C]neuraminate. Second, when the CMP-N-acetyl-$^{14}$C]neuraminate was replaced by 450 pmol of N-acetyl-$^{14}$C]neuraminate, only 0.01 pmol/500,000 cells was incorporated. Thus incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C]neuraminate did not take place via N-acetyl-$^{14}$C]neuraminate. In confirmation of this, it was also found that the addition of 25 mM unlabelled N-acetylneuraminic to incubation mixtures containing CMP-N-acetyl-$^{14}$C]neuraminate did not decrease the $^{14}$C incorporation. Because of the high viscosity of N-acetyl-
neuraminic solutions at this concentration the effect of higher concentrations was not investigated.

EFFECTS OF FORSKOLIN AND CYCLIC AMP ANALOGUES ON THE INCORPORATION OF $^{14}$C FROM CMP-N-ACETYL-$^{14}$C NEURAMINATE

If the effects of 1 mM cyclic AMP and 1 mM theophylline are mediated by an increase in intracellular cyclic AMP levels, it would be expected that other agents capable of increasing intracellular cyclic AMP levels would have similar effects. Therefore the effect of forskolin, an activator of adenylate cyclase in cell membranes (Seamon et al., 1981), on the incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C neuraminate into B16 melanoma cells, was compared with the effect of cyclic AMP and theophylline.

Cells from a single subculture were seeded into bottles and divided into five batches. One batch was grown for 72 h in control tissue culture medium and each of the other batches was grown for 48 h with medium supplemented, respectively, with 1 mM cyclic AMP and 1 mM theophylline, with 10 μM forskolin, with 1 mM theophylline, and with 10 μM forskolin and 1 mM theophylline, added 24 h after subculturing. Table 4.4 shows that B16 melanoma cells grown in 1 mM cyclic AMP and 1 mM theophylline incorporated approximately 80% more $^{14}$C from CMP-N-acetyl-$^{14}$C neuraminate than control cells. 10 μM forskolin increased $^{14}$C incorporation by approximately 50% relative to the control and the combination of 10 μM forskolin and 1 mM theophylline was equally effective. 1 mM theophylline also increased the $^{14}$C incorporation by approx-
TABLE 4.4  Effect of forskolin on the incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminic acid into 
B$_{16}$ melanoma cells

As described in Methods, incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C-neuraminate over 2 h was measured using the glycosyltransferase assay. When added as indicated below forskolin, cyclic AMP and theophylline were included in the tissue culture medium for 48 h before harvesting. In each case the incorporation is the mean of five assays ± S.E.M. and the calculations assume that the labelled sugar is incorporated as such. All cells were from a single subculture.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$^{14}$C incorporation (pmol/500,000 cells)</th>
<th>Relative incorporation (% of control)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.30 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>With 1 mM cyclic AMP</td>
<td>0.54 ± 0.01$^1$</td>
<td>180 ± 8</td>
</tr>
<tr>
<td>and 1 mM theophylline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With 10 μM forskolin</td>
<td>0.44 ± 0.01$^1$</td>
<td>147 ± 7</td>
</tr>
<tr>
<td>With 10 μM forskolin and 1 mM theophylline</td>
<td>0.43 ± 0.01$^1$</td>
<td>143 ± 7</td>
</tr>
<tr>
<td>With 1 mM theophylline</td>
<td>0.47 ± 0.01$^1$</td>
<td>156 ± 8</td>
</tr>
</tbody>
</table>

$^1$ P < 0.01 when compared to control cells (Student's 't' test)

$^2$ Propagation of precision indices was carried out according to accepted procedures (Margenau and Murphy, 1956)
imately 50%.

In this context it also seemed appropriate to investigate effects of cyclic AMP analogues capable of entering cells. Thus in a further experiment, the results of which are not shown in the table, B₁₆ melanoma cells from another subculture were grown with 1 mM dibutyryl cyclic AMP and 1 mM theophylline. The resulting stimulation of $^{14}C$ incorporation from CMP-N-acetyl-$[^{14}C]$neuraminic acid, as measured by the glycosyltransferase assay described in Methods, was only 80% of that obtained when 1 mM cyclic AMP and 1 mM theophylline were added to the tissue culture medium. When 1 mM 8-bromo cyclic AMP and 1 mM theophylline were used similarly the stimulation of $^{14}C$ incorporation was only 60% of that obtained with 1 mM cyclic AMP and 1 mM theophylline.

INCORPORATION OF $^3H$ FROM N-$[^3H]$ACETYL-MANNOSAMINE BY B₁₆ MELANOMA CELLS

The sialyltransferase activity in the foregoing experiments, in which N-acetyl-$[^{14}C]$neuraminic acid becomes incorporated into cell material without prior hydrolysis of CMP-N-acetyl-$[^{14}C]$neuraminic acid to CMP and N-acetyl-$[^{14}C]$-neuraminic acid, may be on the cell surface, i.e. it may be an ectosialyltransferase. If it is intracellular the CMP-N-acetyl-$[^{14}C]$neuraminate must enter the cell by some undefined mechanism. To obtain information about possible alterations in total cell sialyltransferase activity a different assay was used involving the incorporation of $^3H$ from N-$[^3H]$acetyl-mannosamine, a precursor of sialic acid (Monaco et al., 1975).


$^3$H incorporation would in this case represent incorporation of both cell surface and intracellular sialic acid. The effects of 1 mM cyclic AMP and 1 mM theophylline on this $^3$H incorporation were investigated. At the same time the opportunity was taken to compare these effects with those of dibutyryl cyclic AMP and 8-bromo cyclic AMP on N-[3H]acetylmannosamine incorporation.

B16 melanoma cells were grown for 48 h with various analogues added to the growth medium 24 h after subculturing, as indicated in Table 4.5, together with N-[3H]acetylmannosamine (2 µCi per 10 ml of tissue culture medium). After harvesting as described in Methods, phosphotungstic acid was used to precipitate cell material, as in the glycosyltransferase assay described in Methods, the incubation procedure being omitted. Table 4.5 shows that 1 mM cyclic AMP and 1 mM theophylline stimulated the incorporation of $^3$H by more than 20%. The effect of 1 mM dibutyryl cyclic AMP and 1 mM theophylline was similar. 1 mM 8-bromo cyclic AMP and 1 mM theophylline had no significant effect on $^3$H incorporation.

**EFFECT ON GLYCOSIDASE ACTIVITIES WHEN B16 MELANOMA CELLS FROM SPARSE AND CONFLUENT CULTURES WERE GROWN WITH CYCLIC AMP AND THEOPHYLLINE**

Levels of degradative enzymes have been considered in studies of implantation and metastasis presumably because they might increase the infiltration capacity of the cell (Bosman, 1972; Poste, 1972). As discussed earlier, Bosman (1973) has studied the glycosidase activities in both sparse
**TABLE 4.5** Effect of cyclic AMP analogues on $^3$H incorporation by B16 melanoma cells grown in N-[2H]acetylmannosamine

Incorporation of $^3$H from N-[2H]acetylmannosamine added, together with analogues as indicated, to the tissue culture medium for 48 h before harvesting as described in Methods, was measured as described in the Text. In each the incorporation is the mean of five assays ± S.E.M. and the calculations assume that the labelled sugar is incorporated as such. All cells were from a single subculture.

<table>
<thead>
<tr>
<th></th>
<th>$^{3}$H incorporation (fmol/500,000 cells)</th>
<th>Relative incorporation (% of control)$^{*2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>With 1 mM cyclic AMP and 1 mM theophylline</td>
<td>117 ± 6$^{*1}$</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>With 1 mM dibutyryl cyclic AMP and 1 mM theophylline</td>
<td>124 ± 4$^{*1}$</td>
<td>129 ± 8</td>
</tr>
<tr>
<td>With 1 mM 8-bromo cyclic AMP and 1 mM theophylline</td>
<td>99 ± 5</td>
<td>103 ± 8</td>
</tr>
</tbody>
</table>

$^{*1}$ P < 0.01 when compared to control cells (Student's 't' test)

$^{*2}$ Propagation of precision indices was carried out according to accepted procedures (Margenau and Murphy, 1956)
and confluent cultures of B16 melanoma cell lines with
different metastatic potentials. Sparse cells from the line
with low metastatic potential had less β-galactosidase,
β-Fucosidase, N-acetyl-β-galactosaminidase and N-acetyl-β-
glucosaminidase than did sparse cultures from the line with
high metastatic potential. Both cell lines had approximately
the same α-mannosidase activity. Levels of these enzymes in
confluent cultures of both low and high metastatic potentials
resembled the levels in sparse cultures of the line with low
metastatic potential. It therefore seemed appropriate to
investigate the effect of growing B16 melanoma cells with 1 mM
cyclic AMP and 1 mM theophylline on the activities of various
glycosidases so that the results obtained could be compared
with the above results of Bosman (1973).

To obtain the supernatant fluid as used by Bosman
(1973) the homogenate was prepared as described in Methods.
Both sparse and confluent cultures were assayed using the
glycosidase assay described in Methods. A preliminary experi-
ment was carried out to determine the necessity of stirring
the homogenate for 16 h before preparing the supernatant. At
the same time a comparison was made between the activity in
the crude homogenate and the supernatant from the homogenates.
For both of these experiments only α-galactosidase activity
was measured. It was found that the fresh homogenate had
approximately 15% more galactosidase activity than homogenates
which had been stirred for 16 h. This was true both for
control cells and cells grown with 1 mM cyclic AMP and 1 mM
theophylline. It was also found that there was a little less
α-galactosidase activity detected when the homogenate was
used as compared to the activity in the supernatant from the homogenate. Since these experiments showed that these variations from the method of Bosman (1972a, 1973) offered no great advantage, supernatants prepared according to his procedure were used for all other experiments so that appropriate comparisons could be made with his work (Bosman, 1973).

If treating cells with cyclic AMP and theophylline affects their metastatic potential, the the glycosidase pattern in cells from sparse cultures treated with cyclic AMP and theophylline would be expected to be comparable to that obtained by Bosman (1973) in sparse cultures with low metastatic potential. In fact as shown in Fig. 4.8 only N-acetyl-β-galactosaminidase was decreased by treatment with cyclic AMP and theophylline, and α-mannosidase activity remained the same, in line with these expectations. However, this treatment increases N-acetyl-glucosaminidase activity; and β-galactosidase activity does not decrease. β-fucosidase activity remained unchanged. Thus no overall correlation with the results of Bosman (1973) on the basis postulated above can be claimed. In addition it was found that β-fucosidase activity and neuraminidase activity, neither tested by Bosman (1973), were unchanged by the treatment, and α-galactosidase activity was increased by the treatment.

Again, Fig. 4.8 shows that homogenates of confluent cells grown with 1 mM cyclic AMP and 1 mM theophylline were similar to cells with low metastatic potential tested by Bosman (1973) in one respect only: they had less β-galactosaminidase. On the other hand the N-acetyl-β-glucosaminidase,
FIG. 4.8 EFFECT ON GLYCOSIDASE ACTIVITY WHEN B16 MELANOMA CELLS WERE GROWN WITH 1 mM CYCLIC AMP AND 1 mM THEOPHYLLINE AND HARVESTED FROM CONFLUENT AND SPARSE CULTURES

B16 melanoma cell homogenates (prepared as described in Methods) from both sparse (a) and confluent (b) cultures were assayed using the glycosidase assay described under Methods. Homogenates were incubated with the following substrates: (A) p-nitrophenyl-N-acetyl-β-D-glucosaminide; (B) p-nitrophenyl-N-acetyl-β-D-galactosaminide; (C) p-nitrophenyl-α-D-galactopyranoside; (D) p-nitrophenyl-β-D-galactopyranoside; (E) p-nitrophenyl-α-L-fucoside; (F) p-nitrophenyl-β-L-fucoside; (G) p-nitrophenyl-α-D-mannoside. Neuraminidase was assayed by the neuraminidase assay using 4-methylumbelliferyl-α-D-N-acetylneuraminate (H) described in Methods. In each case the result represents the mean of 5 separate assays ± S.E.M. (indicated by the bar lines).

 işlemlerleştiren 1 mM cytosine AMP ve 1 mM theophylline

- **without 1 mM cyclic AMP and 1 mM theophylline**

* P < 0.01 when compared to control cells (Student's 't' test)
N-acetyl-β-galactosaminidase and α-mannosidase were all increased by the treatment and β-fucosidase was unchanged, in contradistinction to the results obtained by Bosman (1973) for the confluent cells of low metastatic potential.

Fig. 4.8 also shows that the neuraminidase activity in control cell homogenates (not measured by Bosman, 1973) was the same whether the cells were from confluent or sparse cultures. In homogenates from cells grown in 1 mM cyclic AMP and 1 mM theophylline the neuraminidase activity in those from confluent cells was less than in those from sparse cells. Much of the neuraminidase activity in these homogenates is presumably lysosomal (Corfield et al., 1981). The cell surface neuraminidase might be expected to constitute a small proportion of the total, but the amount is not known. While the total amount of neuraminidase in the homogenate was diminished as a consequence of treatment of the cells with 1 mM cyclic AMP and 1 mM theophylline, it is not known whether this result represents changes to the cell surface neuraminidase activity. Changes in cell surface neuraminidase activity are of interest because the cell surface neuraminidase might be expected to regenerate sialyl acceptors which would be substrates for cell surface sialyltransferase. The increased $^{14}$C incorporation from CMP-N-acetyl-$[^{14}$C]neuraminate (Fig. 4.5a) could in theory result from an increase in the number of sialyl acceptors on the cell surface, resulting from increased cell surface neuraminidase. In fact the total neuraminidase activity in the homogenates has decreased (Fig. 4.8) when treated with 1 mM cyclic AMP and 1 mM theophylline. It was therefore desirable to use a neuraminidase assay which
would if possible selectively assay cell surface neuraminidase. Furthermore, it was necessary to assay the sialyltransferase activity concurrently with the neuraminidase activity in order to establish if they were interdependent. Since sialyltransferase activity was measured in terms of $^{14}$C incorporation from CMP-N-acetyl-$^{14}$C neuraminate it seemed appropriate that an assay system for neuraminidase using another isotope ($^3$H) be introduced so that both sialyltransferase and neuraminidase activities could be measured at the same time.

**SIALLYLTRANSFERASE AND NEURAMINIDASE: COMBINED EFFECTS**

$B_{16}$ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline incorporated more $^{14}$C when incubated with CMP-N-acetyl-$^{14}$C neuraminate than did control cells. This may be attributed to an increased quantity of sialyltransferase enzyme, but it is also possible that the increased $^{14}$C incorporation was a consequence of increased availability of sites to which N-acetyl-$^{14}$C neuraminate could be transferred. In order to investigate this, the removal of sialic acid from the cells during incubation was measured, as described in Methods. The amount of $^3$H sialic acid liberated during incubation of the cells at 37°C was taken as a measure of endogenous neuraminidase activity. The amount was determined by measuring the $^3$H content of phosphotungstate-precipitable material both at the beginning and at the end of incubation and calculating the difference, as described in Methods. The results of an earlier experiment, in which only 0.01 pmol of N-acetyl-$^{14}$C neuraminate was taken up when $5 \times 10^5$ $B_{16}$ melanoma cells were incubated with 450 pmol of N-acetyl-$^{14}$C-
neuraminate at 37°C for 2 h, has already been reported. Thus the small amount of sialic acid liberated by cell surface neuraminidase would not be significantly reincorporated into B<sub>16</sub> melanoma cells. It is possible that some lysosomal activity may be detectable by this assay system, but [<sup>3</sup>H]-sialic acid liberated intracellularly, such as that produced by lysosomal neuraminidase and discharged back into the cytoplasm, would be expected to be readily incorporated via CMP sialic acid into glycoconjugates. In this experiment the sialyltransferase activity was measured at the same time as the endogenous neuraminidase activity, i.e. CMP-N-acetyl-[<sup>14</sup>C]neuraminate was incubated with the cells for the time period over which <sup>3</sup>H release was being measured and the <sup>14</sup>C incorporation into phosphotungstate-precipitable material taken as a measure of sialyltransferase activity. The experiment also included reaction mixtures which were incubated without CMP-N-acetyl-[<sup>14</sup>C]neuraminate, as controls; this was done so that any effects of resialylation on the apparent measurement of neuraminidase activity could be detected.

Three experiments were carried out using the above procedures. In the first experiment, cells from confluent cultures were used. In the second, cells from sparse cultures were used. In the third, cells from confluent cultures were used but the quantity of CMP-N-acetyl-[<sup>14</sup>C]neuraminate added to each incubation was reduced.

An unexpected result was obtained: under certain conditions the neuraminidase was entirely inhibited by the presence of CMP-N-acetyl-[<sup>14</sup>C]neuraminate. Before indicating
the nature of these conditions it is best to consider the results systematically.

In Fig. 4.9(a,c,d) the left hand portion of each diagram gives several values for the \(^3\text{H}\) content of the cells. Of these the first gives the \(^3\text{H}\) content of cell suspensions at the beginning of incubation; the second gives the \(^3\text{H}\) content of the cells after 2 h incubation with no addition of CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate; the third gives the \(^3\text{H}\) content of the cells after 2 h incubation in the presence of CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate. As indicated earlier, in each case the difference between the \(^3\text{H}\) content after 2 h incubation and the \(^3\text{H}\) content at zero time represents the neuraminidase activity. The right hand portion of each diagram gives the \(^{14}\text{C}\) content of the cells after 2 h incubation with CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate.

Fig. 4.9(aA) shows that control cells from confluent cultures incorporated less \(^3\text{H}\) from N-[\(^3\text{H}\)]acetylmannosamine than cells from confluent cultures grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.9aB). These treated cells also lost more \(^3\text{H}\) during a 2 h incubation without CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate than control cells; therefore the neuraminidase activity in treated cells was greater (\(P < 0.01\) Student's 't' test). However when these treated cells (Fig. 4.9aB) were incubated for 2 h in the presence of CMP-N-acetyl-[\(^{14}\text{C}\)]-neuraminate there was no significant loss of \(^3\text{H}\), i.e. there was no significant neuraminidase activity (\(P > 0.1\) Student's 't' test). The neuraminidase activity of control cells was not significantly affected (\(P > 0.1\) Student's 't' test) by
FIG. 4.9 EFFECT OF INCUBATION WITH CMP-N-ACETYL-[\(^{14}\)C]NEURAMINATE ON \(^3\)H RELEASE, AND \(^{14}\)C UPTAKE, BY B\(_{16}\) MELANOMA CELLS GROWN IN N-[\(^3\)H]ACETYLMANNOSESAMINE

B\(_{16}\) melanoma cells were grown as described in Methods with N-[\(^3\)H]acetylmannosamine (2 \(\mu\)Ci per 10 ml of tissue culture medium) and with and without 1 mM cyclic AMP and 1 mM theophylline. After harvesting as described in Methods, B\(_{16}\) melanoma cells were incubated with CMP-N-acetyl-[\(^{14}\)C]neuraminate for 2 h at 37\(^\circ\)C according to the procedure for the glycosyltransferase assay described in Methods. The \(^3\)H and \(^{14}\)C content of phosophotungstate precipitates was determined as described in Methods for radioactive counting. In each case the result represents the mean of 6 separate assays \( \pm \) S.E.M. (indicated by the bar lines).

(a) \(^3\)H and \(^{14}\)C content of the phosphotungstate precipitates from B\(_{16}\) melanoma cells from confluent cultures, incubated with 450 pmol of CMP-N-acetyl-[\(^{14}\)C]neuraminate as indicated.

A without 1 mM cyclic AMP and 1 mM theophylline
B with 1 mM cyclic AMP and 1 mM theophylline
\[\square\] without CMP-N-acetyl-[\(^{14}\)C]neuraminate
\[\square\] with CMP-N-acetyl-[\(^{14}\)C]neuraminate
FIG. (continued) 4.9

EFFECT OF INCUBATION WITH CMP-N-ACETYL-[14C]NEURAMINATE ON 3H RELEASE, AND 14C UPTAKE, BY B16 MELANOMA CELLS GROWN IN N-[3H]ACETYLMANNOSAMINE


(bii) 3H distribution obtained by TLC of the supernatant from cells grown with N-[3H]acetylmannosamine, 1 mM cyclic AMP and 1 mM theophylline and incubated for 2 h at 37°C without CMP-N-acetyl-[14C]neuraminate. The solvent used was isopropanol:acetone:0.1 M lactic acid (2:2:1 v/v/v). The position of a sialic acid marker, detected by spraying with aniline-diphenylamine as described in Methods for TLC, is shown (●●●).
FIG. (continued) 4.9

EFFECT OF INCUBATION WITH CMP-N-ACETYL-[\textsuperscript{14}C]NEURAMINATE ON \textsuperscript{3}H RELEASE, AND \textsuperscript{14}C UPTAKE, BY B\textsubscript{16} MELANOMA CELLS GROWN IN N-[\textsuperscript{3}H]ACETYLMANNOSAMINE

(c) \textsuperscript{3}H and \textsuperscript{14}C content of the phosphotungstate precipitates from B\textsubscript{16} melanoma cells from sparse cultures, incubated with 450 pmol of CMP-N-acetyl-[\textsuperscript{14}C]-neuraminate as indicated.

(d) \textsuperscript{3}H and \textsuperscript{14}C content of the phosphotungstate precipitates from B\textsubscript{16} melanoma cells from confluent cultures, incubated with 4.5 pmol of CMP-N-acetyl-[\textsuperscript{14}C]-neuraminate as indicated.

\begin{itemize}
  \item [A] without 1 mM cyclic AMP and 1 mM theophylline
  \item [B] with 1 mM cyclic AMP and 1 mM theophylline
  \item [□] without CMP-N-acetyl-[\textsuperscript{14}C]neuraminate
  \item [◼] with CMP-N-acetyl-[\textsuperscript{14}C]neuraminate
\end{itemize}
(c) SPARSE

- 3H incorporation (fmol/500,000 cells)
  - Time (min)
  - 0 120

- 14C incorporation (pmol/500,000 cells)
  - 0 0.1 0.2 0.3 0.4

(d)

- 3H incorporation (fmol/500,000 cells)
  - Time (min)
  - 0 120

- 14C incorporation (pmol/500,000 cells)
  - 0 0.02 0.04 0.06
the presence of CMP-N-acetyl-[\textsuperscript{14}C]neuraminate. Thus CMP-N-acetyl-[\textsuperscript{14}C]neuraminate had totally inhibited the neuraminidase but only in cells which had been grown in 1 mM cyclic AMP and 1 mM theophylline. As in a previous experiment (Fig. 4.5a) B\textsubscript{16} melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.9aB) incorporated more \textsuperscript{14}C from CMP-N-acetyl-[\textsuperscript{14}C]neuraminate than control cells (Fig. 4.9aA). In order to check whether B\textsubscript{16} melanoma cells from confluent cultures grown in 1 mM cyclic AMP and 1 mM theophylline had the capacity to alter the neuraminidase activity in control cells from confluent cultures this experiment was extended as follows. Incubation mixtures were prepared as for the control cells above, except that half the number of control cells in the incubation were replaced with an equal number of cells that had been grown in 1 mM cyclic AMP and 1 mM theophylline. The results (mean of 6 assays) are not shown in the figure but it was found that the \textsuperscript{3}H lost during incubation was the mean of the other two results; these being, in turn, the \textsuperscript{3}H lost from the reaction mixture containing only control cells, and the \textsuperscript{3}H lost from the reaction mixture containing only cells which had been grown with 1 mM cyclic AMP and 1 mM theophylline. This was true also for the \textsuperscript{14}C incorporation. It was concluded that the B\textsubscript{16} melanoma cells from confluent cultures grown with 1 mM cyclic AMP and 1 mM theophylline are unable to alter the neuraminidase activity of control cells from confluent cultures and vice versa.

The above conclusions are based on the assumption that N-\textsuperscript{[3}H]acetylmannosamine is incorporated exclusively into sialic acid residues of B\textsubscript{16} melanoma cells. To check this assumption, the supernatant from B\textsubscript{16} melanoma cells which had
been grown with 1 mM cyclic AMP and 1 mM theophylline and which had been incubated without CMP-N-acetyl-[\(^{14}\)C]neuraminic acid for 2 h at 37°C (as shown in the lower part of Fig. 4.9a) was, after removal of the precipitate obtained in the course of the glycosyltransferase procedure, subjected to TLC, and the resulting distribution of radioactivity determined, all as described in Methods for TLC. Fig. 4.9(bi) shows the distribution of CMP-N-acetyl-[\(^{14}\)C]neuraminic acid and N-acetyl-[\(^{14}\)C]neuraminic acid markers on the TLC plate. Fig. 4.9(bii) shows the distribution of \(^3\)H derived from the N-[\(^3\)H]mannosamine supplied and the position of marker N-acetylneuraminic. It can be seen that material labelled with \(^3\)H, which has disappeared from the phosphotungstate-precipitable material (as shown in the lower part of Fig. 4.9a), has appeared in the supernatant; and the principal peak of radioactivity has an \(R_f\) which coincides with that of N-acetylneuraminic. While there is some trailing of the \(^3\)H in the area behind this principal peak, it is clear that this closely resembles the trailing of the \(^{14}\)C in the area behind the principal peak of the N-acetyl-[\(^{14}\)C]neuraminic acid standard in Fig. 4.9(bi). There is a small peak of \(^3\)H on the plate which runs ahead of the N-acetylneuraminic but this was not identified. It is clear however that the release, during incubation, of \(^3\)H from B16 melanoma cells labelled from N-[\(^3\)H]acetylmannosamine, has a high correlation with neuraminidase activity.

The experimental procedure used in the first part of the above experiment (Fig. 4.9a) was followed in two further experiments, but on each occasion a variation was introduced. The variation in the first of these two experiments was that
sparse cells were used instead of confluent cells. The purpose of this was to see if the results obtained could be correlated with those of Bosman (1973) who studied glycosidase activities of lines of B16 melanoma cells with differing metastatic potentials. The variation introduced in the second experiment was that the concentration of CMP-N-acetyl-[\(^{14}\)C]neuraminic acid was reduced by a factor of one hundred in order to investigate if there was any correlation between the concentration of CMP-N-acetyl-[\(^{14}\)C]neuraminic acid and its effect on neuraminidase activity. Fig. 4.9(c) shows that the uptake of \(^{14}\)C in each group of cells from sparse cultures appeared to be less than that in the corresponding group of cells from confluent cultures, but cells grown with 1 mM cyclic AMP and 1 mM theophylline still incorporated more \(^{14}\)C than did control cells (P < 0.01 Student's 't' test). The addition of CMP-N-acetyl-[\(^{14}\)C]neuraminic acid prevented significant loss of \(^{3}\)H from control cells (P > 0.1 Student's 't' test), and decreased the loss of \(^{3}\)H from cells grown with 1 mM cyclic AMP and 1 mM theophylline. Thus in sparse cells the correlation between the total inhibition of neuraminidase and growth of cells with 1 mM cyclic AMP and 1 mM theophylline seems to be the reverse of the situation found with confluent cells.

The next experiment differed from that shown in Fig. 4.9(a) only in that the concentration of CMP-N-acetyl-[\(^{14}\)C]-neuraminic acid was reduced by a factor of one hundred for the reason explained above. Fig. 4.9(d) shows that this had the effect of reducing the \(^{14}\)C incorporation by both relevant groups of cells five- to seven-fold. The loss of \(^{3}\)H from control cells during incubation was not significantly reduced
by this amount of CMP-N-acetyl-[\(^{14}\)C]neuraminate (P > 0.1 Student's 't' test), but the loss of \(^3\)H from cells grown with 1 mM cyclic AMP and 1 mM theophylline was reduced by approximately 60%. Thus while neuraminidase activity in control cells appears to have been unaffected (as in the experiment, the results of which are shown in Fig. 4.9a), there is still inhibition of neuraminidase activity in cells from confluent cultures which have been grown with 1 mM cyclic AMP and 1 mM theophylline, by levels of CMP-N-acetyl-[\(^{14}\)C]neuraminate one hundred fold less, even though here the inhibition is incomplete.

The above series of results indicate that whatever the mechanism whereby CMP-N-acetyl-[\(^{14}\)C]neuraminate effects the total inhibition of neuraminidase, it is fully effective in cells grown with 1 mM cyclic AMP and 1 mM theophylline only in confluent cultures, and in control cells only in sparse cultures.

CELL SURFACE SIALIC ACIDS AND EXOGENOUS NEURAMINIDASES

In order to obtain information about the total amount of \(^3\)H (and, by inference, \([^{3}\text{H}]\text{sialic acid}\)) that could be released from the cell surface, control B\(_{16}\) melanoma cells labelled from N-[\(^3\)H]acetylmannosamine as in the above experiments were incubated at 37°C with Vibrio cholerae neuraminidase (0.01 I.U. of enzyme per 10\(^6\) cells in 210 \(\mu\)l of 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose). After 1 h of incubation 44% of the \(^3\)H content of the B\(_{16}\) melanoma cells had been released into the supernatant,
and after 2 h 48% had been released. Similar results were obtained when Clostridium perfringens neuraminidase was used. In a similar experiment when the cells were labelled with N-acetyl-[\textsuperscript{\textit{14}}C]mannosamine (2 µCi/10 ml of tissue culture medium), Vibrio cholerae neuraminidase and Clostridium perfringens neuraminidase once again released approximately 50% of the total B₁₆ melanoma cell \textsuperscript{\textit{14}}C, and it made no difference if the cells had been grown with 1 mM cyclic AMP and 1 mM theophylline. When influenza virus neuraminidase was used only 24% of the total cell \textsuperscript{\textit{14}}C had been removed after 2 h of incubation, and again it made no difference whether or not the cells had been grown with 1 mM cyclic AMP and 1 mM theophylline. Influenza virus neuraminidase is known to cleave \textit{α}(2-3)glycosidic linkages of oligosaccharides at a much greater rate than it cleaves \textit{α}(2-6) linkages (Corfield and Schauer, 1982b). Clostridium perfringens neuraminidase and Vibrio cholerae neuraminidase are only slightly more efficient at cleaving \textit{α}(2-3) linkages than influenza virus neuraminidase. They are, however, 100 times more efficient at cleaving \textit{α}(2-3) linkages, and ten times more efficient at cleaving \textit{α}(2-8) linkages, than influenza virus neuraminidase. The differences encountered in the above experiment may be a consequence of the specificity of the glycosidic linkages. The amount of sialic acid removed by endogenous enzyme during 2 h incubation is a little less than that removed by influenza virus neuraminidase in the same time (Fig. 4.9aA), but it is not possible to conclude from these results whether the endogenous enzyme may have a similar specificity to influenza virus neuraminidase or not.
EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON GLYCOPROTEIN

PROFILES OBTAINED BY GEL FILTRATION

To investigate whether 1 mM cyclic AMP and 1 mM theophylline induced any alterations in the oligosaccharide chains of membrane glycoproteins, B₁₆ melanoma cells were grown with and without 1 mM cyclic AMP and 1 mM theophylline. Membrane glycoproteins were labelled with one or more of the following precursors: CMP-N-acetyl-[¹⁴C]neuraminate, [³H]-threonine, N-[³H]acetylmannosamine and N-acetyl-[¹⁴C]mannosamine. When cells were to be labelled from CMP-N-acetyl-[¹⁴C]neuraminate, 11.25 nmol (1.85 μCi) of this substrate were incubated with 2.5 x 10⁷ B₁₆ melanoma cells for 2 h at 37°C. When cells were labelled from other labelled substrates, they were added to the tissue culture medium for 48 h before harvesting. The number of μCi added per 10 ml of tissue culture medium was as follows: 2 μCi of [³H]threonine; 2 μCi of N-[³H]acetylmannosamine; 10 μCi of N-acetyl-[¹⁴C]-mannosamine. Glycoproteins were extracted by 1 mM Triton X-100 as described in Methods. These Triton X-100 extracts were fractionated by gel filtration on a Bio-Gel A-0.5 m agarose column (70 cm x 2.5 cm); 2 ml fractions were collected and 0.5 ml of each fraction was counted as described in Methods for radioactive counting.

Fig. 4.10(a) shows the two elution profiles obtained by gel filtration of Triton X-100 extract on agarose, when cells were labelled only with CMP-N-acetyl-[¹⁴C]neuraminate. There were four major peaks (I-IV). The Triton X-100 extract from B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM
FIG. 4.10  EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE CAPACITY OF B₁₆ MELANOMA CELLS TO INCORPORATE $^{14}$C FROM CMP-N-ACETYL-$^{[14]}$CNEURAMINATE

Distribution of $^{14}$C in gel filtration elution profiles of tritonates obtained from B₁₆ melanoma cells which had been grown with and without 1 mM cyclic AMP and 1 mM theophylline harvested and incubated for 2 h at 37°C with CMP-N-acetyl-[1⁴C]-neuraminate as described in Methods, with 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose.

- with 1 mM cyclic AMP and 1 mM theophylline
- without 1 mM cyclic AMP and 1 mM theophylline

(a) $^{14}$C distribution in the elution profile of a Triton X-100 extract fractionated on a Bio-Gel A-0.5 m agarose column (2.5 cm x 70 cm).
FIG. (continued) 4.10

EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE
CAPACITY OF B16 MELANOMA CELLS TO INCORPORATE
$^{14}\text{C}$ FROM CMP-\textit{N}-ACETYL-$^{[14}\text{C}]$NEURAMINATE

(b) $^{14}\text{C}$ distribution in the elution profile of peak I in
(a) above which had been digested with pronase as
described in Methods and fractionated on a Sephadex
G-50 (superfine) column (0.8 cm x 90 cm).

(c) $^{14}\text{C}$ distribution in the elution profile of peak II
in (a) above obtained after pronase treatment and
gel filtration as for that in (b) above.

(d) $^{14}\text{C}$ distribution in the elution profile of peak III
in (a) above obtained after pronase treatment and
gel filtration as for that in (b) above.

[Graph showing distribution with symbols for 1 mM cyclic AMP and 1 mM theophylline and symbols for without 1 mM cyclic AMP and 1 mM theophylline]
theophylline incorporated relatively more $^{14}$C into peaks I and II and less into peak III than the corresponding Triton X-100 extracts from control cells. Peak IV contains excess CMP-N-acetyl-$^{14}$C]neuraminic acid from the reaction mixtures. Peaks I, II and III from each profile were each separately pooled and digested with pronase and each pronase digest was separately fractionated by gel filtration chromatography on Sephadex G-50 (superfine), as described in Methods. The profiles obtained from peaks I, II and III are shown in Fig. 4.10(b, c and d respectively). While significant quantities of $^{14}$C-labelled material from peaks I and II appear to have been digested by the pronase, very little $^{14}$C-labelled material from Peak III had been digested.

Fig. 4.11(a and b) show the two elution profiles obtained by gel filtration of Triton X-100 extracts on A-0.2 m agarose when $B_{16}$ melanoma cells grown with and without 1 mM cyclic AMP and 1 mM theophylline were each labelled with both [3H]threonine and CMP-N-acetyl-[14C]neuraminic acid as described in Methods. As in Fig. 4.10(a) there are four major peaks, peaks I-III being labelled with both $^3$H and $^{14}$C and peak IV containing excess CMP-N-acetyl-[14C]neuraminic acid. Fig. 4.11(c) shows the profiles obtained when pronase digests of peak III were subjected to gel filtration on Bio-Gel P-300 polyacrylamide, which has a fractionation range of 60,000-400,000 ($M_r$). It can be seen that there is a broad peak in the middle fractionation range, but very little low molecular weight material, indicating that little pronase digestion has taken place. No investigation was carried out to determine whether the resistance of these preparations to proteolysis
FIG. 4.11 EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE CAPACITY OF B16 MELANOMA CELLS TO INCORPORATE $^3$H FROM [$^3$H]THREONINE AND $^{14}$C FROM CMP-N-ACETYL-$[^{14}$C]-NEURAMINATE

Distribution of $^3$H and $^{14}$C in gel filtration profiles of Triton X-100 extract obtained from B16 melanoma cells which had been grown with [$^3$H]-threonine and with and without 1 mM cyclic AMP and 1 mM theophylline, and incubated for 2 h at 37°C with CMP-N-acetyl-$[^{14}$C]neuraminate as described in the Text.

(a) $^3$H and $^{14}$C distribution in the elution profile of a Triton X-100 extract from cells grown with 1 mM cyclic AMP and 1 mM theophylline, and fractionated on a Bio-Gel A-0.5 m column (2.5 cm x 70 cm).

- $^{14}$C from CMP-N-acetyl-$[^{14}$C]neuraminate
- $^3$H from [$^3$H]threonine

(b) $^3$H and $^{14}$C distribution of a Triton X-100 extract from control cells fractionated as in (a) above.

- $^{14}$C from CMP-N-acetyl-$[^{14}$C]neuraminate
- $^3$H from [$^3$H]threonine

(c) $^3$H and $^{14}$C distribution in the elution profiles obtained when pronase digests of peak III in each of (a) and (b) above were separately subjected to gel filtration on a Bio-Gel P-300 polyacrylamide column (2.5 cm x 70 cm). The symbols used relate to those in (a) and (b) above.
was caused by inhibition of pronase by residual Triton X-100 detergent.

Fig. 4.12(a) shows two elution profiles of Triton X-100 extracts on Bio-Gel A-0.5 m agarose when the B₁₆ melanoma cells were labelled from N-acetyl-[¹⁴C]mannosamine and grown with and without 1 mM cyclic AMP and 1 mM theophylline. Peak III from B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline is significantly lower relative to peaks I and II, than the corresponding peak from control B₁₆ melanoma cells. Fig. 4.12(b) shows the profiles obtained when pronase digests of peak III were subjected to gel filtration on Sephadex G-50 (superfine). All of the labelled material is now in the excluded peak showing that as before no pronase digestion has occurred. Fig. 4.12(c and d) show the elution profiles obtained by gel filtration of Triton X-100 extracts on Bio-Gel A-0.5 m agarose when the B₁₆ melanoma cells were grown in N-[³H]acetylmannosamine and incubated with CMP-N-acetyl-[¹⁴C]-neuraminate. Peaks I, II and III in the ³H and ¹⁴C elution profiles are similarly aligned. Relative to the ¹⁴C in peak I, there is less ¹⁴C in peak III from the B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.12c) than in the corresponding profile from control B₁₆ melanoma cells (Fig. 4.12d). Peak IV contains excess of CMP-N-acetyl- [¹⁴C]neuraminate.

The above experiments have shown that ¹⁴C from CMP-N-acetyl-[¹⁴C]neuraminate which had been incorporated into cells may be extracted into the Triton X-100 extract. In order to investigate whether sialyltransferase activity might be present in the Triton X-100 extract, which may permit the
FIG. 4.12 EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE CAPACITY OF B16 MELANOMA CELLS TO INCORPORATE $^3$H OR $^{14}$C FROM N-[$^3$H]ACETYLMANNOSAMINE, N-ACETYL-$^{14}$C]MANNOSAMINE AND CMP-N-ACETYL-$^{14}$C]NEURAMINATE

Distribution of $^3$H and $^{14}$C in gel filtration profiles of Triton X-100 extracts obtained from B16 melanoma cells which had been grown with N-[$^3$H]acetylmannosamine or N-acetyl-$^{14}$C]mannosamine, with and without 1 mM cyclic AMP and 1 mM theophylline and sometimes incubated with CMP-N-acetyl-$^{14}$C]neuraminate as described in the Text.

(a) $^{14}$C distribution in the elution profile of a Triton X-100 extract from cells grown with N-acetyl-$^{14}$C]mannosamine with and without 1 mM cyclic AMP and 1 mM theophylline and fractionated on a Bio-Gel A-0.5 m agarose column (2.5 cm x 70 cm). (○—○) with 1 mM cyclic AMP and 1 mM theophylline (●—●) without 1 mM cyclic AMP and 1 mM theophylline

(b) $^{14}$C distribution in the elution profiles obtained when pronase digests of peak III from each of the profiles in (a) above were separately subjected to gel filtration on a Sephadex G-50 (superfine) column (0.8 cm x 90 cm). The symbols used are as in (a) above.
FIG. (continued) 4.12

EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE CAPACITY OF B16 MELANOMA CELLS TO INCORPORATE $^3$H OR $^{14}$C FROM N-[$^3$H]ACETYLMANNOSAMINE, N-ACETYL-[1$^4$C]MANNOSAMINE AND CMP-N-ACETYL-[1$^4$C]NEURAMINATE

(c) $^3$H and $^{14}$C distribution in the elution profile of a Triton X-100 extract obtained from cells grown with N-[$^3$H]acetylmannosamine, 1 mM cyclic AMP and 1 mM theophylline and incubated for 2 h at 37°C with CMP-N-acetyl-[1$^4$C]neuraminate, and fractionated on a Bio-Gel A-0.5 m agarose column (2.5 cm x 70 cm).

( O——O ) $^{14}$C from CMP-N-acetyl-[1$^4$C]neuraminate

( ■——■ ) $^3$H from N-[$^3$H]acetylmannosamine

(d) $^3$H and $^{14}$C distribution in the elution profile of a Triton X-100 extract, as in (c) above except that the cells had been grown without 1 mM cyclic AMP and 1 mM theophylline. The symbols used are as in (c) above.
incorporation of $^{14}$C from CMP-N-acetyl-$^{14}$C neuraminate into glycoproteins, a Triton X-100 extract was prepared as described in Methods and incubated with CMP-N-acetyl-$^{14}$C neuraminate. A quantity of Triton X-100 extract equivalent to that derived from $10^7$ B$_{16}$ melanoma cells was incubated with 9 nmol (1.48 µCi) of CMP-N-acetyl-$^{14}$C neuraminate in 50 mM Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose, and applied to a Sephadex G-50 (superfine) column as described in Methods for gel filtration chromatography. It was found that no $^{14}$C was incorporated into glycoprotein fractions (almost all in the excluded volume) eluted from the column. In further experiments reaction mixtures were prepared as for the glycosyltransferase assay described in Methods, using 450 pmol of CMP-N-acetyl-$^{14}$C neuraminate as substrate, and incubated for 2 h at 37°C with various concentrations of Triton X-100. The Triton X-100 concentrations used (assuming a mean molecular weight of 646) were: 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM and 1 mM; the inhibition of $^{14}$C incorporation into phosphotungstate precipitable material, brought about by the addition of Triton X-100, was respectively 49%, 67%, 82%, 91% and 100%.

Thus, in summary, when B$_{16}$ melanoma cells are grown with 1 mM cyclic AMP and 1 mM theophylline the uptake of $^{14}$C from CMP-N-acetyl-$^{14}$C neuraminate in peak III, relative to peaks I and II, is less than that in the corresponding profiles from control cells (Figs. 4.10a, 4.11a, 4.11b, 4.12c and 4.12d). When labelled from CMP-N-acetyl-$^{14}$C neuraminate, peak I and peak II increased relative to controls when cells were grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.10a, 4.11a, 4.12c and 4.12d). When B$_{16}$ melanoma cells
were grown with labelled N-acetylmannosamine, the addition of 1 mM cyclic AMP and 1 mM theophylline to the tissue culture medium produced a small decrease in incorporation of label into peak I (Figs. 4.12a, 4.12c and 4.12d. This is not true for peak II (Figs. 4.12a, 4.12c and 4.12d). However these results suggest that the metabolic pool labelled from CMP-N-acetyl-[\(^{14}\)C]neuraminic is not identical with the metabolic pool labelled from labelled N-acetylmannosamine. Peaks I and II are to a large extent degraded by pronase (Figs. 4.10b and 4.10c). Peak III was not degraded by pronase (Fig. 4.10d and Fig. 4.12b), as confirmed by the position of the \(^{3}\text{H}^{2}\)-threonine in the P-300 polyacrylamide elution profile (Fig. 4.11c), which coincided with the position of \(^{14}\)C from CMP-N-acetyl-[\(^{14}\)C]neuraminic. The reason for the resistance to pronase degradation of the above fraction was not investigated.

**SIALYLTRANSFERASE OF B_{16} MELANOMA CELL SURFACE MEMBRANES**

**PREPARATIONS**

In an attempt to investigate whether the findings of the preceding experiments still applied if cell surface membranes instead of whole cells were incubated with CMP-N-acetyl-[\(^{14}\)C]neuraminic, cell surface membranes were prepared as described in Methods. The initial experiment examined the sialyltransferase activity. B_{16} melanoma cells used in this experiment had been grown with and without 1 mM cyclic AMP and 1 mM theophylline. Since the sialyltransferase has been shown to be inhibited by Mn\(^{2+}\) (Fig. 4.4a) and the procedure for membrane preparation uses the divalent cation Zn\(^{2+}\), a
preliminary study was carried out to test the effect of EDTA on the sialyltransferase activity in the membrane preparation obtained. The glycosyltransferase assay described in Methods was used except that each 1.2 ml reaction mixture contained, instead of 5 x 10^5 B16 melanoma cells, the cell surface membranes obtained from 8 x 10^6 B16 melanoma cells; moreover, EDTA was substituted for Mn^{2+}. Two concentrations of EDTA were investigated, namely 0.83 mM and 83 μM, and the results with each did not differ (P > 0.1, Student's 't' test, n=5). The addition of each of the above concentrations of EDTA increased the uptake of ^14C into the phosphotungstate precipitate by two thirds compared to the uptake by cells incubated without EDTA. It was concluded that EDTA should be added to reaction mixtures in experiments involving sialyltransferase in membrane preparations.

It was found that when B16 melanoma cells were incubated with CMP-N-acetyl-[^14C]neuraminic acid in accordance with the procedure for the glycosyltransferase assay as described in Methods (except that Mn^{2+} was omitted), they incorporated 85 times more ^14C into the phosphotungstate precipitate than a quantity of cell surface membranes, derived from the same number of cells, incubated with either 0.83 mM or 83 μM EDTA instead of Mn^{2+} and assayed similarly. Fig. 4.13 shows the ^14C distribution in elution profiles obtained by gel filtration on Bio-Gel A-0.5 m agarose as described in Methods. The Triton X-100 extracts applied to the column were prepared as described in Methods from B16 melanoma cell surface membranes. The membranes from 9 x 10^7 cells had been incubated for 2 h at 37°C with 13.5 nmol of
FIG. 4.13 EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON THE CAPACITY OF B₁₆ MELANOMA CELL SURFACE MEMBRANES TO INCORPORATE $^{14}$C FROM CMP-N-ACETYL-$[^14]$C-NEURAMINATE

(a) $^{14}$C distribution in the elution profile of a Triton X-100 extract obtained from surface membranes of B₁₆ melanoma cells which had been grown with 1 mM cyclic AMP and 1 mM theophylline and incubated for 2 h at 37°C with CMP-N-acetyl-$[^14]$C-neuraminate as described in the Text, and fractionated on a Bio-Gel A-0.5 m agarose column (2.5 cm x 70 cm).

(b) $^{14}$C distribution in the elution profile of a Triton X-100 extract as in (a) above except that the surface membranes were obtained from control B₁₆ melanoma cells.
CMP-N-acetyl-[\(^{14}\)C]neuraminate in 1.5 ml of 0.05 M Tris-maleate buffer, pH 7.4, containing 0.25 M sucrose and 0.83 mM EDTA. The distribution obtained in Fig. 4.13(a), where the membranes were derived from cells grown with 1 mM cyclic AMP and 1 mM theophylline, and in Fig. 4.13(b), where the membranes were derived from control cells, may be compared respectively with the \(^{14}\)C distribution in comparable profiles in Fig. 4.10(a). While the comparable profiles are not grossly dissimilar, the positions of the principal peaks in Fig. 4.13 are not exactly the same as those in Fig. 4.10(a), and the total incorporation in the former is very small indeed. A sufficient number of counts were obtained to give a 98% level of confidence (Aronoff, 1956) for the c.p.m. values of the tubes coinciding with the principal peaks in the profile. While treatment with 1 mM cyclic AMP and 1 mM theophylline appears to bring about small changes in some of these peaks, the time required for counting and the number of cells needed rendered this approach impractical. For this reason the approach was not pursued further.

**INHIBITION OF INFLUENZA VIRUS NEURAMINIDASE BY B\(_{16}\) MELANOMA CELLS INCUBATED WITH CMP-N-ACETYL-[\(^{14}\)C]NEURAMINATE: THE MODEL SYSTEM**

It was shown (Fig. 4.9a) that B\(_{16}\) melanoma cell neuraminidase was inhibited by the addition of CMP-N-acetyl-[\(^{14}\)C]neuraminate to incubation mixtures. While mammalian neuraminidase has been reported to occur in a number of intracellular sites, namely, lysosomes (Horvat and Touster, 1968), Golgi apparatus (Kishore et al., 1975) and cytosol
(Tulsiani and Carubelli, 1970), it is possible that the B₁₆ melanoma cell neuraminidase enzyme inhibited was bound to the plasma membrane, as explained in the results relating to Fig. 4.9(a, c and d). However, to prove this contention it would be necessary to isolate neuraminidases from the various cell locations in order to test the inhibitory effects of CMP-N-acetylneuraminate on each. These enzymes have not been isolated and purified from B₁₆ melanoma cells and in view of the limitation in the quantity of material available it is possible that purification of these enzymes might not be readily accomplished. For this reason it seemed appropriate to investigate whether B₁₆ melanoma cells supplied with CMP-N-acetylneuraminate might be able to inhibit an exogenous neuraminidase, particularly when the cells had been grown with 1 mM cyclic AMP and 1 mM theophylline.

The advantage of such a model system is that a greater quantity of enzyme can be used and thus the effects obtained are more easily studied. The fact that CMP-N-acetylneuraminate is the substrate for sialyltransferase suggests that the inhibitory effect may result from a sialylation reaction, as for example sialylation of the neuraminidase itself, or sialylation of some other molecule with the production of the inhibitor.

Influenza virus neuraminidase was chosen as the enzyme to be tested because it is known that it contains five potential glycosylation sites (Fields et al., 1981), none of which are sialylated in the active enzyme isolated (Blok et al., 1982). While such a system (i.e. incubation of mammalian cells such as B₁₆ melanoma cells with influenza
virus neuraminidase) can be regarded as a model system, it is nevertheless related to one that has a known biological function. Fields et al. (1981) have suggested that neuraminidase which is embedded in the membrane of the influenza virus functions by desialylating a second viral membrane-bound glycoprotein, namely haemagglutinin. Virus neuraminidase has two possible roles related to the function of the haemagglutinin, which binds the influenza virus to sialic acid-containing receptors on the cells. One is to remove sialic acid from the virus envelope itself, thus preventing self-aggregation of virus particles during their release from the host cells by eliminating receptors for the haemagglutinin (Palese et al., 1974). The other is to remove sialic acid from the haemagglutinin, thus facilitating its proteolytic cleavage, which is essential for infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

Therefore three separate experiments were carried out to investigate whether B16 melanoma cells could inhibit influenza virus neuraminidase when supplied with CMP-N-acetyl-neuraminate. In fact CMP-N-acetyl-[14C]neuraminate was used because unlabelled CMP-N-acetylneuraminate was not commercially available when these experiments were undertaken.

In the first experiment, using the model system for studying neuraminidase inhibition, influenza virus neuraminidase was incubated with 10⁷ B16 melanoma cells grown with and without 1 mM cyclic AMP and 1 mM theophylline and 4.5 nmol of CMP-N-acetyl-[14C]neuraminate per 1 ml incubation mixture, in Hanks's buffered saline, pH 7.4, for 2 h as described in
Methods. Two control incubations were also carried out. In the first, cells were omitted; and in the second, both cells and CMP-N-acetyl-[\textsuperscript{14}C]neuraminate were omitted. After incubation all cells were spun off and the supernatant fluid and controls were each incubated with 2-(3'-methoxyphenyl)-\textalpha-D-neuraminate for further periods up to 2 h, according to the procedure for the neuraminidase assay using the above substrate as described in Methods.

Fig. 4.14(a) shows that CMP-N-acetyl-[\textsuperscript{14}C]neuraminate alone did not inhibit influenza virus neuraminidase. However, the influenza virus neuraminidase that had been incubated for 2 h with CMP-N-acetyl-[\textsuperscript{14}C]neuraminate and control cells was inhibited for the first hour, but this inhibition subsequently decreased with time. The quantity of methoxyphenol released during the last 25 min of incubation was approximately the same as that released by enzyme alone over the same time period, i.e. the neuraminidase activity was the same in both. The influenza virus neuraminidase which had been incubated with CMP-N-acetyl-[\textsuperscript{14}C]neuraminate and cells grown with 1 mM cyclic AMP and 1 mM theophylline was totally inhibited for the first hour, but some activity was regained over the second hour.

The second experiment was carried out in order to determine how rapidly the inhibition of neuraminidase was brought about during incubation with B₁₆ melanoma cells and CMP-N-acetyl-[\textsuperscript{14}C]neuraminate. Only cells grown in 1 mM cyclic AMP and 1 mM theophylline were used, because these had been shown to produce more inhibition than had untreated cells. CMP-N-acetyl-[\textsuperscript{14}C]neuraminate was omitted from a
FIG. 4.14 INHIBITION OF INFLUENZA VIRUS NEURAMINIDASE INCUBATED WITH B16 MELANOMA CELLS AND CMP-N-ACETYL-[14C]NEURAMINATE

(a) Release of methoxyphenol, measured as described in Methods, during incubation of supernatants with 2-(3'-methoxyphenyl)-N-acetyl-\(\alpha\)-D-neuraminate for various times up to 2 h. Supernatants were obtained, as described in Methods for the model system for studying neuraminidase inhibition, from 2 h incubation mixtures containing B16 melanoma cells, CMP-N-acetyl-[14C]neuraminate and influenza virus neuraminidase. Each point represents a single determination. Cells used were grown with (△—△) and without (Δ—Δ) 1 mM cyclic AMP and 1 mM theophylline. Control incubations in which cells were omitted (●—●) and in which both cells and CMP-N-acetyl-[14C]neuraminate were omitted (○—○) were included.

(b) Release of methoxyphenol, measured as described in Methods, during incubation of supernatants with 2-(3'-methoxyphenyl)-N-acetyl-\(\alpha\)-D-neuraminate for 1 h. Supernatants were obtained, as described in Methods for the model system for studying neuraminidase inhibition, from incubation mixtures containing B16 melanoma cells, CMP-N-acetyl-[14C]neuraminate and influenza virus neuraminidase which had been incubated for various times up to 1 h as shown. Each point represents a single determination. Cells grown with 1 mM cyclic AMP and 1 mM theophylline were incubated with (●—●) and without (○—○) CMP-N-acetyl-[14C]neuraminate.
FIG. (continued) 4.14

INHIBITION OF INFLUENZA VIRUS NEURAMINIDASE
INCUBATED WITH B16 MELANOMA CELLS AND CMP-N-ACETYL-[\(^{14}\)C]NEURAMINATE

(c) Release of methylumbelliferone, measured as described in Methods, during incubation of supernatants with 4-methylumbelliferyl-\(\alpha\)-D-N-acetylneuraminide for 1 h. Supernatants were obtained, as described in Methods for the model system for studying neuraminidase inhibition, from incubation mixtures containing B16 melanoma cells, CMP-N-acetyl-[\(^{14}\)C]neuraminate and influenza virus neuraminidase which had been incubated for various times up to 2 h as shown. Each point represents a single determination. Cells grown with 1 mM cyclic AMP and 1 mM theophylline were incubated with (\(\triangle\)) and without (\(\square\)) CMP-N-acetyl-[\(^{14}\)C]neuraminate. Control cells were also incubated with (\(\square\)) or without (\(\bigcirc\)) CMP-N-acetyl-[\(^{14}\)C]neuraminate. A series of control incubations (\(\bigcirc\)) containing influenza virus neuraminidase alone were included.

(d) Release of methylumbelliferone, measured as described in Methods, during incubation of supernatants with 4-methylumbelliferyl-\(\alpha\)-D-N-acetylneuraminide for various times up to 3 h. Supernatants were obtained as described in Methods for the model system for studying neuraminidase inhibition, from 2 h incubation mixtures containing B16 melanoma cells, CMP-N-acetyl-[\(^{14}\)C]neuraminate and influenza virus neuraminidase. Each point represents a single determination. The symbols are as for (c) above.
second series of incubation mixtures which provided a control. The experiment was carried out in the same way as the first, except that instead of incubation for the initial 2 h period the influenza virus neuraminidase was incubated with the cells for various times up to one hour. At the conclusion of each period cells were spun off and the supernatants were assayed by incubating for 1 h with 2-(3'-methoxyphenyl)-α-D-neuraminate substrate as described in Methods. It is important to note the essential difference between the first and second experiments. In the first experiment the mixtures were incubated for a fixed time before the preparation of supernatants, but the time of incubation of the supernatant with the assay substrate was varied. In the second experiment the mixtures were incubated for various times before the preparation of the supernatant, but the time of the subsequent incubation of the supernatant with the assay substrate was constant.

Fig. 4.14(b) shows that cells without exogenously supplied CMP-N-acetyl-[\(^{14}\)C]neuraminate nevertheless had some capacity to inhibit influenza virus neuraminidase. The neuraminidase activity, measured in terms of methoxyphenol released per hour, decreased over the first 10 minutes of incubation with cells grown with 1 mM cyclic AMP and 1 mM theophylline. After incubation for 1 h the neuraminidase activity was not less than the activity measured after 10 minutes incubation. When exogenous CMP-N-acetyl-[\(^{14}\)C]neuraminate was added as well, the neuraminidase activity decreased at a faster rate over the first 10 minutes, but again the neuraminidase activity after incubation for 1 h
differed very little from the activity measured after 10 minutes.

At this time the 2-(3'-methoxyphenyl)-N-acetyl-α-D-neuraminate substrate became unavailable and subsequent experiments were carried out with an alternative substrate, 4-methylumbelliferyl-α-D-N-acetylneuraminide.

The third experiment was designed to verify the effect of CMP-N-acetyl-[\(^{14}\)C]neuraminate found in the second experiment, using cells grown with 1 mM cyclic AMP and 1 mM theophylline. In addition the effect of using cells grown without 1 mM cyclic AMP and 1 mM theophylline was investigated. Appropriate controls without CMP-N-acetyl-[\(^{14}\)C]neuraminate were included as well as a series of control incubations containing influenza virus alone. Furthermore, in addition to measuring the activity of neuraminidase in mixtures incubated for various times, this experiment was extended to include a study of the effect on neuraminidase activity when supernatants were incubated for various times, as in the first experiment. This used both control cells and cells grown with 1 mM cyclic AMP and 1 mM theophylline with and without CMP-N-acetyl-[\(^{14}\)C]neuraminate. A series of incubations using influenza virus alone was also included.

Fig. 4.14(c) confirms the results of the previous experiment (Fig. 4.14b) in that the amount of inhibition achieved by cells grown in 1 mM cyclic AMP and 1 mM theophylline was greater when CMP-N-acetyl-[\(^{14}\)C]neuraminate was added. A similar result was obtained for control cells. For almost all time periods cells grown in 1 mM cyclic AMP and
1 mM theophylline were more effective than control cells at inhibiting influenza virus neuraminidase. In this experiment the inhibitory effect obtained when supernatants were prepared from incubation mixtures containing CMP-N-acetyl-[^14C]neuraminate and cells grown with 1 mM cyclic AMP and 1 mM theophylline, was greater than that shown in Fig. 4.14(a), and after 40 minutes of incubation there was no further increase in inhibition. It can also be seen that in the absence of cells there was some inactivation of the enzyme but this was less than when cells were present - presumably loss of activity by enzyme alone was due to heat inactivation.

Fig. 4.14(d) shows the results of the second part of this third experiment, in which the incubation of influenza virus neuraminidase was reversed when supernatants, obtained after removal of the cells, were incubated for three hours. The reversal of inhibition is clearly seen only for the influenza virus neuraminidase which was inhibited by incubation in the presence of both CMP-N-acetyl-[^14C]-neuraminate and B16 melanoma cells grown for 48 h with 1 mM cyclic AMP and 1 mM theophylline. The methylumbelliferone released between 120 and 180 minutes by the supernatants was approximately the same as that released by control enzyme incubated alone for the corresponding times. The slope of the line joining these two points is a function of the enzyme activity (methylumbelliferone released per hour) and the two lines are approximately parallel. In other words the activity of the neuraminidase in these supernatants was the same as that of enzyme alone; the inhibition, clearly seen over the
first 90 minutes, was no longer present.

**H.P.L.C. ELUTION PROFILES OF SUPERNATANTS**

(a) **H.p.l.c. elution profiles of supernatants from the model system**

Using the model system described in Methods CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate incubated with B\(_{16}\) melanoma cells has been shown to inhibit influenza virus neuraminidase. It therefore seemed appropriate to investigate whether the \(^{14}\text{C}\) from CMP-N-acetyl-[\(^{14}\text{C}\)]neuraminate became incorporated into influenza virus neuraminidase when this was inhibited under these conditions. Supernatants obtained after removal of the cells from such incubation mixtures were examined by h.p.l.c. using the procedure described in Methods for gel filtration chromatography.

Before undertaking this investigation the reproducibility of the procedure was examined. H.p.l.c. elution profiles (absorption at 280 nm) obtained from each of a number of samples from a single supernatant were found to be very similar with respect to the height and time of elution of each of the respective peaks. Profiles of supernatants prepared from different batches of cells by the same procedure were very similar providing conditions were standardised. (The relative heights of some peaks did vary with time after preparation.)

**Fig. 4.15(a)** shows the h.p.l.c. elution profile (absorption at 280 nm) of a supernatant obtained from a reaction mixture which contained 10\(^{7}\) B\(_{16}\) melanoma cells
FIG. 4.15  H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM 
B16 MELANOMA CELLS INCUBATED WITH INFLUENZA VIRUS 
NEURAMINIDASE AND CMP-N-ACETYL-[\(^{14}\)C]NEURAMINATE

(a) H.p.l.c. elution profile (absorption at 280 nm) of a 
supernatant obtained when influenza virus neuraminid-
ase was inhibited, as described for the model system 
in Methods, by incubating for 2 h at 37°C with B16 
melanoma cells grown with 1 mM cyclic AMP and 1 mM 
theophylline. The distribution of \(^{14}\)C (-------) 
supplied as CMP-N-acetyl-[\(^{14}\)C]neuraminate was 
determined by counting 0.5 ml fractions as described 
in Methods for radioactive counting.

(b) H.p.l.c. elution profile (absorption at 280 nm) 
obtained as in (a) above except that influenza virus 
neuraminidase and CMP-N-acetyl-[\(^{14}\)C]neuraminate were 
omitted.

(c) H.p.l.c. elution profile (absorption at 280 nm) of 
1.25 units of influenza virus neuraminidase in Hanks's 
buffered saline, pH 7.4.

(d) H.p.l.c. elution profile (absorption at 280 nm) of the 
following marker proteins (i) \(\gamma\) globulin (bovine, 
160,000 \(M_r\)), (ii) albumin (bovine, 68,000 \(M_r\)), 
(iii) albumin (egg, 45,000 \(M_r\)) and (iv) cytochrome C 
(horse heart, 12,384 \(M_r\)).
(which had been grown with 1 mM cyclic AMP and 1 mM theophylline), 5 I.U. (3.1 mg) of influenza virus neuraminidase and 4.5 μM CMP-N-acetyl-[\textsuperscript{14}C]neuraminate, and had been incubated for 2 h at 37°C (in a ml of Hanks's buffered saline, pH 7.4). Fig. 4.15(a) also shows the h.p.l.c. \textsuperscript{14}C elution profile superimposed on the absorption (280 nm) profile obtained.

Fig. 4.15(b) shows the control elution profile (absorption at 280 nm) of the supernatant obtained when a reaction mixture without influenza virus neuraminidase and without CMP-N-acetyl-[\textsuperscript{14}C]neuraminate was incubated for 2 h at 37°C. Fig. 4.15(c) shows the elution profile (absorption at 280 nm) when an amount of influenza virus neuraminidase equal to that in the profile of Fig. 4.15(a) was added (in Hanks's buffered saline, pH 7.4) to the h.p.l.c. column. Fig. 4.15(d) shows the profile at 280 nm of the following marker proteins: γ globulin (bovine), albumin (bovine), albumin (egg) and cytochrome C (horse heart).

Peak 1 (Fig. 4.15a) is greater than peak 1 (Fig. 4.15b) since it contains influenza virus neuraminidase (200,000 M\textsubscript{x}) (Varghese et al., 1983). Peaks 2 and 3 (Fig. 4.15b) appear to be largely unaltered by the omission of influenza virus neuraminidase and CMP-N-acetyl-[\textsuperscript{14}C]neuraminate, although peak 4 increases and peak 5 decreases slightly. It can be seen that in Fig. 4.15(a) the \textsuperscript{14}C profile elutes where residual CMP-N-acetyl-[\textsuperscript{14}C]neuraminate or its breakdown products would be expected to elute. The ascending limb of this peak may contain some small proteins
since cytochrome C elutes in the same position. It was found that the activity of the influenza virus neuraminidase (Fig. 4.15c) was lost on passage through the h.p.l.c. column.

(b) **H.p.l.c. profiles of supernatants from B₁₆ melanoma cells grown with labelled precursors**

(i) **Elution profile labelled from N-[^3]H]acetylmannosamine**

It has been shown in Fig. 4.15(a) that influenza virus neuraminidase incubated for 2 h at 37°C with CMP-N-acetyl-[¹⁴]C]neuraminate and B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline does not contain incorporated ¹⁴C. To investigate whether the material responsible for the absorption peaks in an h.p.l.c. profile, obtained by similar procedures, contained endogenously derived sialic acid, the CMP-N-acetyl-[¹⁴]C]neuraminate was omitted from reaction mixtures and N-[^3]H]acetylmannosamine was added to the growth medium, together with 1 mM cyclic AMP and 1 mM theophylline, for 48 h before harvesting. In the same experiment, instead of incubating for 2 h at 37°C, reaction mixtures were incubated both for 10 min and 3 h, in order to compare the h.p.l.c. profiles obtained with that from the 2 h incubation mixture previously described, and gain some idea of the changes in the h.p.l.c. profile with time. It has already been noted in the Results section for the model system (Fig. 4.15a-d) that h.p.l.c. profiles carried out were highly reproducible when conditions were standardised. Thus the changes obtained after incubation at various times may be regarded as time related.
Fig. 4.16(a) shows that small amounts of $^3$H are incorporated into material co-eluting with the material responsible for the peaks of absorption at 280 nm. Fig. 4.16(b) shows small increases in the $^3$H incorporation into peaks 2 and 3 and a major increase into peak 5. There is no change in the $^3$H incorporation into peak 1, co-eluting with the influenza virus neuraminidase. The $^3$H incorporation into peak 5 probably indicates the presence of sialic acid as demonstrated by the results of Fig. 4.9(bii). Fig. 4.16(b) shows, relative to the profile in Fig. 4.16(a), that incubation of the reaction mixture for 3 h resulted in a considerable increase in the absorption at 280 nm in the region of the peaks eluting after peak 3, and a considerable diminution in peak 1. In a number of preliminary experiments, (results not shown) a similar pattern of change with time in the peaks was observed: the $A_{280}$ peak 1 decreased and the $A_{280}$ in peak 5 increased.

(ii) Elution profile labelled from DL-$[^3$H]threonine

To investigate the effect of incubation on the distribution of protein in h.p.l.c. profiles, obtained by similar procedures to those in Fig. 4.16(a) and Fig. 4.16(b), reaction mixtures containing influenza virus neuraminidase and B16 melanoma cells grown with DL-$[^3$H]threonine, 1 mM cyclic AMP and 1 mM theophylline were incubated for 10 min and 2 h without CMP-N-acetyl-$[^1$C]neuraminate. Control reaction mixtures prepared similarly, except that the neuraminidase was omitted, were incubated for the same periods. Fig. 4.17 shows that in all cases $^3$H incorporation into
FIG. 4.16  H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM B₁₆ MELANOMA CELLS GROWN WITH N-[³H]ACETYL-
MANNOSAMINE

(a) H.p.l.c. elution profile (absorption at 280 nm) of a supernatant obtained when influenza virus neuraminidase was incubated, as described in Methods for the model system for studying neuraminidase inhibition, for 10 min at 37°C without CMP-N-acetyl-[¹⁴C]neuraminic acid but with B₁₆ melanoma cells grown with N-[³H]acetyl-
mannosamine (2 µCi per 10 ml of tissue culture medium), 1 mM cyclic AMP and 1 mM theophylline. The distribution of ³H incorporated (-- -- --) from N-[³H]acetylmannosamine was determined by counting 0.5 ml fractions as described in Methods for radioactive counting.

(b) H.p.l.c. elution profile (absorption at 280 nm) and ³H incorporation obtained as in (a) above except that the period of incubation was for 3 h at 37°C.
FIG. 4.17 H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM B_{16} MELANOMA CELLS GROWN WITH DL-[^{3}H]THREONINE

(a) H.p.l.c. elution profile (absorption at 280 nm) of a supernatant obtained when influenza virus neuraminidase was incubated, as described in Methods for the model system for studying neuraminidase inhibition, for 10 min at 37°C without CMP-N-acetyl-[^{14}C]neuraminate but with B_{16} melanoma cells grown with DL-[^{3}H]threonine (2 µCi per 10 ml tissue culture medium), 1 mM cyclic AMP and 1 mM theophylline. The distribution of ^{3}H incorporated (-----) from [^{3}H]threonine was determined by counting 0.5 ml fractions as described under Methods for radioactive counting.

(b) H.p.l.c. elution profile (absorption at 280 nm), showing distribution of ^{3}H incorporation, obtained as in (a) above except that influenza virus neuraminidase was not included in the reaction mixture.

(c) H.p.l.c. elution profile (absorption at 280 nm), showing distribution of ^{3}H incorporation obtained as in (a) above except that the period of incubation was for 2 h at 37°C.

(d) H.p.l.c. elution profile (absorption at 280 nm), showing distribution of ^{3}H incorporation obtained as in (a) above except that influenza virus neuraminidase was not included in the reaction mixture and the period of incubation was for 2 h at 37°C.
peak 1 is relatively small by comparison with $^3$H incorporation into peak 3. The addition of influenza virus neuraminidase decreases the incorporation of $^3$H into all peaks including that in the low molecular weight range, peak 5. It can be seen also that for cells, incubated both with and without influenza virus neuraminidase, the longer incubation time results in a decreased $^3$H incorporation into peak 1 (the excluded peak) and an increased incorporation into most peaks eluting subsequently. Thus it appears that influenza virus neuraminidase prevents the release of material from the cell but does not prevent the conversion of material to lower molecular weight products during incubation. While the bulk of this material is likely to be protein, some conversion of threonine to lipid and carbohydrate cannot be excluded.

(iii) Elution profiles labelled from $[^3]$Hserine and from $[^{14}]$Cpalmitic acid

It appeared worthwhile to compare these results with those obtained when label from $[^3]$Hserine was incorporated into similar profiles; serine may be incorporated into glycolipids by more direct pathways than those used by threonine, since it is a precursor of sphingosine. A region in the profile containing label from serine but not label from threonine would suggest that glycolipids might be present. Since sphingolipids contain fatty acyl moieties, the incorporation of label from palmitic acid was also investigated. Only control cells were studied. Control cells were grown as described in Methods in medium to which $[^3]$Hserine (2.5 $\mu$Ci/
10 ml) and, in separate tissue culture bottles, \([^{14}\text{C}]\text{palmitic acid}\) (1 \(\mu\text{Ci/10 ml}\)), were added. H.p.l.c. profiles, obtained in a similar manner to those above, but not shown in a figure, showed that the distribution of palmitic acid and the distribution of serine were similar to the distribution of threonine. The incorporation of palmitic acid was very low. From this it was concluded that the \(^{14}\text{C}\) in peak 5, supplied as CMP-N-acetyl-\([^{14}\text{C}]\text{neuraminate}\), did not represent a major incorporation into sphingolipids.

**INHIBITION OF INFLUENZA VIRUS NEURAMINIDASE BY CELL FREE SUPERNATANTS**

Preceding experiments have shown that \(^{14}\text{C}\) from CMP-N-acetyl-\([^{14}\text{C}]\text{neuraminate}\) is not incorporated into influenza virus neuraminidase or other material in peak 1 of the h.p.l.c. elution profiles, even though the influenza virus neuraminidase is inhibited under these conditions. It is possible that the presence of CMP-N-acetyl-\([^{14}\text{C}]\text{neuraminate}\) leads to the release of material from the cell surface which does not contain \(^{14}\text{C}\) but which brings about the inhibition of the neuraminidase. To investigate this possibility, influenza virus neuraminidase was not incubated directly with the cells. Supernatants were prepared from \(B_{16}\) melanoma cells, grown with and without 1 mM cyclic AMP and 1 mM theophylline, which had been incubated for 15 min at 37° with CMP-N-acetyl-\([^{14}\text{C}]\text{neuraminate}\) according to the model system for studying neuraminidase inhibition described in Methods, but this time without the addition of influenza virus neuraminidase to the incubation mixture. The supernatants were then added to an
amount of influenza virus neuraminidase equal to that which had been omitted in the above procedure, and incubated for 20 min at 37°. The influenza virus neuraminidase was then assayed by incubating for 1 h at 37° using 2-(3'-methoxyphenyl)-N-acetyl-α-D-neuraminate as described in Methods.

Fig. 4.18 shows that, while supernatants did contain material which inhibited the neuraminidase, the effect was relatively small. Using the mean values shown in Fig. 4.18, it was calculated that in the case of control B₁₆ melanoma cells there was 11% inhibition and in the case of B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline there was 14% inhibition. These effects may be compared with those obtained in a previous experiment where comparable amounts of enzyme and CMP-N-acetyl-[¹⁴C]neuraminate incubated with cells in similar conditions, also for 1 h at 37° (Fig. 4.14c), inhibited influenza virus neuraminidase.

From the data shown in Fig. 4.14(c) it can be calculated that the addition of CMP-N-acetyl-[¹⁴C]neuraminate inhibited the influenza virus neuraminidase by 16% when added to control B₁₆ melanoma cells and by 34% when added to cells grown with 1 mM cyclic AMP and 1 mM theophylline. It appears therefore that in order to obtain the full inhibition brought about by CMP-N-acetyl-[¹⁴C]neuraminate the influenza virus neuraminidase must be incubated directly with B₁₆ melanoma cells in the presence of CMP-N-acetyl-[¹⁴C]neuramin-ate. Material released from B₁₆ melanoma cells incubated with CMP-N-acetyl-[¹⁴C]neuraminate in the absence of influenza virus neuraminidase does bring about some inhibition but does
FIG. 4.18 INHIBITION OF INFLUENZA VIRUS NEURAMINIDASE BY CELL FREE SUPERNATANTS

Supernatants were obtained from B16 melanoma cells, grown with or without 1 mM cyclic AMP and 1 mM theophylline, which had been incubated for 15 min according to the procedure for the model system for studying neuraminidase inhibition described in Methods, except that influenza virus neuraminidase was omitted. The supernatants were then incubated for 20 min at 37°C with an amount of influenza virus neuraminidase equal to that which had been omitted, and then assayed for neuraminidase activity using 2-(3'-methoxyphenyl)-N-acetyl-α-D-neuraminic acid, as described in Methods, by incubating for 1 h at 37°C. Each value represents the mean of 5 determinations ± S.E.M.

■ with CMP-N-acetyl-[14C]neuraminate

□ without CMP-N-acetyl-[14C]neuraminate

CMP-N-acetyl-[14C]neuraminic acid has a significant effect (P < 0.01, Student's 't' test) on both control B16 melanoma cells and B16 melanoma cells treated with 1 mM cyclic AMP and 1 mM theophylline.
3-Methoxyphenol released (pmol/10^6 cells)

- Control
- + 1 mM Cyclic AMP
- + 1 mM Theophylline
not account for the full effect, at least in the case of B₁₆ melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline. A limitation of this interpretation is that the inhibitory effects of the supernatants were not studied using the same batch of B₁₆ melanoma cells that was used to study inhibition in the complete model system.

CHANGES INDUCED BY CMP-N-ACETYL-[¹⁴C]NEURAMINATE IN H.P.L.C. ELUTION PROFILES

In preceding experiments it was shown that ¹⁴C from CMP-N-acetyl-[¹⁴C]neuraminate was not incorporated into the inhibited influenza virus neuraminidase, or into other material co-eluting with it, in peak 1 of the h.p.l.c. elution profile. It has been shown also that much less inhibition of influenza virus neuraminidase was found when supernatants from cells incubated with CMP-N-acetyl-[¹⁴C]-neuraminate in the absence of influenza virus neuraminidase were subsequently added to the influenza virus neuraminidase. It therefore seemed appropriate to investigate whether or not the above effects might result from the incorporation of the CMP moiety. Although CMP-N-acetylneuraminate was not commercially available radioactively labelled in the CMP moiety, thus precluding ¹⁴C labelling experiments, any incorporation of CMP into material in peak 1 would be associated with a change in the u.v. spectrum and consequently could be detected.

Using the procedure described in Methods for the model system for studying neuraminidase inhibition, cells which had been grown with and without 1 mM cyclic AMP and
1 mM theophylline were incubated with CMP-N-acetyl-[\textsuperscript{14}C]-neuraminate. Control incubations without the latter were also included. H.p.l.c. elution profiles of supernatants from all groups (Fig. 4.19) were obtained as described in Methods for gel filtration chromatography. The absorption spectrum of the CMP moiety of CMP-N-acetyl-[\textsuperscript{14}C]neuraminate, has an absorption peak at 271 nm at pH 7.0 (Dunn and Hall, 1975).

The elution profile data (Fig. 4.19) were examined. Changes in $A_{271}$ brought about by the addition of CMP-N-acetyl-[\textsuperscript{14}C]neuraminate were calculated, as a percentage of the absorption at 271 nm in the whole profile, for each elution profile peak. It was found that the percentage increase in peak 1 for cells grown with 1 mM cyclic AMP and 1 mM theophylline (9.2%; Fig. 4.96b compared to Fig. 4.19a) was very similar to the percentage increase in peak 1 for control cells (9.7%; Fig. 4.19d compared to Fig. 4.19c). The respective changes in peak 5 for cells grown with 1 mM cyclic AMP and 1 mM theophylline (3.8%; Fig. 4.19b compared to Fig. 4.19a) was also similar to those from control cells (3.2%; Fig. 4.19d compared to Fig. 4.19c).

The results of this experiment are consistent with the concept that the CMP moiety is removed from CMP-N-acetyl-[\textsuperscript{14}C]neuraminate and bound, presumably covalently, to material in peak 1 where it is responsible for the inhibition of neuraminidase. It is not however consistent with the Results of the experiment shown in Fig. 4.14c which shows that the resulting inhibition of neuraminidase was greater with cells grown with 1 mM cyclic AMP and 1 mM theophylline. However other interpretations of these results are possible; for
FIG. 4.19  H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM B16 MELANOMA CELLS INCUBATED WITH AND WITHOUT CMP-N-ACETYL-[¹⁴C]NEURAMINATE

(a) H.p.l.c. elution profile (absorption at 271 nm) of a supernatant obtained when influenza virus neuraminidase was incubated, as described in Methods for the model system for studying neuraminidase inhibition, for 20 min at 37°C, with B16 melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline, but without CMP-N-acetyl-[¹⁴C]neuraminate.

(b) H.p.l.c. elution profile (absorption at 271 nm) obtained as in (a) above except that CMP-N-acetyl-[¹⁴C]neuraminate was included in the incubation mixture.

(c) H.p.l.c. elution profile (absorption at 271 nm) obtained as in (a) above except that control cells were used.

(d) H.p.l.c. elution profile (absorption at 271 nm) obtained as in (a) above except that control cells were used and CMP-N-acetyl-[¹⁴C]neuraminate was included in the incubation mixture.
example, the change in absorption in peak 1, contingent on the addition of CMP-N-acetyl-[\(^{14}\text{C}\)]neuramate, may be due to the release of some other molecule derived from the cell and bound to peak 1, or eluting in peak 1 as a consequence of its gel filtration characteristics. This interpretation gained support when the u.v. absorption spectra were examined in the following experiment.

ULTRAVIOLET ABSORPTION SPECTRA OF PEAKS FROM H.P.L.C. ELUTION PROFILES OF SUPERNATANTS

The preceding experiment has demonstrated that the addition of CMP-N-acetyl-[\(^{14}\text{C}\)]neuramate to incubation mixtures containing influenza virus neuraminidase and B\(_{16}\) melanoma cells resulted in an increased absorption at 271 nm in peak 1 of the h.p.l.c. elution profile. In order to examine the u.v. absorption spectrum of each of the principal peaks in the h.p.l.c. elution profile, B\(_{16}\) melanoma cells which had been grown in 1 mM cyclic AMP and 1 mM theophylline were incubated with influenza virus neuraminidase for 20 min at 37° according to the procedure for the model system for studying neuraminidase inhibition described in Methods. Control incubations without influenza virus neuraminidase or without CMP-N-acetyl-[\(^{14}\text{C}\)]neuramate were included. In another incubation mixture the concentration of CMP-N-acetyl-[\(^{14}\text{C}\)]neuramate was doubled.

Fig. 4.20 shows (I) the h.p.l.c. elution profiles of supernatants derived from the reaction mixtures (a-d) and (II) the u.v. absorption spectra of the four principal peaks (A-D) respectively, measured against eluting buffer as des-
FIG. 4.20 ULTRAVIOLET ABSORPTION SPECTRA OF PEAKS FROM
H.P.L.C. ELUTION PROFILES OF SUPERNATANTS

I. H.p.l.c. elution profiles (absorption at 271 nm) of supernatants obtained when influenza virus neuraminidase was incubated, as described in Methods for the model system for studying neuraminidase inhibition (with variations as set out below), for 20 min at 37° with cells which had been grown in 1 mM cyclic AMP and 1 mM theophylline.

(a) Control profile obtained by the procedure above except that both influenza virus neuraminidase and CMP-N-acetyl-[14C]neuraminate were omitted from the incubation mixture.

(b) Control profile obtained by the procedure above except that CMP-N-acetyl-[14C]neuraminate was omitted from the incubation mixture.

(c) Profile obtained by the procedure above.

(d) Profile obtained by the procedure above except that the concentration of CMP-N-acetyl-[14C]-neuraminate was doubled.

II. Ultraviolet absorption spectra of peaks labelled 1, 2, 3 and 5 in each of the above profiles obtained by the procedure described in Methods for gel filtration chromatography. The spectra (A-D) were obtained from the profiles (a-d) respectively, using from the beginning of peak 1: 2 ml, 1.5 ml, 2 ml, 2 ml and 4 ml fractions for peaks 1, 2, 3, 4 (this region was not measured) and 5 respectively.
cibed in Methods for gel filtration chromatography. The u.v. absorption spectra of peak 1 from each elution profile (Fig. 4.20 A-D) had a maximum (at pH 7.4) at 259 nm. Maxima for peaks 2, 3 and 5 (at pH 7.4) were all in the range of 261 to 262 nm. There would be some contribution to these spectra from a variety of proteins and there may also be some light scattering from protein in the solution. Nevertheless these spectra are consistent with the presence of nucleotides. However, these maxima are different from the maximum at 271 nm in the spectrum of CMP; they more closely approximate the maxima of spectra of AMP ($\lambda_{\text{max}}^{259}$ at pH 7.0) and UMP ($\lambda_{\text{max}}^{262}$ at pH 7.0) (Dunn and Hall, 1975).

**H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM B16 MELANOMA CELLS GROWN WITH [$^{14}$C]CYTIDINE, [$^{14}$C]RIBOSE, [$^{32}$P]PHOSPHATE OR [$^{14}$C]URIDINE**

The preceding experiment has demonstrated that the u.v. absorption spectra of peaks in h.p.l.c. elution profiles of supernatants from B16 melanoma cells incubated with influenza virus neuraminidase have absorption maxima in the region of 260 nm. This suggests that the material in the peaks might contain nucleotides or related compounds. To further investigate this possibility various labelled precursors of nucleotides were added to the growth medium of B16 melanoma cells which were then harvested and incubated for 20 min at 37°C as described in Methods for the model system for studying neuraminidase inhibition, except that CMP-N-acetyl-[14C]neuraminate was omitted and influenza virus neuraminidase was sometimes omitted. Supernatants obtained were subjected to h.p.l.c. as described in Methods
for gel filtration chromatography, and 0.5 ml fractions of the eluate were collected for radioactive counting as described in Methods.

Fig. 4.21(a-g) show the distribution of label (\(^{14}\)C or \(^{32}\)P) in h.p.l.c. elution profiles of supernatants from such incubation mixtures. Fig. 4.21(a,c,e and g) show the distributions of label found when cells grown with 1 mM cyclic AMP and 1 mM theophylline were used. Fig. 4.21(b,d and f) show the distribution of label found when control cells were used. Labelled substrates added to the growth medium were: \([U-^{14}\)C]cytidine (Fig. 4.21a and b); D-[1-\(^{14}\)C]-ribose (Fig. 4.21c and d); \([^{32}\)P]orthophosphoric acid (Fig. 4.21e and f); and [2-\(^{15}\)C]uridine (Fig. 4.21g), which was present in the medium for only 24 h before harvesting. A different subculture of B\(_{16}\) melanoma cells was used for each different labelled substrate.

Fig. 4.21 shows that supernatants from B\(_{16}\) melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline incorporated less labelled material, as shown in the h.p.l.c. profiles, than control cells.

It can also be seen that the addition of influenza virus neuraminidase to reaction mixtures has decreased the incorporation of \(^{14}\)C into peak 1 in profiles obtained from cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.21a,c and g). At the same time there is little change in incorporation of \(^{14}\)C into peak 5, although this is decreased when the substrate is \([^{14}\)C]ribose. In control cells the addition of influenza virus neuraminidase to reaction
FIG. 4.21 DISTRIBUTION OF LABEL IN THE H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM $B_{16}$ MELANOMA CELLS GROWN WITH VARIOUS LABELLED SUBSTRATES

Distribution of label in h.p.l.c. elution profiles of supernatants obtained when influenza virus neuraminidase was incubated as described in Methods for inactivation of influenza virus neuraminidase for 10 min at 37°C, but without CMP-N-acetyl-[14C]-neuraminate. A control reaction mixture without influenza virus neuraminidase was included.

( ) with influenza virus neuraminidase
( ) without influenza virus neuraminidase

(a) 14C distribution when $B_{16}$ melanoma cells used had been grown with 1 mM cyclic AMP and 1 mM theophylline, together with 1 μCi of [U-14C]cytidine per 10 ml of tissue culture medium for 48 h, added 24 h after subculturing.

(b) 14C distribution when $B_{16}$ melanoma cells used had been grown with 1 μCi of [U-14C]cytidine per 10 ml of tissue culture medium for 48 h, added 24 h after subculturing.

(c) 14C distribution when $B_{16}$ melanoma cells used had been grown with 1 mM cyclic AMP and 1 mM theophylline, together with 10 μCi of [1-14C]ribose per 10 ml of tissue culture medium for 48 h, added 24 h after subculturing.

(d) 14C distribution when $B_{16}$ melanoma cells used had been grown with 10 μCi of [1-14C]ribose per 10 ml of tissue culture medium for 48 h, added 24 h after subculturing.
FIG. (continued) 4.21

DISTRIBUTION OF LABEL IN THE H.P.L.C. ELUTION PROFILES OF SUPERNATANTS FROM B16 MELANOMA CELLS GROWN WITH VARIOUS LABELLED SUBSTRATES

(———) with influenza virus neuraminidase
(-----) without influenza virus neuraminidase

(e) $^{32}$P distribution when B16 melanoma cells used had been grown with 1 mM cyclic AMP and 1 mM theophylline, together with 200 µCi of $[^{32}\text{P}]$orthophosphoric acid per 10 ml of tissue culture medium, for 48 h, added 24 h after subculturing.

(f) $^{32}$P distribution when B16 melanoma cells used had been grown with 200 µCi of $[^{32}\text{P}]$orthophosphoric acid per 10 ml of tissue culture medium, for 48 h, added 24 h after subculturing.

(g) $^{14}$C distribution when B16 melanoma cells used had been grown with 1 mM cyclic AMP and 1 mM theophylline for 48 h, added 24 h after subculturing, together with 1 µCi of $[2-^{14}\text{C}]$uridine per 10 ml of tissue culture medium for 24 h, added 48 h after subculturing.
mixture has increased the incorporation of $^{14}$C into peak 1 in the profile obtained when the substrate was $[^{14}$C]cytidine (Fig. 4.21b) but has decreased the incorporation of $^{14}$C into peak 1 from control cells when the substrate was $[^{14}$C]ribose. Little change can be seen in h.p.l.c. profiles labelled with $[^{32}$P]orthophosphoric acid (Fig. 4.21e and f), for both control cells and cells treated with 1 mM cyclic AMP and 1 mM theophylline. The significance of the changes found above depend upon their reproducibility. Because of financial constraints these experiments were not repeated. However, the conclusions suggested by these results could sometimes be checked in other ways. For example, since uridylic acid is a precursor of cytidylic acid, effects on incorporated CMP (Fig. 4.21a) would be the same if $[^{14}$C]uridine was the CMP precursor (Fig. 4.21g); in fact the effects in peak 1 are very similar.

In another experiment, to which the same criticisms apply, B$_{16}$ melanoma cells were labelled with $[^{14}$C]asparagine (2 μCi/10 ml of tissue culture medium) and h.p.l.c. elution profiles were obtained by procedures similar to those above. Cells grown with 1 mM cyclic AMP and 1 mM theophylline incorporated more $^{14}$C material into the h.p.l.c. profile than control cells; the $^{14}$C incorporated into peaks 1, 2 and 3 increased by 21%, 16% and 5% respectively, while the incorporation into peaks 4 and 5 was approximately the same in both.

These experiments suggest that when influenza virus neuraminidase is present less protein is released from the cells grown with 1 mM cyclic AMP and 1 mM theophylline. Moreover, less material labelled from $[^{14}$C]cytidine, $[^{14}$C]-uridine and $[^{14}$C]ribose is released.
EFFECT OF INFLUENZA VIRUS NEURAMINIDASE ON PEAKS FROM H.P.L.C. ELUTION PROFILES OF SUPERNATANTS

The preceding experiment demonstrated (Fig. 4.21a) that \(^{14}\text{C}\), supplied as \([^{14}\text{C}]\)cytidine, was incorporated into peak 1 of an h.p.l.c. elution profile of a supernatant from B16 melanoma cells, grown with 1 mM cyclic AMP and 1 mM theophylline, on incubation in buffer. When influenza virus neuraminidase was added to the incubation mixture the \(^{14}\text{C}\) incorporation into peak 1 was decreased. This suggested that there may be a relationship between the quantity of influenza virus neuraminidase added and the amount of cytidine-containing (and thus u.v. absorbing) material which, as a consequence of some effect of influenza virus neuraminidase, did not appear in peak 1 of the h.p.l.c. elution profile of the supernatant. In order to investigate this, the effect of adding various quantities of influenza virus neuraminidase to reaction mixtures, otherwise prepared as described in Methods for the model system for studying neuraminidase inhibition, was examined. Reaction mixtures were incubated for 20 min at 37°C.

Fig. 4.22 shows (I) the h.p.l.c. elution profiles (absorption at 280 nm) of supernatants derived from the above reaction mixtures (a–d) and (II) the u.v. absorption spectra of peak 1 from each of these profiles. H.p.l.c. elution profile (a) was derived from a control reaction mixture, where influenza virus neuraminidase (usually 5 I.U.) was omitted. Reaction mixtures from which profiles (b), (c) and (d) were derived contained respectively 1.7, 5 and 15 I.U. of
influenza virus neuraminidase. It can be seen in Fig. 4.22 (II) that the amount of u.v. absorbing material in the supernatants decreased as the amount of influenza virus neuraminidase added to the incubation mixture increased. The spectral changes are greatest in the region between 240 and 265 nm.

This result lends support to the tentative conclusions drawn from the results in Fig. 21(a and g) in which the addition of influenza virus neuraminidase to incubation mixtures decreased the amount of label, in peak 1 of the h.p.l.c. elution profile, incorporated from $[^{14}C]$cytidine and $[^{14}C]$uridine respectively.

**N-ACETYL-$[^{14}C]$NEURAMINATE FORMATION BY THE MODEL SYSTEM**

To investigate whether N-acetyl-$[^{14}C]$neuraminate was produced from CMP-N-acetyl-$[^{14}C]$neuraminate when cells grown with 1 mM cyclic AMP and 1 mM theophylline were incubated with influenza virus neuraminidase, material from peak 5 of the h.p.l.c. elution profiles obtained in the preceding experiment (Fig. 4.22) was examined by descending paper chromatography as described in Methods. The material examined was derived from those reaction mixtures which contained 5 and 15 I.U. of influenza virus neuraminidase. In each case only material from the first 4 ml of peak 5 was applied to the paper, i.e. material from the tail of the peak was omitted. Controls were prepared, using a different subculture of B$_{16}$ melanoma cells. Fig. 4.23(a and b) show the $^{14}C$ distribution in descending paper chromatograms when the 10 μl of supernatant applied came from a 120 μl reaction mixture, containing 5 x $10^5$ B$_{16}$ melanoma cells in 50 mM Tris-
FIG. 4.22 EFFECT OF INFLUENZA VIRUS NEURAMINIDASE ON PEAKS FROM H.P.L.C. ELUTION PROFILES OF SUPERNATANTS

I. H.p.l.c. absorption profiles (absorption at 280 nm) of supernatants obtained when various quantities of influenza virus neuraminidase were incubated, as described in Methods for the model system for studying neuraminidase inhibition, for 20 min at 37°C with cells which had been grown with 1 mM cyclic AMP and 1 mM theophylline.

(a) Control profile obtained by the procedure above except that influenza virus neuraminidase was omitted from the incubation mixture.

(b) Profile obtained by the procedure above, where 1.7 units of influenza virus neuraminidase were added to the incubation mixture instead of the prescribed amount.

(c) Profile obtained by the procedure above, where 5 units of influenza virus neuraminidase (the prescribed amount) were added to the incubation mixture.

(d) Profile obtained by the procedure above, except that 15 units of influenza virus neuraminidase were added to the incubation mixture instead of the prescribed amount.

II. Ultraviolet absorption spectra of peak 1 in each of the above profiles, obtained by the procedure described in Methods for gel filtration chromatography. The spectra (a-d) were obtained from the profiles I(a-d) respectively.
FIG. 4.23 N-ACETYL-[\textsuperscript{14}C]NEURAMINATE FORMATION BY THE MODEL SYSTEM

(a) \textsuperscript{14}C distribution on a descending paper chromatogram obtained when 10 \textmu l of a supernatant prepared as described in the Text was applied to Whatman 3MM paper, using ethyl acetate:pyridine:glacial acetic acid:water (5:5:1:3 \textit{v/v/v/v}) as solvent, as described in Methods. The supernatant was from control cells which had been incubated with CMP-N-acetyl-[\textsuperscript{14}C]neuraminate for 1 h at 37\textdegree C. As a marker non-radioactive N-acetylneuraminate (●—●) was simultaneously chromatographed and stained with silver nitrate.

(b) As for (a) above except that the reaction mixture contained 8.3 mM Mn\textsuperscript{2+}.

(c) As for (a) above except that the B\textsubscript{16} melanoma cells had been grown with 1 mM cyclic AMP and 1 mM theophylline.

(d) As for (c) above except that the reaction mixture contained 8.3 mM Mn\textsuperscript{2+}.

(e) As for (a) above except that B\textsubscript{16} melanoma cells were omitted.

(f) As for (e) above except that the mixture had not been incubated.

(g) and (h) \textsuperscript{14}C distribution on descending paper chromatograms when 100 \textmu l of the first 4 ml eluted in peak 5 of the profiles shown in Fig. 4.22 (c and d respectively) were applied to Whatman 3MM paper and run in the same solvent as in (a) above.
maleate buffer, pH 7.4, 0.25 M sucrose, 450 pmol of CMP-N-acetyl-[\(^{1}^{4}\)C]neuraminate and (for the result in Fig. 4.23b only) 8.3 mM Mn\(^{2+}\), which had been incubated for 1 h at 37°C. Fig. 4.23(c and d) are as for Fig. 4.23(a and b respectively) except that the B_{16} melanoma cells used had been grown with 1 mM cyclic AMP and 1 mM theophylline. Fig. 4.23(e) is as for Fig. 23(a) except that B_{16} melanoma cells were omitted. Fig. 4.23(f) shows the \(^{1}_{4}\)C distribution in a descending paper chromatogram when 0.14 \(\mu\)Ci of CMP-N-acetyl-[\(^{1}^{4}\)C]neuraminate was applied with 10 \(\mu\)l of the same buffer used in incubation mixtures for the other controls. Fig. 4.23(g and h) show the \(^{1}_{4}\)C distribution on descending paper chromatograms when 100 \(\mu\)l of the first 4 ml eluted in peak 5 of the profiles shown in Fig. 4.22(c and d respectively) were applied to the paper. Fig. 4.23(f) shows that a small amount of non-enzymic hydrolysis of CMP-N-acetyl-[\(^{1}^{4}\)C]neuraminate has occurred before incubation or during paper chromatography. This amount has increased after 1 h incubation (Fig. 4.23e). Incubation with control cells has increased the amount slightly (Fig. 4.23a) as has incubation with cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.23b). The addition of 8.3 Mn\(^{2+}\) (Fig. 4.23b and d) has in each case resulted in a further slight increase. Fig. 4.23(g and h) show that the presence of influenza virus neuraminidase has resulted in total hydrolysis of the CMP-N-acetyl-[\(^{1}^{4}\)C]-neuraminate, with the production of N-acetyl-[\(^{1}^{4}\)C]neuraminate, regardless of whether the amount of influenza virus neuraminidase added to reaction mixtures was 5 or 15 I.U. The hydrolysis of CMP-N-acetyl-[\(^{1}^{4}\)C]neuraminate (Fig. 4.23g)
cannot be explained by the combined action of sialyltransferase and influenza virus neuraminidase since the activity of the sialyltransferase (Fig. 4.5a) and the activity of the influenza virus neuraminidase (Fig. 4.14d) are each less than 1% of the activity required to bring about the hydrolysis in the time available.
CHAPTER FIVE

DISCUSSION

Morphological changes (Fig. 4.1, Fig. 4.2 and Fig. 4.3), seen when sparse and confluent B16 mouse melanoma cells were grown with 1 mM cyclic AMP and 1 mM theophylline, are difficult to quantify. The extent to which the changes observed represent reversion of malignant characteristics is uncertain. Certainly the rate of cell division was reduced (Table 4.1) by growing B16 melanoma cells in a combination of 1 mM cyclic AMP and 1 mM theophylline. The work of Sharmeen (1985) has shown that treatment of B16 melanoma cells with 1 mM cyclic AMP and 1 mM theophylline for 48 h results in a doubling of the intracellular cyclic AMP level. This suggests that the changes observed following this treatment are mediated by a rise in the intracellular cyclic AMP level. However, other mechanisms of action of exogenous cyclic AMP and theophylline may be responsible for the observed effects and must always be considered. While it may be argued that more direct methods of stimulating adenylate cyclase could have been used, the use of exogenous cyclic AMP is of particular interest because cells are known to secrete cyclic AMP into the extracellular medium; this extracellular cyclic AMP may in turn be responsible for effects on neighbouring cells (Barber and Butcher, 1983). Theophylline was introduced because early work (Goswell et al., 1977) suggested and the work of Sharmeen (1985) has confirmed that cyclic AMP and theophylline produced a consistent rise in the intracellular cyclic AMP level only when combined. As discussed
in the introduction theophylline may have other effects mediated by a number of different mechanisms of action, a disadvantage which was accepted in view of the advantage conferred, i.e. a reproducible doubling of intracellular cyclic AMP.

Although glycosyltransferases, with the exception of the sialyltransferases, are said to require divalent cations (e.g. Mg$^{2+}$ or Mn$^{2+}$) (Schacter, 1978; Schacter and Roseman, 1980), Beyer and Hill (1980) have described a fucosyltransferase which is active in the absence of divalent metal ions, but is stimulated upon addition of Mn$^{2+}$. The purpose of the experiment, the results of which are reported in Fig. 4.4, was to ensure that divalent cations were not inhibiting or limiting factors in the glycosyltransferase assay system. The absolute requirement for Mn$^{2+}$ found in the incorporation of label from UDP-[$^{14}$C]galactose, UDP-N-acetyl-[$^{14}$C]galactosamine and UDP-N-acetyl-[$^{14}$C]glucosamine suggests that the incorporation obtained represents ectoglycosyltransferase activity. It seems unlikely that the cell washing procedures would deplete the cells of divalent cations. There is no divalent cation requirement for incorporation of label from GDP-[$^{14}$C]mannose or UDP-[$^{14}$C]glucose where the same washing procedures have been used. The incorporation of label from GDP-[$^{14}$C]fucose did not have an absolute requirement for Mn$^{2+}$ although the addition of Mn$^{2+}$ did stimulate the incorporation (Fig. 4.4c).

As expected there was no divalent cation requirement in the case of label from CMP-N-acetyl-[$^{14}$C]neuraminic and
Mn\(^{2+}\) has been found to inhibit this incorporation (Table 4.2) as well as the incorporation of label from GDP-[\(^{14}\)C]mannose and UDP-[\(^{14}\)C]glucose (Fig. 4.4). Perhaps in the case of B\(_{16}\) melanoma cells the reason why there are no divalent cation requirements for the uptake of label from GDP-[\(^{14}\)C]mannose and UDP-[\(^{14}\)C]glucose is that the enzymes responsible are not ectoglycosyltransferases and intracellular Mn\(^{2+}\) would be available.

This raises the question of the mechanism by which label from nucleotide-sugars can enter the cell. Some workers (Patt and Grimes, 1976; Arnold et al., 1976) have suggested a mechanism in which polyprenol derivatives are involved. The interpretation placed upon this work by these authors was criticised by Deppert and Walter (1978). However, Hoflak et al. (1980, 1982) have characterised cell surface glycosyltransferases in the dolichol pathway in rat-spleen lymphocytes and found that the utilisation of the intracellular pool of precursors by these enzymes is prevented. They showed, using UDP-N-acetylglucosamine, that the exogenous labelled nucleotide-sugar was utilised without any isotopic dilution from the cellular pool. The enzymes responsible for the incorporation of label are directly accessible from the outside of the cell so that the type of neosynthesised product can be easily controlled by the type of nucleotide-sugar added. Hoflak et al. (1982) also concluded that this metabolic pathway might represent a second route for the N-glycosylation of protein.

More recent work suggests an additional possibility. Cecchelli et al. (1983) have shown that when mouse thymocytes
are treated with iso-osmotic NH₄Cl, the sugar incorporated into the endogenous acceptors from labelled nucleotide-sugar is largely increased compared with that in control thymocytes. This effect was obtained with labelled GDP-mannose, UDP-galactose and CMP-N-acetylneuraminic acid. Thus NH₄Cl treatment allowed the labelled nucleotide-sugar to penetrate into the cell and to behave as the cellular pool to be utilised for glycosylation by intracellular vesicles. Cacan et al. (1984) extended this work to include GDP-fucose and showed that CMP-N-acetylneuraminic acid and GDP-fucose accumulated in vesicles. UDP-galactose did not accumulate in vesicles, suggesting that for this substrate the transport and transfer were simultaneous events. Ceccelli et al. (1983) pointed out that these permeability studies did not contradict the earlier work of Hoflak et al. (1982). They considered that there were two cellular sites of utilisation of nucleotide-sugars: the first one, exhibited by whole cells, presumably corresponding to cell surface glycosyltransferase activities; and the second one, representing the major proportion of the activity, being located inside the cell but accessible to nucleotide-sugars owing to cell leakage after NH₄Cl treatment. Evidence that the two sites were cytologically separated was that the first site was saturatable without affecting the second one. This work suggests the possibility that the increased glycosyltransferase activity of B₁₆ melanoma cells in the presence of Mn²⁺ may be due to increased permeability of the cells to nucleotide-sugars such as UDP-galactose, UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. Cell membranes may already be permeable to other nucleotide-sugars,
i.e. GDP-mannose, UDP-glucose and GDP-fucose, so that, with these, activity is detected in the absence of Mn$^{2+}$. This possibility remains conjectural because the effect of Mn$^{2+}$ on nucleotide-sugar uptake was not further investigated. However, pits of the type seen in Fig. 4.2, might eventually form vesicles, although it is also possible that the pits may be artefacts.

The presence of ectoglycosyltransferase activities in the plasma membrane of cells nevertheless remains controversial (Corfield and Schauer, 1982b). Not all uptake of label from labelled nucleotide-sugars can be attributed to ectoglycosyltransferase activity. Hirschberg et al. (1976) have shown that after incubation of intact NIL, BHK and 3T3 cells with $^3$H- or $^{14}$C-labelled CMP-N-acetylneuraminate, at least 78% of the label incorporated by the cells was the result of uptake of free labelled N-acetylneuraminate derived from the labelled CMP-N-acetylneuraminate by extracellular hydrolysis. The apparent $K_M$ of uptake of N-acetylneuraminate by these cells was approximately 10 mM. When the cells were incubated with 20 mM unlabelled N-acetylneuraminate the incorporation of label fell by 94%. From the data supplied it appears that for BHK cells the maximum amount of N-acetylneuraminate that could be directly incorporated into phosphotungstate-precipitable material by a cell surface transferase would be 0.175 pmol/90 min incubation at 37°C/500,000 cells, which is about half of the incorporation found in control B₁₆ melanoma cells (Fig. 4.4a). These cells did not incorporate label from CMP-N-acetyl-$^{14}$Cneuraminate via N-acetyl-$^{14}$Cneuraminate under the conditions used since the
quantity of $^{14}$C incorporated from the latter was very small compared to that incorporated from the CMP-N-acetyl-$[^{14}\text{C}]$-neuraminate. Moreover, the addition of unlabelled N-acetyl-neuraminate (25 mM) to incubation mixtures containing CMP-N-acetyl-$[^{14}\text{C}]$neuraminate did not decrease the $^{14}$C incorporation; assuming that the $K_M$ value for uptake of N-acetyl-neuraminate in B16 melanoma cells is approximately 10 mM as in NIL, BHK and 3T3 cells then, if CMP-N-acetyl-$[^{14}\text{C}]$-neuraminate was first hydrolysed to N-acetyl-$[^{14}\text{C}]$neuraminate before being incorporated, a decrease in the $^{14}$C incorporation as found by Hirschberg et al. would have been expected. Thus it seems reasonable to conclude that incorporation of CMP-N-acetyl-$[^{14}\text{C}]$neuraminate was not preceded by hydrolysis of the nucleotide-sugar. Similarly the results given in Fig. 4.7 show that the incorporation of label from UDP-$[^{14}\text{C}]$-glucose was not preceded by hydrolysis of the nucleotide-sugar.

The effect of Mn$^{2+}$ is independent of the combined effect of cyclic AMP and theophylline and, as would be expected from the role of theophylline as a phosphodiesterase inhibitor, the effect of theophylline potentiates that of cyclic AMP (Table 4.4). The fact that 10 μM forskolin has led to an increase in sialyltransferase activity, which is 50% of the increase obtained with 1 mM cyclic AMP and 1 mM theophylline, supports the conclusion that the increase in sialyltransferase activity results from an increase in endogenous cyclic AMP levels. It is not clear why theophylline and forskolin do not potentiate each other; perhaps one interferes with the other (Table 4.4).
With one exception activities of glycosyltransferases in confluent cultures of B₁₆ melanoma cells treated with 1 mM cyclic AMP and 1 mM theophylline did not differ substantially from those in control cells (Fig. 4.5). The one exception was sialyltransferase (Fig. 4.5a). Following on work in this thesis Sharmeen (1985) confirmed that the incorporation of N-acetyl-[¹⁴C]neuraminate from CMP-N-acetyl-[¹⁴C]neuraminate by B₁₆ melanoma cell suspensions was doubled if the cells were grown with 1 mM cyclic AMP and 1 mM theophylline. It was shown that in untreated cells the number of acceptor sites were limiting, because introduction of an exogenous acceptor further increased the activity. In treated cells the number of acceptors were no longer limiting, suggesting that the increased sialyltransferase activity in the treated cells was, at least in part, due to an increase in the number of acceptor sites. The increase in sialyltransferase brought about by the addition of asialofetuin in untreated cells was only one third of the increase obtained by treating the B₁₆ melanoma cells with 1 mM cyclic AMP and 1 mM theophylline. But it was not possible to exclude, in addition to the number of acceptors, a small increase in the quantity of enzyme or in the activity of the enzyme, although such increases could not have been of sufficient magnitude to cause the number of acceptors to be rate-limiting. Furthermore, since the extracellular acceptor asialofetuin was sialylated by suspensions of whole cells, this sialyltransferase activity appears to be on the cell surface, or on the side of vesicles formed by endocytosis (Schauer, 1985).

The activities of all glycosyltransferases in sparse
control cells (Fig. 4.6) were each about half of the activities found in confluent control cells. The effect of 1 mM cyclic AMP and 1 mM theophylline in sparse cultures (Fig. 4.6) is to double the sialyltransferase activity in the absence of Mn$^{2+}$; this is also what happens in confluent cultures (Table 4.2). At the same time, the increases in the activities of all other glycosyltransferases were considerably larger than any corresponding increases seen in confluent cultures. Although the level of cyclic AMP in sparse cells has not been measured, the changes in glycosyltransferase activity brought about by the confluence of the cells are the same as those brought about by the addition of cyclic AMP and theophylline to the tissue culture medium and this suggests that the levels of cyclic AMP in sparse control cells might be lower than those in confluent control cells.

The significance of the empirical relationship between glycosidase activities of B$_{16}$ melanoma cells and metastatic potential studied by Bosman (1973) is not clear. The relationship may be fortuitous rather than logically correlated. Similar changes brought about in the glycosidase activities of B$_{16}$ melanoma cells when they are treated with 1 mM cyclic AMP and 1 mM theophylline show no clear correlation with the differences seen between B$_{16}$ melanoma cells with high and low metastatic potentials respectively as described by Bosman (1973). Thus the changes seen in Fig. 4.8(a and b) lend no support to the view that the addition to the growth medium of 1 mM cyclic AMP and 1 mM theophylline brings about a change to a more normal phenotype.
In view of the increased incorporation of label from CMP-N-acetyl-[\textsuperscript{14}C]neuraminate when cells were grown in 1 mM cyclic AMP and 1 mM theophylline, protein fractions which contained this incorporated label were isolated (Fig. 4.10a). The separation into three major peaks may be a reflection of their dispersion in Triton X-100 which is not likely to be completely removed by dialysis. One of the peaks obtained could not be digested by pronase (Fig. 4.10d), and the fact that this peak contained glycoprotein was supported by its incorporation of threonine (Fig. 4.12b). The two other major peaks obtained were not completely digested by pronase. One possible reason for the lack of digestion of these peaks is that the pronase was inhibited by Triton X-100 which was not completely removed by dialysis. Perhaps the pronase did not have full access to the proteins, if together with the Triton X-100 they are incorporated into micelles. It is possible that peak III (Fig. 4.10d) contains more Triton X-100 than the other two peaks (Fig. 4.10b and c) but this was not investigated further. On the other hand it is important to note that the profiles in Fig. 4.10 are based on incorporation of N-acetylneuraminate, presumably by ectosialyltransferases; to what extent this would represent sialylation of particular proteins is unknown. It may be possible that the resistance to pronase is related to the increased sialylation.

In confirming the above results the opportunity was taken to label the glycoproteins with either \textsuperscript{3}H- or \textsuperscript{14}C-labelled N-acetylmannosamine, an efficient precursor of sialylconjugate (Corfield and Schauer, 1981). This was done
because it was possible that the sialyltransferase activity which incorporated $^{14}$C from CMP-N-acetyl-$^{[14]}$C-neuraminate was all on the cell surface membrane and might have unrepresentative specificity.

When cells were grown with 1 mM cyclic AMP and 1 mM theophylline, peak I, labelled from CMP-N-acetyl-$^{[14]}$C-neuraminate, increased (Fig. 4.10a, Fig. 4.11a) but decreased slightly when labelled with $^{15}$C or $^3$H supplied as labelled N-acetylmannosamine (Fig. 4.12a, c and d). These changes suggest either that the metabolic pools labelled from each of these labelled substrates are different, or that sialic acid residues which are accessible to labelled N-acetylmannosamine are removed and replaced by sialic acid supplied as CMP-N-acetyl-$^{[14]}$C-neuraminate. Since the addition of 1 mM cyclic AMP and 1 mM theophylline increases the incorporation into peak II regardless of which labelled substrate is supplied, peak II would appear to be from a different metabolic pool to peak I. With this treatment there is always a fall in peak III relative to peak I and peak II. Thus peak III also appears to belong to a different metabolic pool to peak I and peak II. It is not clear why each particular elution profile peak is associated with a different metabolic pool but it is possible that this is a reflection of the fact that the capacity of a protein to be removed by Triton X-100 is a function of localisation in the membrane, and that the metabolic pools are also a reflection of the membrane organisation.

The fall (relative to peak II) of incorporation of label from CMP-N-acetyl-$^{[14]}$C-neuraminate into peak III
(Fig. 4.10a) suggests that there is less replacement of sialic acid residues during the 2 h incubation period, which is consistent with less neuraminidase activity. Fig. 4.8 shows that the neuraminidase activity in cells grown with 1 mM cyclic AMP and 1 mM theophylline was 10\% less than that of control cells if the cells were from confluent cultures and was unchanged if the cells were from sparse cultures. More importantly, Fig. 4.9(a) shows that the effect of cyclic AMP and theophylline in this system is to permit CMP-N-acetyl-[^14]C]neuraminic to totally inhibit the cell neuraminidase. (Unlabelled CMP-N-acetylneuraminic was not available for these experiments.) Therefore it follows that the ^14C-labelled elution profile in Fig. 4.12(c), which was labelled from CMP-N-acetyl[^14]C]neuraminic, will be obtained when the neuraminidase was totally inhibited. Moreover, a second effect of 1 mM cyclic AMP and 1 mM theophylline is to double the sialyltransferase activity (Fig. 4.5a, Fig. 4.9a, Table 4.2 and Table 4.3) in cell suspensions incubated with CMP-N-acetyl[^18]C]neuraminic. The effect on peak III of the elution profile, relative to peak I and peak II from treated cells, in Fig. 4.10(a) suggests the involvement of a component of sialyltransferase activity which was not stimulated by 1 mM cyclic AMP and 1 mM theophylline. The decreased incorporation of label from labelled N-acetylmannosamine (Fig. 4.12a) in profiles from B16 melanoma cells grown with 1 mM cyclic AMP and 1 mM theophylline suggests either that there is less synthesis of sialoconjugate from N-acetylmannosamine in the metabolic pool containing peak III (Fig. 4.12a), or that more sialic acid residues so
derived are removed and not replaced. Furthermore, the profiles labelled from labelled N-acetylmannosamine (Fig. 4.12a and b) in the presence of 1 mM cyclic AMP and 1 mM theophylline would have been obtained in the presence of 90% (Fig. 4.8) of the control level of neuraminidase. Therefore, it is possible that the sialyltransferase stimulated by the addition of 1 mM cyclic AMP and 1 mM theophylline was an ectosialyltransferase and that the sialyltransferase not stimulated under these conditions (which contributed to labelling peak III) was in the Golgi apparatus.

Two other points are worth noting. First, CMP-N-acetyl-[^14]C]neuraminate inhibits the neuraminidase in cells from sparse cultures. It is not clear from these experiments why the capacity to completely inhibit the neuraminidase is, in the case of sparse cells, a characteristic of cells grown without 1 mM cyclic AMP and 1 mM theophylline; whereas in cells from confluent cultures it was a characteristic only of cells grown with 1 mM cyclic AMP and 1 mM theophylline. Secondly when the concentration of CMP-N-acetyl-[^14]C]neuraminate was reduced one hundred-fold (Fig. 4.9c), the sialyltransferase activity was reduced five-fold. At this concentration of CMP-N-acetyl-[^14]C]neuraminate the inhibition of neuraminidase activity in cells grown with 1 mM cyclic AMP and 1 mM theophylline was no longer complete but was still greater than in control cells (Fig. 4.9cB).

The demonstration of the effect of CMP-N-acetyl-[^14]C]neuraminate on neuraminidase is based upon the premise that
the removal of $^3$H during incubation of cells labelled from N-$[^3]$H acetylmannosamine is a measure of neuraminidase activity. That this is substantially true is demonstrated by Fig. 4.9(bi and bii) which show that most of the $^3$H released was incorporated into sialic acid. A small proportion of the label was incorporated into another peak. While this might possibly be a sialic acid derivative it might also be another sugar, derived from N-$[^3]$H acetylmannosamine according to the scheme shown in Fig. 1.1 and liberated by the appropriate glycosidase activity.

Methods for the purification of sialyltransferase are elaborate, laborious, and require large amounts of starting material (Paulson et al., 1977a and b; Sadler et al., 1979a and b; Beyer et al., 1981; Alhadeff and Holzinger, 1982b). For these reasons the mechanism whereby 1 mM cyclic AMP and 1 mM theophylline brought about an increase in sialyltransferase activity was not further investigated. It seemed more appropriate to investigate the mechanism whereby CMP-N-acetylneuraminic acid completely inhibits neuraminidase in confluent cells grown with 1 mM cyclic AMP and 1 mM theophylline (Fig. 4.9a). In order to avoid the problem of purifying B16 melanoma cell neuraminidase for such studies, a model system using influenza virus neuraminidase was adopted, since this enzyme was available in quantity in a reasonably pure form, and considerable information on the structure of influenza virus neuraminidase has recently become available (Blok et al., 1982; Blok and Air, 1982a and b; Cabezas et al., 1982; Jackson and Webster, 1982; Laver et al., 1982; Colman et al., 1983; Varghese et al.,
1983). The complete structure of the neuraminidase gene of both human influenza viruses A and B is known (Fields et al., 1981; Markoff and Lai, 1982; Shaw et al., 1982). It must be emphasised that the inhibitory effects demonstrated in the model system with influenza virus neuraminidase may in fact bear no relationship to the inhibitory effects found with the B_{16} melanoma neuraminidase. Nevertheless an understanding of the inhibition of influenza virus neuraminidase may at least provide a basis for further study of the B_{16} melanoma system. Some authorities (Schauer, 1985) have speculated that there may be a relationship between the neuraminidase genes of microorganisms and animal cells and consider it is possible that the neuraminidase gene may have been acquired by microorganisms from their host during evolution. The common occurrence of neuraminidase in pathogenic microorganisms, and comparison of the properties of neuraminidases from animal and microbial origin, support this assumption (Schauer and Vliegenthart, 1982). Schauer (1985) points out that, in addition, the genes for the synthesis of sialic acid observed in only a few pathogenic bacteria could have been transferred from higher organisms (guided by natural selection). This hypothesis is supported by the observations that the biosynthetic pathways of sialic acids are almost identical in bacteria and mammals (Corfield and Schauer, 1982a).

The work of Blok and Air (1982a) and Varghese et al. (1983) has shown that solubilised influenza virus neuraminidase is a tetramer (200,000 M_r) with a box-shaped head attached to a slender stalk with a hydrophobic knob at the end, which anchors the neuraminidase to the membrane.
Neuraminidase molecules aggregate via this hydrophobic region to form rosettes which may contain six tetramers.

Each monomer (50,000 M\(_r\)) contains approximately 397 amino acid residues and 31 carbohydrate residues, probably all in glycosidic linkage to asparagine residues (Blok et al., 1982). Sugar residues are attached to four of the five potential glycosylation sequences, and in one case contribute to interaction of subunits in the tetramer. Influenza A2 virus neuraminidase cleaves \(\alpha-2,3\) linked sialic acids, and (at a much slower rate) \(\alpha-2,8\) linkages; but \(\alpha-2,6\) linkages are only poorly attacked (Corfield and Schauer, 1982b).

The model system proved to be a suitable one for studying the effect of CMP-N-acetyl-[\(^{14}\)C]neuraminate in bringing about inhibition of neuraminidase, whether or not the mechanism is the same as that occurring in B\(^{16}\) melanoma cells. Most pertinently, influenza virus neuraminidase incubated with substrate (2-(3-methoxyphenol)-N-acetyl-\(\alpha\)-D-neuraminic acid) and CMP-N-acetyl-[\(^{14}\)C]neuraminate in the absence of cells was not inhibited (Fig. 4.14a), and clearly the mechanism whereby cells utilise CMP-N-acetyl-[\(^{14}\)C]neuraminate to bring about inhibition of neuraminidase is a more complex one.

In Fig. 4.14(c) it may be calculated that when B\(^{16}\) melanoma cells which had been grown with 1 mM cyclic AMP and 1 mM theophylline were incubated for 2 h with CMP-N-acetyl-[\(^{14}\)C]neuraminate the inhibition of the influenza virus neuraminidase was 50%, as compared to the inhibition of 33% obtained when such cells had been incubated without CMP-N-
acetyl-[\textsuperscript{14}C]neuraminate. It can be seen also that, although viral neuraminidases show a greater thermal stability than do bacterial neuraminidases (Corfield and Schauer, 1982), after 2 h incubation without cells 20% of the influenza virus neuraminidase appears to have been inactivated. It is possible that the inhibition obtained in the absence of exogenous CMP-N-acetyl-[\textsuperscript{14}C]neuraminate, over and above the inactivation occasioned by instability of the enzyme during incubation, may result from production of endogenous CMP-N-acetylneuraminate. This inhibition appears to be limited. Although the cells are incubated with a plentiful supply of glucose it may be that some other component, such as glutamine, required for synthesizing CMP-N-acetylneuraminate, is limiting.

At this point it appeared that the most likely explanation was that the neuraminidase was inhibited by becoming sialylated by the sialyltransferase. Had this been so, small amounts of unsialylated neuraminidase might still have been available to remove the N-acetylneuraminate added and thus remove the inhibition. However, while an effect of this kind was achieved, the above explanation was not the correct one. Fig. 4.14(d) shows that influenza virus neuraminidase that has been incubated with cells grown with 1 mM cyclic AMP and 1 mM theophylline and CMP-N-acetyl-[\textsuperscript{14}C]neuraminate (i.e. under conditions which produce inhibition, Fig. 4.14c) can on prolonged incubation in the absence of cells recover activity comparable to that of the controls. Fig. 4.15(a) shows that when influenza virus neuraminidase, which had been incubated with CMP-N-acetyl-[\textsuperscript{14}C]neuraminate and cells
grown with 1 mM cyclic AMP and 1 mM theophylline, was fractionated on an h.p.l.c. column designed to separate molecules with relative molecular weights between 12,000 and 200,000, no radioactivity was associated with the peak containing influenza virus neuraminidase. This enzyme would, according to the results in Fig. 4.14(c), have been 50% inhibited when placed on the column, and would have remained at least partly inactivated for 2 h (Fig. 4.14d). Clearly the inactivation cannot be due to sialylation of the influenza virus neuraminidase. Neither is N-acetyl-[14C] neuraminate attached to any molecule with a relative molecular weight greater than 12,000 (Fig. 4.15).

Fig. 4.16(a and b) shows that small peaks of 3H-labelled material, presumably sialic acid derived from N-[3H]acetylmannosamine, coincided with the peak I and also appeared in other parts of the profiles. Some sialic acid would have been released from the cells by the influenza virus neuraminidase. This enzyme would have been only partly inhibited by the cells since CMP-N-acetylneuraminate had not been added (Fig. 4.14c). Fig. 4.17(b and d) shows that protein has also been removed from the cell and degraded in the course of prolonged incubation. However, less material is removed from the cell if influenza virus neuraminidase is present in the incubation mixture (Fig. 4.17a and c compared to Fig. 4.17b and d, respectively). Thus it is possible that desialylated glycoproteins are less readily removed from the cell during incubation, or, alternatively, that the influenza virus neuraminidase may bind and remove a molecule necessary for protein release from the cell. It should also be noted
that the $A_{280}$ in the peak I in Fig. 4.17(a) and Fig. 4.17(b) is much greater in relation to the $[^3H]$threonine content of the peak than it is in the other peaks in these figures, suggesting that a significant part of the $A_{280}$ is not due to protein but to some other material which largely disappears on prolonged incubation. A similar decrease in $A_{280}$ is also shown in Fig. 4.16(a and b).

After it had been demonstrated (Fig. 4.15(a) that CMP-N-acetyl-$[^14C]$neuraminate does not inhibit influenza virus neuraminidase as a consequence of direct sialylation, it seemed appropriate to investigate whether an inhibitor was released from the cell when some other molecule was sialylated. Obviously the inhibitor itself would not be sialylated because such an inhibitor would have bound to the influenza virus neuraminidase and would have appeared in the first h.p.l.c. elution peak, which it did not. If inhibitor release resulted from sialylation of some other molecule, then desialylation and consequent diminution of inhibitor release would be brought about by neuraminidase, provided that the sialic acid was attached by a linkage capable of being hydrolysed by the neuraminidase. This is consistent with the concept that the presence of influenza virus neuraminidase leads to a decrease of protein release from the cell (Fig. 4.17). However, the results in Fig. 4.18, as previously interpreted, do not support this relatively straightforward hypothesis. This is because the degree of inhibition brought about by the release of material from the cell, in the case of the cells grown with 1 mM cyclic AMP and 1 mM theophylline, was less than half that obtained when the
influenza virus neuraminidase was incubated directly with the cells.

Since a mechanism such as the above, involving sialylation, can account for only part of the effect, perhaps there is some other explanation for the results in Fig. 4.18. As the results cannot be explained by sialylation of the influenza virus neuraminidase, the possibility of inhibitory cytideylation of influenza virus neuraminidase or some associated protein needs to be considered. Fig. 4.21(a and b) shows that \([^{14}C]\)cytidine may be incorporated in the material which elutes in peak 1 of the h.p.l.c. profile, but the addition of influenza virus neuraminidase led to a decrease in the amount to \([^{14}C]\)cytidine-containing material in this peak when the cells had been grown with 1 mM cyclic AMP and 1 mM theophylline. Although no exogenous CMP-N-acetyl-\([^{14}C]\)neuraminate was added in this experiment the incubation was carried out under conditions which had been shown to bring about some inhibition of the influenza virus neuraminidase by the cells (Fig. 4.14(b)). These results are not consistent with the concept that cytidylolation of influenza virus neuraminidase, or of any other protein associated with influenza virus neuraminidase in peak 1 of the h.p.l.c. elution profiles, is responsible for the inhibitory effect. Moreover when CMP-N-acetyl-\([^{14}C]\)neuraminate was added to incubation mixtures the associated increase in u.v. absorption (Fig. 4.19) had a \(\lambda_{max}\) at 260 nm (Fig. 4.20) and not at 271 nm which would be expected if cytideylation of material in peak 1 resulted from the addition of CMP-N-acetyl-\([^{14}C]\)neuraminate. However 260 nm is the \(\lambda_{max}\) of the u.v.
absorption spectra of DNA (MacHatti and Thomas, 1975). It is not impossible that the solution contains DNA from cells which may have lysed during incubation, or DNA originally liberated from dead cells in the course of tissue culture, which has not been completely removed by the washing procedures. The results shown in Fig. 4.17(a and b), which suggest that a significant part of the $A_{260}$ in peak 1 is not due to protein but to some other material which decreases on prolonged incubation, are consistent with this possibility. Furthermore Fig. 4.22 shows that the material with a maximum absorbance at 260 nm is decreased as the amount of influenza virus added to the incubation mixture is increased. As indicated by Fig. 4.17(a and b) some of this decrease is probably due to protein but much of it is due to other material absorbing at 280 nm.

If the removal of negatively charged sialic acid residues from the cell surface by the neuraminidase had permitted increased binding to the cell surface of negatively charged DNA derived from broken cells, then the quantity of DNA remaining in the supernatant would be decreased. Thus the amount of material with a $\lambda_{max}$ 260 nm in peak 1 of the h.p.l.c. profile derived from the supernatant would be decreased (Fig. 4.22). On the other hand such an explanation is not consistent with the results shown in Fig. 4.21(b) where the addition of neuraminidase, known to be only partly inactivated by incubation with cells, has led to an increase in material containing $^{14}$C incorporated from $[^{14}\text{C}]$cytidine. Even if DNA is present this does not preclude the possibility that some nucleotide such as AMP ($\lambda_{max}$ 259 nm) is also
present, covalently or noncovalently bound to protein. The absorption maximum of 261-262 nm for material in peak 5 (Fig. 4.20) suggests that some nucleotides may be present.

If CMP is indeed transferred to some cell surface protein from CMP-N-acetyl-[^1^4]C]neuraminic then free N-acetyl-[^1^3]C]neuraminic would be released. Unless the presence of a cytidylylated product was demonstrated it would not be possible to distinguish the enzymic activity, which leads to the production of free N-acetyl-[^1^3]C]neuraminic from CMP-N-acetyl-[^1^4]C]neuraminic, from that of the enzyme CMP-sialic acid hydrolase (Kean and Bighouse, 1974). The activity of this enzyme was determined by measuring the disappearance of the substrate CMP-N-acetyl-[^1^4]C]neuraminic and/or the appearance of N-acetyl-[^1^4]C]neuraminic. The fate of the CMP moiety was not followed by these workers although Shoyab and Bachhawat (1974), who purified the enzyme 173-fold from ovine liver, demonstrated the presence of cytidine in their preparation, which was contaminated by CMP-phosphohydrolase. Kean and Bighouse (1974) showed that the plasma membrane was the predominant cellular site of this enzyme and Van Dijk et al. (1977) demonstrated that the functional centre of the enzyme was directed towards the outside of the cell. Havercamp et al. (1979) showed that the enzyme was specific for sialic acid in β-glycosidic linkage, a type for which CMP-N-acetylneuraminic provides the only naturally occurring example. CMP-sialic acid hydrolase has been found to be elevated in serum of patients with ovarian cancer and has proved useful in detection and management of ovarian neoplasia. Kean and Bighouse (1974) also found that the action of the
CMP-sialic acid hydrolase was not reversible, that there was no metal ion requirement, and that the enzyme was inhibited by nucleosides, nucleotides and sugar-nucleotides; in particular UDP-galactose and UDP-N-acetylglucosamine acted as competitive inhibitors. They suggested that the location of CMP-sialic acid hydrolase on the plasma membrane may be one of the means by which the cell regulates the extent of attachment of sialic acid to its component surface heteropolymers, but they did not in any way suggest a specific role for the enzyme in relation to neuraminidase.

Results in Fig. 4.23 clearly demonstrate that the production of free N-acetyl-[\textsuperscript{14}C]neuraminate from CMP-N-acetyl-[\textsuperscript{14}C]neuraminate is dependent on the presence of influenza virus neuraminidase. Since CMP-N-acetyl-[\textsuperscript{14}C]-neuraminate supplied to B16 melanoma cells leads to the inhibition of influenza virus neuraminidase the possibility that CMP-sialic acid hydrolase has a role in bringing about the inhibition of influenza virus neuraminidase may be considered. Such a hypothesis would assume that the CMP moiety was first transferred to form a cytidylylated intermediate which was subsequently hydrolysed with the production of free CMP.

An example of regulation of enzyme activity by covalent attachment of nucleotide to protein is known. This system is the bicyclic cascade regulating glutamine synthetase of \textit{E. coli} (Stadtman and Ginsburg, 1974; Chock \textit{et al.}, 1980) shown in Fig. 5.1. The uridylylation-deuridylylation and the adenylylation-deadenylylation reactions illustrated
FIG. 5.1 REGULATION OF GLUTAMINE SYNTHETASE IN E. coli

(a) Schematic representation of the interrelationship between the covalent modifications of glutamine synthetase and of the regulatory protein $P_{II}$.

(b) Glutamine synthetase adenylylation (inactivation) in the cascade control of glutamine synthetase activity.

(c) Glutamine synthetase deadenylylation (activation).

Abbreviations: G, glutamine synthetase; KG, α-keto-glutarate; EP, end products of glutamine metabolism; $\text{Me}^{2+}$, divalent metal ions; UT, UTase; UR or URenz, UR-enzyme. UTase, ATase and UR-enzyme are defined in the Text; $+$ sign, stimulation; $-$ sign, inhibition.

(After Stadtman and Ginsberg, 1974)
in Fig. 5.1 are the basis of two oppositely directed cascade systems leading on the one hand to activation of glutamine synthetase, and to its inactivation on the other hand. The regulatory protein $P_{II}$ is capable of covalently binding a nucleotide (UMP) supplied from UTP; this reaction is catalysed by the enzyme UTase. The same enzyme operating in a different mode (UR-enzyme) brings about the hydrolysis of the complex, liberating free UMP. Each subunit of the glutamine synthetase dodecamer is capable of covalently binding a nucleotide (AMP) supplied from ATP; this reaction is catalysed by the enzyme ATase. The same enzyme operating in a different mode brings about the hydrolysis of the complex, liberating free AMP.

The inactivation cascade shown in Fig. 5.1(b) begins with the UR-enzyme-catalysed deuridylylation of $P_{II}$-UMP to form $P_{II}'$, which in turn stimulates ATase to catalyse the adenylylation of glutamine synthetase. This converts it from a $\text{Mg}^{2+}$-dependent more active form to a $\text{Mn}^{2+}$-dependent less active form. Running counter to this is the activation cascade depicted in Fig. 5.1(c). This is initiated by metabolite activation of UTase which catalyses the uridylylation of $P_{II}$. The $P_{II}$-UMP then stimulates UTase-catalysed deadenyllylation of glutamine synthetase converting it back to the more active form. Stadtman and Ginsburg (1974) point out that, except in situations where all enzymes of the cascade are present in similar concentrations (as might occur if they are all organised in a single multienzyme complex), the principal function of cascade systems may be amplification of the effects of primary stimuli. In addition, however, cascade
systems increase the potential for allosteric control of the regulatory system.

To what extent can this cascade system for regulating the synthesis of the sialic acid precursor glutamine be considered as a model for a hypothesis explaining the regulation of neuraminidase? In framing such a hypothesis the concept that CMP-N-acetyl-[\(^{14}\)C]neuraminate availability increases the capacity of an inhibitor to inhibit neuraminidase (Fig. 4.14c) is an appropriate starting point. While the addition of CMP-N-acetyl-[\(^{14}\)C]neuraminate results in an increase in the u.v. absorption (Fig. 4.19), the u.v. absorption spectrum of peak 1 (Fig. 4.20) does not exhibit an absorption maximum at 271 nm. This absorption maximum at 271 would be expected if CMP-N-acetyl-[\(^{14}\)C]neuraminate or any CMP derivatives were bound to the influenza virus neuraminidase or to any inhibitor associated with influenza virus neuraminidase. This suggests that CMP-N-acetyl-[\(^{14}\)C]neuraminate binds instead to some molecule which remains bound to the surface of the cell. The presence of label from \([^{14}\)C]cytidine in peak 1 (Fig. 4.21) suggests that a small amount of material with bound endogenous CMP-N-acetyl-[\(^{14}\)C]neuraminate or a bound CMP moiety might be released from the cell. Any such release, from cells grown with 1 mM cyclic AMP and 1 mM theophylline, would appear to be inhibited by the addition of influenza virus neuraminidase (Fig. 4.21a), although the release was increased when influenza virus neuraminidase was added to control cells (Fig. 4.21b). Material labelled from \([^{14}\)C]cytidine is still liable to be present as \([^{14}\)C]cytidine, i.e. it is unlikely to be incorpor-
ated into UMP (Maley and Maley, 1964; Tomchick et al., 1968). In both instances (Fig. 4.21a and b) most of the added CMP-N-acetyl-[14C]neuraminate or its derivatives (CMP moieties), would not be released but would be bound to the cell surface.

The protein which binds CMP-N-acetyl-[14C]neuraminate may be CMP-sialic acid hydrolase. In support of this is the finding that hydrolysis of CMP-N-acetyl-[14C]neuraminate occurs (Fig. 4.23) with the release of free N-acetyl-[14C]-neuraminate. If the CMP moiety is first covalently attached to another protein, which normally regulates the availability of the inhibitor, it is possible that some conformational change results. This in turn could facilitate the release (from the regulatory protein) of the inhibitor which could then bind to neuraminidase. If hydrolysis of the covalently attached CMP on the regulatory protein is then in turn facilitated by release of the inhibitor, the decytidylated regulatory protein would then be available once again to bind the inhibitor. This would remove the inhibitor from the influenza virus neuraminidase, thus reversing the process. The CMP-sialic acid hydrolase may catalyse both the transfer and removal of the CMP moiety. The hydrolysis could take place during incubation of the supernatant; CMP-sialic acid hydrolase is known to be liberated from cell membranes into serum (Chatterjee and Kim, 1978). The postulated dual function of CMP-sialic acid hydrolase is analogous to that of the enzymes which both transfer and remove, in one case UMP, and in the other case AMP, in the course of the operation of the glutamine synthetase regulatory cascade (Fig. 5.1). Further
the increase in the u.v. absorption at 271 nm (Fig. 4.19), in peak 1 of the h.p.l.c. elution profile, on addition of CMP-N-acetyl-[14C]neuraminate, proved to be due to the presence of material with a $\lambda_{\text{max}}$ at 260 nm (Fig. 4.20). This raises the question of whether an adenylylated protein, of the kind found in the glutamine synthetase cascade, is the influenza virus neuraminidase inhibitor. However there may be other explanations for this increase in absorption at 260 nm. It must be emphasised that this hypothesis is based on a limited number of experimental findings. No experiments have been carried out to test its validity but it has been proposed with a view to further work.

The difference between sparse and confluent cells in their capacities for neuraminidase inhibition by the addition of CMP-N-acetyl-[14C]neuraminate may have a rationale consistent with the above postulated regulatory mechanism. If sparse cells have a high level of CMP-sialic acid hydrolase they would be capable of fully inhibiting their cell surface neuraminidase when supplied with CMP-N-acetyl-[14C]neuraminate even if they had limited quantities of inhibitor protein. The effect of 1 mM cyclic AMP and 1 mM theophylline may be to lower the levels of CMP-sialic acid hydrolase without increasing the level of inhibitor protein (assuming that they differ in this respect from confluent cells). Exogenous CMP-N-acetyl-[14C]neuraminate may then no longer compensate for this difference and the level of active inhibitor protein may be too low for complete inhibition. This hypothetical explanation likewise requires experimental validation.

Recent studies (Sharmeen, 1985) carried out subsequent
to and based on the work in this thesis have confirmed and extended a number of the findings presented herein. A brief outline of some of these results is presented in the appendix. One finding is of particular interest. Removal of the inhibition of influenza virus neuraminidase by incubation of supernatants at 37°C was shown to result from the liberation of N-acetyleneuraminate. Either exogenous N-acetyleneuraminate or N-acetyleneuraminate generated in reaction mixtures as a consequence of hydrolysis of 4-methylumbelliferyl-N-acetyl-α-D-neuraminate was sufficient to restore influenza virus neuraminidase activity. Similar treatment with N-acetyleneuraminate was found to restore B₁₆ melanoma neuraminidase activity. These results support the concept that the mechanism of inhibition in the model system is similar to that bringing about the inhibition of B₁₆ melanoma neuraminidase.

Recently the known biological roles of sialic acid have been summarised by Schauer (1985). Sialic acids mask specific recognition sites on molecules and cells. They have been found to be antigenic determinants in various mammalian and microbial systems. They are essential components of receptors for peptide hormones, toxins and viruses. They are involved in binding and transport and in the aggregation and disaggregation of cells. They influence the conformation of glycoproteins, which is important for the correct arrangement of glycoprotein molecules in cell membranes, for resistance of glycoproteins to proteases and for maintenance of the activity of glycoprotein enzymes. In this thesis it has been shown that B₁₆ melanoma cells that have been grown
in 1 mM cyclic AMP and 1 mM theophylline have an increased sialyltransferase activity and an increased capacity to inhibit cell surface neuraminidase. CMP-N-acetylneuraminate, the substrate for sialyltransferase, mediates the reversible inhibition of the neuraminidase. Clearly these findings are of considerable importance in view of the known biological roles of sialic acid.
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APPENDIX I

Recent studies, presented in the thesis of Sharmeem (1985), carried out subsequent to and based on the work in this thesis (Jarvis, 1986), have confirmed and extended a number of the findings presented in the latter. A brief outline of some of these results is set out below.

When $B_{16}$ melanoma cells were grown with 1 mM cyclic AMP and 1 mM theophylline the amount of u.v. absorbing material with $\lambda_{max}$ of 260 nm in peak 1 of h.p.l.c. elution profiles of supernatants of cell incubation mixtures was twice that in corresponding controls. After incubation of peak 1 fractions from cells grown with 1 mM cyclic AMP and 1 mM theophylline at 37°C for 30 min free adenosine and inosine could be recovered by h.p.l.c.. Furthermore $^3$H from cyclic [$^3$H]AMP which had been present in the growth medium of cells for 48 h could be recovered in the above adenosine and inosine fractions.

15 µM Inosine incubated with CMP-N-acetylneuraminic acid, influenza virus neuraminidase and $B_{16}$ melanoma cells in the model system for short time periods (15 min) resulted in 90% inhibition of the influenza virus neuraminidase. It appeared that the effect of growing cells with 1 mM cyclic AMP and 1 mM theophylline in studies of the inhibition of neuraminidase was mediated by the production of inosine from cyclic AMP.

Removal of the inhibition of influenza virus neuraminidase by incubation of supernatants at 37°C was shown to result from the liberation of N-acetylneuraminic acid. Either
exogenous N-acetylneuraminic acid or N-acetylneuraminic acid generated in reaction mixtures as a consequence of hydrolysis of 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid was sufficient to restore influenza virus neuraminidase activity. Similar treatment with N-acetylneuraminic acid was found to restore B16 melanoma neuraminidase activity.

Treatment of B16 melanoma cells with influenza virus neuraminidase appeared to alter the specificity of receptors for inosine and adenosine. Before treatment with influenza virus neuraminidase incubation with inosine led to an increase in intracellular cyclic AMP levels but incubation with adenosine had no effect. After treatment with influenza virus neuraminidase incubation with adenosine led to an increase in intracellular cyclic AMP levels but inosine had no effect.
APPENDIX II

REVERSIBLE INHIBITION OF NEURAMINIDASE INVOLVING CMP-SIALIC ACID HYDROLASE IN B16 MELANOMA CELLS GROWN WITH CYCLIC AMP AND THEOPHYLLINE

Suspensions of B16 melanoma cells grown with N-[^3H]-acetylmannosamine released [^3H]sialic acid when incubated at 37°C. This neuraminidase activity was inhibited when suspensions of cells were incubated with 4.5 µM CMP-N-acetylneuraminate. In suspensions of cells from confluent cultures the inhibition was total only if the cells had been grown with cyclic AMP and theophylline. In suspensions of cells from sparse cultures (2 x 10^4 ± 2 x 10^3 cells per cm^2) the inhibition was total only if the cells had been grown without cyclic AMP and theophylline.

Inhibition of neuraminidase was studied in a model system in which influenza virus neuraminidase was incubated at 37°C with B16 melanoma cells from confluent cultures. Cells alone had the capacity to inhibit the enzyme, but the inhibition was several times greater when CMP-N-acetylneuraminate was added to the incubation mixture. In each case cells grown with cyclic AMP and theophylline had a greater capacity to inhibit the neuraminidase than did control cells. In the absence of cells CMP-N-acetylneuraminate did not inhibit the neuraminidase. Cells incubated with only CMP-N-acetylneuraminate produced a neuraminidase inhibitor, but the overall inhibition was greater when the cells, neuraminidase, and CMP-N-acetylneuraminate were incubated together. Influenza
virus neuraminidase which had been inhibited, by incubation either with cells or with cells plus CMP-N-acetylneuraminate, could be reactivated by incubation at 37°C after the cells had been removed by centrifugation. When cells which had been grown with cyclic AMP and theophylline were incubated with influenza virus neuraminidase and CMP-N-acetyl-[14C]-neuraminate there was complete hydrolysis of the latter to free N-acetyl-[14C]neuraminate by the time the maximum inhibitory effect was achieved. Therefore CMP-sialic acid hydrolase may have a role in inhibition of neuraminidase. Very little hydrolysis occurred in the absence of influenza virus neuraminidase. UDP-galactose was as effective as CMP-N-acetylneuraminate as an inhibitor of neuraminidase in the system.

APPENDIX III

THE EFFECT OF CYCLIC AMP AND THEOPHYLLINE ON GLYCOSYL-
TRANSFERASES AND GLYCOSIDASES OF B16 MELANOMA CELLS*

When B16 mouse melanoma cells are grown in tissue culture with 1 mM cyclic AMP and 1 mM theophylline, changes in growth and morphology are observed. To investigate whether these alterations may be associated with changes in the biosynthesis of the sugar chains of glycoproteins or glycolipids, glycosidases and glycosyltransferases were assayed in both treated and untreated cells.

Glycosidases for removing sialic acid, N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose and fucose were assayed in homogenates from both sparse and confluent cultures. Neuraminidase activity was assayed spectrofluorometrically (450 nm) using 4-methylumbelliferyl-\(\alpha\)-D-N-acetylneuraminide as substrate. Other glycosidases were assayed spectrophotometrically (500 nm) using the appropriate \(p\)-nitrophenylglycosides. When cells had been grown with 1 mM cyclic AMP and 1 mM theophylline, there was, compared to controls, a small decrease in neuraminidase activity in homogenates from confluent cultures, but not in those from sparse cultures. There were also small (20-30\%) increases in N-acetyl-\(\beta\)-D-galactosaminidase and \(\alpha\)-D-galactosidase in homogenates from both confluent and sparse cultures. Similar increases were found in N-acetyl-\(\beta\)-D-galactosaminidase and \(\alpha\)-D-mannosidase, but only in homogenates from confluent cultures. Both \(\alpha\)- and \(\beta\)-L-fucosidases, and \(\beta\)-D-galactosidase,
were unchanged.

Glycosyltransferases for transferring N-acetyl-glucosamine, N-acetylgalactosamine, galactose, mannose, fucose, glucose, xylose and sialic acids from the corresponding nucleotide-[\(^{14}\)C]sugars were assayed in suspensions of cells from both sparse and confluent cultures by measuring the incorporation of label into phosphotungstate-precipitable material. The possibility that incorporation of label supplied as nucleotide-[\(^{14}\)C]sugar might be preceded by hydrolysis to free [\(^{14}\)C]sugar was investigated in two cases (UDP-[\(^{14}\)C]glucose and CMP-N-acetyl-[\(^{14}\)C]neuraminic acid) and the possibility excluded. When B16 melanoma cells were grown with 1 mM cyclic AMP and 1 mM theophylline, then in confluent cultures the incorporation of label from CMP-N-acetyl-[\(^{14}\)C]-neuraminic acid was doubled, and the incorporation of label from UDP-[\(^{14}\)C]glucose eventually decreased; other glycosyltransferase activities changed very little. In sparse cultures all glycosyltransferase activities increased to twofold or less. In confluent cultures added Mn\(^{2+}\) appeared to be essential for incorporation of label from UDP-N-acetyl-[\(^{14}\)C]-galactosamine, UDP-N-acetyl-[\(^{14}\)C]glucosamine and UDP-[\(^{14}\)C]-galactose, suggesting that the incorporation of label was not dependent on cell damage. While the enzymes responsible may be exoglycosyltransferases, it is also possible that there exists some mechanism for the uptake of label from these nucleotide-[\(^{14}\)C]sugars, not necessarily involving hydrolysis to free sugars, so that the label would be available to intracellular glycosyltransferases.
