

Getting the glycosylation right: Implications for the biotechnology industry

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Glycosylation is the most extensive of all the posttranslational modifications, and has important functions in the secretion, antigenicity and clearance of glycoproteins. In recent years major advances have been made in the cloning of glycosyltransferase enzymes, in understanding the varied biological functions of carbohydrates, and in the accurate analysis of glycoprotein heterogeneity. In this review we discuss the impact of these advances on the choice of a recombinant host cell line, in optimizing cell culture processes, and in choosing the appropriate level of glycosylation analysis for each stage of product development.

Keywords: glycosylation, recombinant, antigenicity, clearance, review, carbohydrates

A better understanding of glycoprotein biosynthetic pathways and cloning of many of the key enzymes involved, together with progress in assigning functions to specific carbohydrate structures, has provided the groundwork for exploitation of glycobiology within the biotechnology industry. The carbohydrate components of glycoproteins can play crucial roles in protein folding, oligomer assembly and secretion processes, and in the clearance of glycoproteins from the bloodstream. Certain carbohydrate structures have also been found to be antigenic, and regulatory authorities such as the US Food and Drug Administration are demanding increasingly sophisticated carbohydrate analyses as part of the product or process validation^{1,2}.

It is not the purpose of this review to provide a general update on advances in glycobiology, which have already been covered by several excellent reviews published on biosynthetic pathways³⁻⁶, the biological properties of carbohydrates⁷, and cellular influences on the glycosylation process^{8,9}. Instead, this review will focus on the differences in carbohydrate structures that may arise from choosing alternative gene expression systems and culture conditions, their physiological significance, and the level of carbohydrate analysis that is appropriate at each stage of product development.

Oligosaccharide structures found on glycoproteins

Oligosaccharides can attach to proteins in three ways (Fig.1): (1) Via an *N*-glycosidic bond to the *R*-group of an Asn residue within the consensus sequence Asn-X-Ser/Thr (*N*-glycosylation). All mature *N*-linked glycan structures have a common core of Man₅GlcNAc₂, which can form part of simple oligomannose structures or be extensively modified by the addition of other residues such as fucose, galactose, and sialic acids. Hybrid structures also exist where one or more arms of the glycan are modified and the remaining arm(s) contain only mannose. (2) Via an *O*-glycosidic

bond to the *R*-group of Ser or Thr (*O*-glycosylation). *O*-linked glycosylation is extensive in structural proteins such as proteoglycans. Small glycan structures can also be *O*-linked to the side chain of hydroxylysine or hydroxyproline. (3) Carbohydrates are also components of the glycoposphatidylinositol anchor used to secure some proteins to cell membranes.

The presence of these consensus sequences by no means guarantees their glycosylation. They show varying degrees of occupancy with oligosaccharides (macroheterogeneity) dependent on their position within the protein and its conformation, the host cell type used for expression, and its physiological status. These three factors also determine the extent of variation in the type of sugar residues found within each oligosaccharide (microheterogeneity).

Choice of expression system

Bacteria. Common bacterial expression systems such as *Escherichia coli* have no capacity to glycosylate proteins in either *N*- or *O*-linked conformations¹². Although other bacterial strains such as *Neisseria meningitidis* have recently been shown to *O*-glycosylate certain of their endogenous proteins, the trisaccharide added is different from *O*-linked sugars found in eukaryotes¹³.

Yeast. Hypermannosylation (the addition of a large number of mannose residues to the core oligosaccharide), is a common property of most yeast strains¹⁴ and can compromise the efficacy of recombinant proteins such as the hepatitis B vaccine¹⁵ (Table 1). But hypermannosylation can be prevented by expressing the polypeptide in mutant yeast strains (e.g., *mnn-9* or the temperature-sensitive *ngd-29*) in which *N*-glycosylation is confined to core oligosaccharide residues with a limited mannose content (up to Man₃GlcNAc₂), resulting in more effective vaccines^{15,16}. There is also evidence to suggest that different *O*-glycosylation sites are used by yeast and mammalian cells¹⁷.

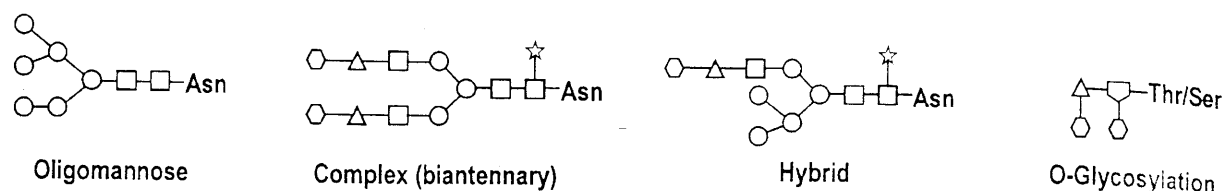


Figure 1. Common oligosaccharide structures found on glycoproteins: ○ Man, □ GlcNAc, △ Gal, ○ NeuAc, ☆ Fucose, ▽ GalNAc.

Plants. The few studies reporting the production of human proteins in plants have suggested that simple *N*-glycan structures that lack sialic acids are added, which may compromise activity. For example, erythropoietin (EPO) produced in tobacco cells has no biological activity in vivo, presumably because of its high clearance rate¹⁸. Another obstacle may be the presence of potentially allergenic residues such as core α 1-3 linked fucose¹⁹ or xylose β 1-2 mannose²⁰.

Insects. The baculovirus-infected insect cell expression system has become a popular route for recombinant protein synthesis because of its short process development time and potentially high yields. Most evidence to date indicates that the *N*-glycosylation capabilities of this system are limited to producing only simple oligomannose-type oligosaccharides (Man₃₋₆GlcNAc)²¹⁻²³; only a few studies have demonstrated complex *N*-linked glycans²⁴. However, most of these data are derived from *Spodoptera frugiperda* (Sf9 and Sf21 lines), and other baculovirus-infected insect cell lines may differ. For example, the Ea4 line (derived from *Estigmene acrea*) can add some terminal galactose residues to recombinant interferon- γ ²⁵, and secreted alkaline phosphatase produced in *Trichoplusia ni* (TN-368 and BT1-Tn-5B1-4 lines) has

both galactose and terminal sialic acids²⁶.

Mammals. Species that are phylogenetically closer to humans may be expected to have more elements of the glycosylation machinery in common. Nevertheless, there are some surprising differences between the glycosylation characteristics of the rodent cell lines (routinely used for recombinant glycoprotein synthesis) and human tissues.

Mouse cells. Most mammals express the enzyme α 1-3-galactosyltransferase, which generates Gal α 1,3-Gal β 1,4-GlcNAc residues on membrane and secreted glycoproteins. The notable exceptions are in humans, apes, and Old World monkeys where the gene has become inactivated through frameshift mutations²⁷. Certain mouse lines such as hybridomas, mouse-human heterohybridomas, and C127 cells synthesize some glycans terminating in Gal α 1,3-Gal β 1,4-GlcNAc^{28,29}, particularly when grown in nonagitated flasks^{30,31}. But other rodent lines such as mouse NS0 or rat Y0 myeloma, producing humanized antibodies, do not add Gal α 1,3-Gal β 1,4-GlcNAc^{32,33}, and only induce a mild human immune response³⁴. These residues are more likely to occur in hybridoma-derived antibodies where glycosylation occurs in the light chain, compared to the partially buried Fc glycosylation site³⁵. Over 1% of

Table 1. Summary of the major glycosylation attributes for different cell expression systems.

Organism	Cell type	Type of Glycosylation				Fucose		Galactose			Sialic acids		Bisecting GlcNAc	Glycosidases
		O-linked	Oligo-mannose	Hyper-mannose	Complex	α 1-6 linked	α 1-3 linked	Gal α 1-3	SO ₂ -GalNAc	α 2,6 linked	α 2,3 linked	NeuGc		
Bacterium	<i>E. coli</i>	0	0	0	0	0	0	0	0	0	0	0	0	?
Yeast	<i>Saccharomyces</i>	++	0	++++	0	0	0	0	0	0	0	0	0	?
Plant	Tobacco BY2	?	++	0	?	?	++	?	0	0	0	0	0	?
Insect	<i>S. frugiperda</i> Sf9	++	++++	0	D	++	+	0	0	0	0	0	0	++
	<i>S. frugiperda</i> Sf21	++	++++	0	D	++	?	?	?	D	D	?	0	?
	<i>Trichoplusia ni</i>	?	++	0	++	?	?	?	?	++	++	?	0	?
	<i>E. acrea</i> Ea4	?	+++	0	++	++	0	0	0	0	0	0	0	?
	<i>M. brassicae</i>	++	++++	0	0	++	?	?	?	?	?	?	0	?
Hamster	BHK	++	++	0	++	++	0	+	0	0	++	?	0	?
	CHO	++	++	0	++	++	D	0	0	0	++	+	0	++
Mouse	Hybridoma	++	++	0	++	++	0	++	0	0	++	+++	0	+
	Myeloma	++	++	0	++	++	0	++	0	+	+	+++	0	+
	C127	++	++	0	++	++	0	++	++	++	++	+++	0	?
	J558L	++	?	0	++	++	0	++	?	++	++	+++	0	?
	Transgenic	++	++	0	++	++	0	?	?	+	+	?	?	?
Rat	Y0 Myeloma	?	?	0	++	?	0	?	?	+	+	?	++	?
Goat & Sheep	Transgenic	?	++	0	++	++	0	?	?	+	+	?	?	?
Human	Liver	++	+	0	++	++	0	0	0	++	++	0	0	?
	Brain	++	++	0	++	++	++	0	0	++	++	0	+	?
	Pituitary	++	++	0	++	++	0	0	+++	+	+	0	++	?
	B-lymphocyte	++	0	0	+	++	0	0	0	+	+	0	++	?
	Namalwa	++	++	0	++	++	0	0	0	++	++	?	?	?
Human-Mouse	Heterohybridoma	?	++	0	++	++	0	0	0	+	+	+	0	?

Note that a few studies have analyzed the glycosylation profile of the same glycoprotein expressed in more than one system, therefore some characteristics may be protein-specific. Further details of individual cell lines are given in the text. 0=not detected; ?=not tested; D=disputed (conflicting results reported in different publications). + to ++++: an approximation of the levels of oligosaccharides detected.

human serum IgG is directed against the Gal(α 1,3)-Gal- β 1,4-GlcNAc epitope³⁶, which may be a consequence of its presence on enteric bacteria. Specific removal of this epitope from porcine endothelial cells substantially diminishes their reaction with the natural cytotoxic antibodies found in human serum³⁷, but comparisons of mouse C127 and chinese hamster ovary (CHO)-derived tissue plasminogen activator (tPA) reveal only minor differences in the pharmacokinetics induced by interaction with these antibodies³⁸.

N-glycolylneuraminic acid (NeuGc) is a derivative of the sialic acid N-acetylneuraminic acid (NeuAc), and NeuGc levels have been shown to be more prevalent than NeuAc in antibodies derived from mouse or human-mouse hybridomas³⁹. In contrast, glycoproteins in adult humans do not normally contain NeuGc, which is an oncofetal antigen⁴⁰. Low levels of NeuGc (1% of total sialic acids) are tolerated in recombinant proteins such as EPO, but higher levels (e.g., in fetuin containing 7% NeuGc) can elicit an anti-NeuGc immune response⁴⁰. Furthermore, high levels of terminal NeuGc on a chimeric CT4-IgG fusion protein are correlated with a rapid removal of the molecule from circulation, compared to the same protein bearing terminal NeuAc residues⁴¹. The hydroxylase enzyme that converts cytidine 5'-phosphate (CMP)-NeuAc to CMP-NeuGc has recently been cloned⁴².

Hamster cells. Most of the CHO cell lines used for recombinant protein synthesis, such as Dux-B11, have fortuitously inactivated the gene for α 1-3-galactosyltransferase⁴³ and make low levels of NeuGc (NeuAc is the dominant sialic acid found on CHO-derived glycoproteins)⁴⁴. But cell lines such as CHO and baby hamster kidney (BHK) lack a functional α 2,6-sialyltransferase

(ST) enzyme, and synthesize exclusively α 2,3-linked terminal sialic acids via α 2,3-ST, in contrast to human and mouse cells, which have both enzymes⁴⁵. Most rodent cells, including the CHO cell line, can be genetically modified to resemble the human glycan profile by transfection of appropriate glycosyltransferases. For example, the gene for rat α 2,6-ST has been cloned into a recombinant CHO cell line to produce glycoproteins with both α 2,6-linked and α 2,3-linked NeuAc⁴⁶⁻⁴⁷. In addition, many mutants of CHO cells that display altered glycosylation properties that may prove useful hosts for expressing glycoproteins with minimal heterogeneity have been isolated⁴⁸.

Human cells. Other host cell lines have not been studied in sufficient depth to precisely define their glycosylation capabilities. Even the use of human host cell lines is not perfect, since the transformation event required in most cases to produce a stable cell line may itself result in altered glycosylation profiles⁴⁹. However, the human lymphoblastoid Namalwa cell line performs O-linked and N-linked glycosylation efficiently, and preliminary studies on Namalwa-derived recombinant tPA have demonstrated all the human-type glycosylation characteristics^{50,51}. All mouse-human heterohybridomas examined thus far have been found to follow the glycosylation characteristics of the mouse parental line^{38,52}.

Transgenic animals. Relatively few studies have been reported on the glycosylation of recombinant proteins expressed in the milk of transgenic animals. Those published from experiments in transgenic goats⁵³ suggest that a low level of complex glycans may be achieved. Furthermore, interferon- γ (IFN- γ) expressed in transgenic mice showed a greater proportion of truncated and oligomannose structures at the Asn₇ site compared to IFN- γ that

Table 2. Decisions governing the type of glycosylation analyses appropriate at each stage of production.

Question		Mono-saccharide composition analysis	Glyceran 'finger-printing'	Structural analysis/epitope determination
TARGET GLYCOPROTEIN Choice of Expression System PROKARYOTIC EUKARYOTIC Yeast Insect (Plant) Mammalian SMALL-SCALE EXPRESSION DEVELOPMENT: CULTURE CONDITIONS CULTURE METHOD PRODUCTION/ REGULATION				
DECISION 1				
Q1.1	Is glycosylation necessary for the therapeutic profile of the target glycoprotein?	—	—	—
Q1.2	If glycosylation does not occur, will underlying peptide regions be 'revealed' to the immune system?	—	—	—
DECISION 2				
Q2.1	Are specific glycan structures required for full therapeutic activity?	✓	✓	✓
Q2.2	Are certain glycan structures/epitopes to be avoided?	✓	✓	✓
Q2.3	Is mammalian glycosylation preferred?	✓	✓	✓
DECISION 3				
Q3.1	Is the anticipated glycosylation pattern obtained in practice?	—	✓	(✓)
Q3.2	Is the glycosylation pattern obtained consistent with activity measurements?	—	—	—
Q3.3	Is the glycosylation stable in the chosen formulation?	✓	✓	—
DECISION 4				
Q4.1	Can a reproducible glycosylation pattern be obtained on a lot-to-lot basis?	✓	✓	(✓)
Q4.2	What is the optimum method to monitor glycosylation?	✓	✓	(✓)
Q4.3	What methods/levels of analysis will be submitted to regulatory authorities?	✓	✓	✓
Lot-to-lot analysis on an on-going basis		✓	✓	—

expressed in CHO cells, although the level of glycosylation site occupancy was increased⁷¹. Glycoproteins can be remodelled *in situ* by the transgenic expression of extra glycosyltransferases in the mouse mammary gland⁷².

Cells derived from specialized tissues. A significant proportion of IgG molecules produced by human B-lymphocytes possess a bisecting GlcNAc residue β 1-4 linked to the central β -linked mannose of the core glycan. This residue may play a role in antibody-dependent cell-mediated cytotoxicity, and only certain rodent cell lines such as the rat Y0 myeloma (but not CHO cells or NS0 myelomas) produce recombinant antibodies containing this bisecting residue³¹. The GlcNAc transferase III enzyme which adds the residue has been cloned, thus, the opportunity exists for remodelling chimeric antibodies by transfecting this gene into host cells⁵⁵.

A large body of evidence suggests that natural IgG molecules lacking galactose are associated with rheumatoid arthritis⁵⁶. This has also been tested experimentally by presenting the agalactosyl glycoforms of autoantibodies recognizing type II collagen in T-cell-primed mice, resulting in acute synovitis⁵⁷. Thus, expression systems that result in a large proportion of recombinant therapeutic antibodies lacking galactose should be avoided. Low levels of sialic acids are typically found in both recombinant and natural antibodies³³, probably due to steric hindrance at the Fc glycosylation site.

Sulfated residues (SO₄-GalNAc-GlcNAc) appear on the outer arms of glycans attached to several pituitary glycoprotein hormones such as luteinizing hormone (LH), thyrotropin, pro-opiomelanocortin and certain proteases such as urokinase^{58,59}. A specific GalNAc transferase and a terminal GalNAc sulfotransferase recognize special protein motifs in the nascent peptide (e.g., Pro-Leu-Arg) and are mainly restricted to the anterior pituitary gland. Therefore, only cell lines derived from the pituitary gland or endothelium (such as At20 and 293 cells) are able to perform this sulfation^{60,61}. A hepatic receptor binds oligosaccharides terminating with SO₄-GalNAc residues, and could account for the rapid removal of sulfated LH from the circulation in contrast to the removal of follicle-stimulating hormone and chorionic gonadotropin, which bear terminal NeuAc residues⁶². Mouse C127 cells produce a different pattern of sulfation (NeuAc- α 3SO₄-6Gal), which occurs after the addition of NeuAc⁶³.

The clearance of a given glycoprotein from the blood stream is highly dependent on its oligosaccharides, particularly those that are situated on the outer arms of the glycan structures⁶⁴. Indeed, artificial glycosylation sites have even been introduced into small peptides in order to improve their pharmacokinetic properties or to render them resistant to blood proteases^{64,65}. There are several receptors for specific oligosaccharide structures that contribute to the clearance of glycoproteins from the bloodstream, the most significant being the asialoglycoprotein receptor⁶⁶. Other binding proteins recognize specific structures such as the Man- α 6Man- β 4GlcNAc- β 4GlcNAc found in interleukin-6 (IL-6)⁶⁷, but their significance in the clearance of the majority of glycoproteins remains to be determined⁶⁸. Studies in rats using EPO fractions enriched for particular glycoforms indicate that more highly branched glycans (e.g., triantennary or tetraantennary) are less susceptible to renal clearance than biantennary structures⁶⁹.

Cell culture conditions

Once the host cell line has been chosen, the cell culture conditions should be optimized in order to minimize glycoprotein heterogeneity and prevent deterioration of product quality during fermentation. Pioneering studies analyzing glycoproteins from batch samples^{70,71} demonstrated that the culture environment can influence both the macroheterogeneity and microheterogeneity of

oligosaccharides in recombinant glycoproteins^{60,72}.

Cell culture media. Monoclonal IgG, produced by mouse hybridomas in serum-free medium has higher levels of terminal sialic acid and galactose residues relative to that produced using serum⁷³, but antibody galactosylation is better in serum-containing medium for recombinant CHO cells⁷⁴. Production of antibodies in cell culture results in more consistent glycosylation than achieved in ascites fluid^{75,76}. Adaptation of BHK-21 cells producing a recombinant IL-2 mutant, from serum-containing to serum-free medium, results in substantial changes to its glycosylation, such as the complexity of glycan chains (number of arms, and higher levels of terminal sialylation and proximal α 1-6 fucosylation), and increases the overall level of glycosylation⁷⁷.

The ambient glucose concentration affects the degree of glycosylation of monoclonal antibodies produced by human hybridomas in batch culture⁷⁸, and of IFN- γ produced by CHO cells in continuous culture^{79,80}. Lipids such as dolichol act as key carriers in the glycosylation process, and lipid supplements alone or in combination with lipoprotein carriers can improve the N-glycosylation site occupancy of IFN- γ ^{78,79}. Work on hepatocytes suggests that provision of cytidine and uridine can also alter protein glycosylation by increasing the availability of nucleotide-sugars⁸¹.

The application of sophisticated analytical techniques to analyze glycosylation microheterogeneity has led to some interesting findings. An increase in the percentage of oligomannose (predominantly Man₅GlcNAc₂) and truncated structures has been observed when monitoring both recombinant antibodies made by NS0 myelomas^{32,82} and IFN- γ made by recombinant CHO cells⁸³. In the former study, supplemental nutrients in fed-batch culture did not prevent this deterioration in glycosylation. In perfusion culture of BHK-21 cells producing a recombinant IL-2 mutant, nutrient limitations (glucose, amino acids, dO₂) led to short-term changes in macroheterogeneity, but microheterogeneity was largely unchanged⁸³.

Cell status. Lowering the protein synthetic rate by cycloheximide improves the glycosylation site occupancy of recombinant prolactin produced by C127 cells⁸⁴, but studies on tPA synthesis in CHO cells suggest that the rate of protein synthesis by itself has little influence on protein glycosylation⁸⁵. Folding and disulfide bond formation certainly can influence the efficiency of N-linked glycosylation in some proteins. For example, low concentrations of the reducing agent dithiothreitol prevent cotranslational disulfide bond formation in the endoplasmic reticulum and lead to complete glycosylation of a tPA sequon that normally undergoes variable glycosylation⁸⁶. Cell growth rate influences glycosyltransferase V levels in HepG2 cells⁸⁷. Sodium butyrate is sometimes used to improve protein synthesis, but can change glycosylation by inducing a GlcNAc-transferase involved in O-glycosylation⁸⁸ and increasing sialyltransferase activity in recombinant CHO cells^{89,90}. (Curiously, butyrate has the opposite effect in HepG2 cells⁹¹.)

Bioreactor configuration. Mild hypoxia has minimal effects on the glycosylation of tPA produced by recombinant CHO cells⁹², but influences the level of sialylation of recombinant FSH⁹³. Similarly, pH changes within the range 6.9–8.2 in the cell culture medium do not have a dramatic effect on the glycosylation profile of recombinant placental lactogen expressed in CHO cells, however there was some evidence for underglycosylation outside this range⁹⁴.

Increases in the concentration of ammonium ion in the culture medium above 2 mM may compromise sialyltransferases present in the Golgi, resulting in reduced α 2,6-linked sialic acids in G-CSF produced by recombinant CHO cells⁹⁴. Increased ammonium ions also reduced the extent of recombinant placental lactogen N-glycosylation by CHO cells, but this was dependent on the pH⁹⁵.

Product degradation. Various glycosidase activities have been measured in CHO cell lysates and culture supernatants, the most active of which is sialidase at neutral pH⁹⁷. The soluble enzyme has been purified from culture supernatant of CHO cells⁹⁸ and can degrade glycans from proteins such as recombinant gp120. The CHO cell gene coding for this soluble sialidase was subsequently cloned and showed structural similarities to bacterial sialidases⁹⁹. Although CHO cells also produce an α -L-fucosidase, this enzyme is incapable of releasing core α 1,6-fucose from intact recombinant glycoproteins (gp120 or CD4), or the more peripheral Fuc- α 1,3-GlcNAc linkage from serum α -acid glycoprotein¹⁰⁰. Sialidase, β -galactosidase, β -hexosaminidase, and fucosidase can be detected at low levels in supernatants from mouse 293, NS0, and hybridoma cells, and the sialidase activity is much lower than that found in CHO cells¹⁰¹.

In a contrasting study, purified monoclonal antibody was incubated with supernatants from various mammalian cell lines (CHO K1, BHK-21, mouse C127, P3-X63, Ag8.653, and a human-mouse heterohybridoma) and an insect cell line (SF-21AE), and only the insect line showed evidence of glycosidase activity in the supernatant¹⁰².

Controlled carbohydrate modification. Advances are being made in the chemical production of oligosaccharides (e.g., synthesis of the core N-glycan structure Man₅GlcNAc₆ and oligomannose glycans from monosaccharides has recently been accomplished^{103,104}). This opens the possibility of adding defined glycan structures after recombinant protein synthesis and secretion. In addition, the cloning and expression in *E. coli* of several key glycosyltransferases will facilitate the postharvest remodelling of glycoproteins produced in cell culture^{105,106}, making them more acceptable as human therapeutic proteins.

Conclusions

Improvements in glycosylation analysis have enabled scientists to judge how the glycan structures of recombinant glycoproteins compare to their natural human counterparts. Ultimately, production may go beyond the reiteration of the human glycosylation profile, because it may be desirable to alter the product's bioactivity or pharmacokinetics in vivo by altering specific glycan structures. The initial choice of expression system will continue to be of crucial importance, and as more recombinant proteins are expressed in different cell lines a pattern of glycoform predictions can be assembled (Table 1), although these conclusions must remain speculative until more data are available. The influences of the cell culture process (including the effects of scale-up) are not as well defined at present, but as more studies are published generic protocols may emerge to produce consistent (albeit heterogeneous) glycosylation patterns. A more difficult objective for cell technology will be to produce a single invariant protein glycoform, rather than the current mixture of glycoforms each bearing different characteristics (analogous to chiral separation of small molecule drugs).

An increased awareness of the importance of product glycosylation has led to more detailed carbohydrate analysis at earlier stages of product development than in the past. The choice of a particular cell expression system, and the influence of a different manufacturing process on the biochemical consistency of the product are now being evaluated (Table 2). A small but increasing number of glycosylation analyses are requested by legal departments, who may use the resulting information in patent applications to substantiate claims and to distinguish their organization's material from that of their competitors.

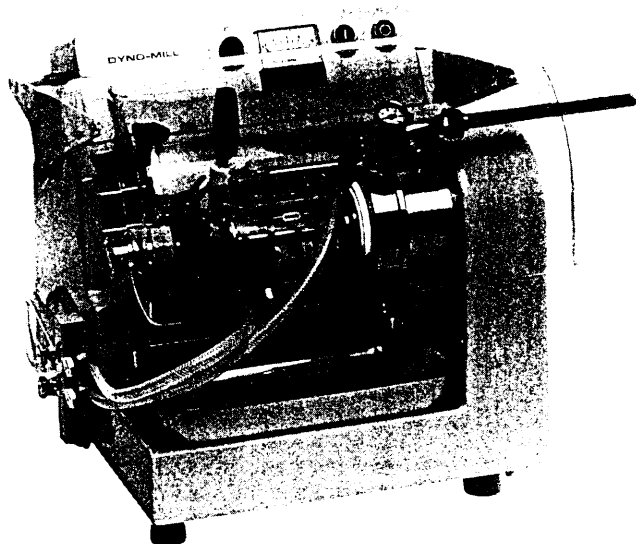
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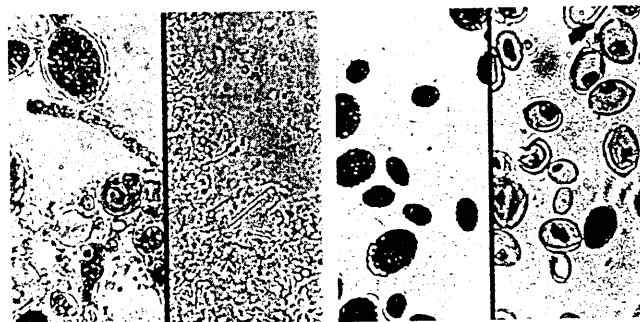
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