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Inhibition of glycoprotein processing by L-fructose and L-xylulose

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A number of unusual and rare carbohydrates were tested as potential inhibitors of various glycosidases, as well as inhibitors of N-linked oligosaccharide processing. The best inhibitors of several arylglycosidases and of glucosidase I were L-xylulose and L-fructose. Both of these sugars showed some inhibitory activity towards yeast α-glucosidase but were inactive against β-glucosidase and other arylglycosidases. The inhibition of yeast α-glucosidase by L-xylulose was of a competitive nature and required a concentration of 1×10^{-5} M for 50% inhibition. Both L-xylulose and L-fructose also inhibited the purified soybean glucosidase I, with 50% inhibition occurring at about 1×10^{-4} M, but showed no inhibitory activity against soybean glucosidase II. When influenza virus–infected MDCK cells were raised in the presence of L-xylulose, there was a dose-dependent inhibition in the formation of complex types of oligosaccharides on the viral glycoproteins consistent with the inhibition of the processing glucosidase I. This inhibition resulted in the occurrence of oligosaccharides on the viral glycoproteins that were characterized as $Glc_3Man_9(GlcNAc)_2$ structures. L-Fructose also inhibited glycoprotein processing in cell culture, and that inhibition resulted in the formation of similar oligosaccharides to those seen with L-xylulose. However, L-fructose was a poorer inhibitor than L-xylulose and required much higher concentrations for the same degree of inhibition. Neither of these compounds inhibited protein synthesis or the formation of lipid-linked saccharides in cultured MDCK cells, even when tested at concentrations of 5 mg/ml (about 30 mM) of culture media.

Key words: oligosaccharides/L-xylulose/L-fructose

Introduction

The asparagine-linked, or N-linked, oligosaccharides are commonly found on many membrane-bound and secretory glycoproteins (Varki, 1993) including a variety of important receptors such as the insulin (Duronio *et al.*, 1986) and the low density lipoprotein receptors (Cummings *et al.*, 1983). The biosynthesis of these oligosaccharide chains involves a complex series of reactions that begin in the endoplasmic reticulum (ER) with the assembly of the 'immature' oligosaccharide chain on the lipid carrier resulting in the formation of $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol (Elbein, 1979; Struck and Lennarz, 1980; Lehrman, 1991). The oligosaccharide portion of this lipid-linked oligosaccharide is then transferred to specific asparagine residues that are present on the protein in the consensus sequence Asn-X-Ser (thr), as the protein is being synthesized on ribosomes bound to the ER (Geetha-Habib *et al.*, 1990; Kelleher *et al.*, 1992). Thus, glycosylation is generally considered to be a cotranslational event (Lennarz, 1987).

Once the protein has been glycosylated, and while it is still in the ER, two enzymes referred to as glucosidase I and glucosidase II remove the three glucose residues. Glucosidase I removes the outermost α1,2 linked glucose (Grinna and Robbins, 1979; Hubbard and Robbins, 1979), whereas glucosidase II cleaves the next two α1,3-linked glucoses (Michael and Kornfeld, 1980; Burns and Touster, 1982). These trimming reactions result in the formation of a $Man_9(GlcNAc)_2$ oligosaccharide on the protein. This oligosaccharide may then be further processed by a number of α-mannosidases that reside in the ER and the Golgi apparatus and which may trim as many as six mannose residues from the $Man_9(GlcNAc)_2$ structure as it is processed to give complex types of oligosaccharides (Kornfeld and Kornfeld, 1985).

Studies on the biosynthesis and function of the oligosaccharide portion of the N-linked glycoproteins have been greatly facilitated by the use of inhibitors that act at specific steps in the processing pathway (Elbein, 1987). Some of the inhibitors that have been widely used over the past 10 years include swainsonine, an alkaloid from various toxic plants that inhibits the Golgi mannosidase II (Colgate *et al.*, 1979; Elbein *et al.*, 1981; Tulsiani *et al.*, 1982); deoxymannojirimycin, a synthetic mannose analog that inhibits Golgi mannosidase I (Elbein *et al.*, 1984; Fuhrmann *et al.*, 1984); kifunensine, a microbial analog of deoxymannojirimycin that is a more potent inhibitor of mannosidase I (Iwami *et al.*, 1987; Elbein *et al.*, 1990); castanospermine and australine, alkaloids from the seeds of the Australian tree, *Castanospermum australe,* that inhibit the ER glucosidase I and glucosidase II (Pan *et al.*, 1983; Tropea *et al.*, 1989); and deoxynojirimycin from microbial sources that also inhibits glucosidase I and glucosidase II (Saunier *et al.*, 1982; Peyrieras, *et al.*, 1983).

Since several of the above inhibitors have structural similarities to sugars, it was of interest to screen a number of unusual sugars and polyols to determine whether any of them would serve as inhibitors of the glycosidases. In addition to the possibility of finding useful inhibitors, such studies should be informative in terms of structure function relationships.

In the present report, we show that the simple sugars, L-fructose and L-xylulose, are reasonably good inhibitors of the purified processing glucosidase, glucosidase I, but have virtually no effect on glucosidase II. In addition, these sugars also inhibit the removal of glucoses from N-linked oligosaccharides in cell culture systems, and therefore prevent the oligosaccharide chains of the influenza viral hemagglutinin from being processed to complex types of oligosaccharides. Since these sugars appear to be relatively nontoxic to cells and do not inhibit protein synthesis or the formation of lipid-linked sac-

Table I. Effect of unusual carbohydrates on the activities of various arylglycosidases

Carbohydrates[a] tested (50 μg/assay)	Enzymes assayed (% of control activity)					
	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	α-Mannosidase	β-Mannosidase
Allitol	—	102	24	107	82	92
L-Fructose	53	106	87	112	82	98
L-Glucose	100	103	91	105	102	94
D-Iditol	96	111	87	110	73	94
L-Iditol	61	107	87	109	88	98
D-Psicose	86	106	82	111	95	98
L-Psicose	39	103	90	111	88	97
D-Sorbose	75	95	77	105	90	102
D-Tagatose	100	108	87	101	78	95
L-Tagatose	100	104	86	101	95	98
L-Xylulose	6	102	88	111	85	95

[a]Other inactive carbohydrates included D- and L-talitol and L-sorbitol.

charides, they should be useful as inhibitors of glycoprotein processing in various cell culture systems.

Results and discussion

Initial tests of rare carbohydrates as inhibitors of various glycosidases

A number of rare carbohydrates were tested as potential inhibitors of the arylglycosidases, as well as of the glycoprotein processing enzymes, glucosidase I and glucosidase II. The results of these initial tests are presented in Tables I and II. Table I shows that most of these unusual sugars were ineffective in inhibiting any of the aryl-glycosidases when tested at concentrations of about 1 mM. However, L-xylulose was an effective inhibitor of the yeast α-glucosidase at that concentration, and almost completely inhibited its activity. This sugar had no inhibitory effect on almond β-glucosidase, or on any other aryl-glycosidase. L-Fructose also showed some inhibitory activity towards the α-glucosidase giving about 50% inhibition at 1 mM concentration, while L-psicose showed about the same activity and L-iditol somewhat less.

Interestingly enough, those sugars that inhibited the yeast α-glucosidase also were effective inhibitors of the glycoprotein processing enzyme, glucosidase I, but they did not inhibit the other processing glucosidase, glucosidase II. Those data are presented in Table II. It can be seen that L-fructose, L-xylulose, and L-iditol were the most effective inhibitors of glucosidase I, while D- and L-psicose and D-sorbose were also reasonably effective inhibitors. Interestingly enough, L-glucose did not show any inhibitory effect on this enzyme at this concentration. Also of considerable interest was the observation that none of these sugars had any inhibitory effect on glucosidase II, even though some of them were effective against the aryl-glucosidase, yeast α-glucosidase. This is of special interest since glucosidase II also catalyzes the hydrolysis of p-nitrophenyl-α-glucoside.

Effects of L-fructose and L-xylulose on processing glucosidases

Since L-fructose and L-xylulose showed the strongest inhibition of glucosidase I, we determined the effects of various concentrations of these sugars on the activities of glucosidase I and glucosidase II. Figure 1 shows that L-xylulose and L-fructose were almost equally effective in inhibiting glucosidase I, with 50% inhibition of this enzyme occurring at a concentration of about 25–50 μg/ml (i.e., 5–10 μg/assay mixture) for either sugar. Although these concentrations (about 1×10^{-4} M) are somewhat high for an inhibitor, these sugars appear to be quite nontoxic, and therefore high concentrations do not adversely affect the cells in culture (see results below). Therefore, they should be useful inhibitors to block glycoprotein processing (see below). On the other hand, as shown in Figure 2, neither of these sugars had any inhibitory effect on glucosidase II, even when tested at concentrations of 200 μg/incubation mixture. Thus, these compounds are fairly selective inhibitors of the processing glucosidase I.

The inhibition of glucosidase activity was examined throughout the time course of the reaction to be certain that inhibition continued during that entire time period. As shown in Figure 3, the inhibition of the reaction was evident during the entire 60 min incubation, and remained at the same level during this time. The inhibition of yeast α-glucosidase by L-xylulose was examined over a range of substrate concentrations (i.e., p-nitrophenyl-α-glucoside) to determine the type of inhibition that it caused. Figure 4 presents Lineweaver–Burk plots of the substrate concentration curves at several different concentrations of L-xylulose. The best computer fit of the data indicate that inhibition is of the competitive type. The amount of L-xylulose required to give about 50% inhibition of the yeast α-glucosidase was about 1×10^{-5} M.

Table II. Effect of unusual carbohydrates on processing glycosidase

Carbohydrates[a] tested (50 μg/assay)	Enzymes tested (% of control activity)	
	Glucosidase I	Glucosidase II
L-Fructose	7.0	107 0
L-Glucose	98.0	124 0
D-Iditol	15.7	111 0
L-Iditol	7.8	104.0
D-Psicose	13.7	108 0
L-Psicose	11.0	104.0
D-Sorbose	10.0	122 0
L-Talitol	22.0	117.0
D-Tagatose	63.0	111.0
L-Tagatose	56.0	102.0
L-Xylulose	6.3	100 0

[a]Inactive carbohydrates include allitol, L-sorbitol, D-talitol

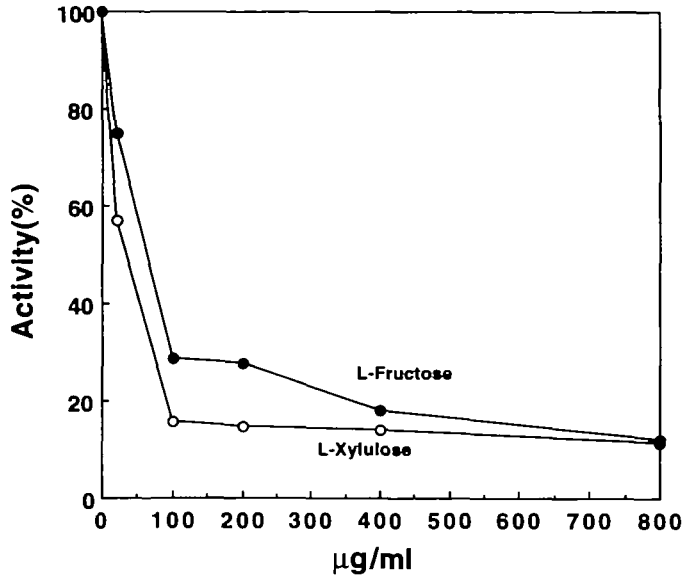


Fig. 1. Effect of concentration of L-xylulose or L-fructose on the *in vitro* enzymatic activity of purified soybean cell glucosidase I. Incubations were as described in the methods, except that various amounts of the inhibitors were added as shown in the figure. The release of radiolabeled glucose from $Glc_3Man_9GlcNAc$ was monitored as a measure of enzymatic activity.

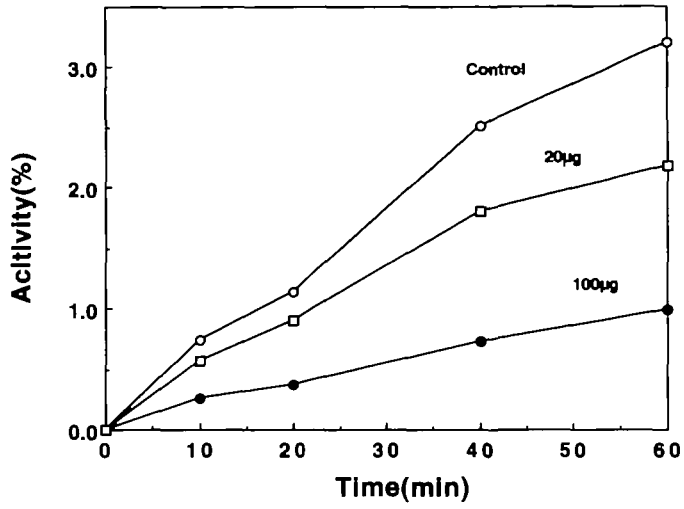


Fig. 3. Effect of time of incubation on the inhibition of yeast α-glucosidase by L-xylulose Incubations contained 10 μmol of p-nitrophenyl-α-D-glucopyranoside, various amounts of L-xylulose as shown, and buffer. The reaction was initiated by the addition of yeast α-glucosidase and the amount of p-nitrophenol released was measured for enzymatic activity.

Effect of L-fructose and L-xylulose on glycoprotein processing in culture

Since L-fructose and L-xylulose were found to be reasonable inhibitors of glucosidase I, we tested them in cell culture experiments to determine whether they could affect the processing of the oligosaccharide chains of the N-linked glycoproteins. For these studies, influenza virus–infected MDCK cells were incubated for 2 h in the absence or presence of various amounts of either L-fructose or L-xylulose, and then the cultures were labeled for 36–48 h with [2–^{3}H]mannose. This virus has an envelope glycoprotein (i.e., the hemagglutinin) that is an N-linked glycoprotein having about seven or eight oligosaccharides mostly of the complex type (Nakamura and Compans, 1979; Deon and Schulze, 1985). The mature virus was isolated by differential centrifugation and digested exhaustively with pronase for 48 h to generate glycopeptides. The glycopeptides

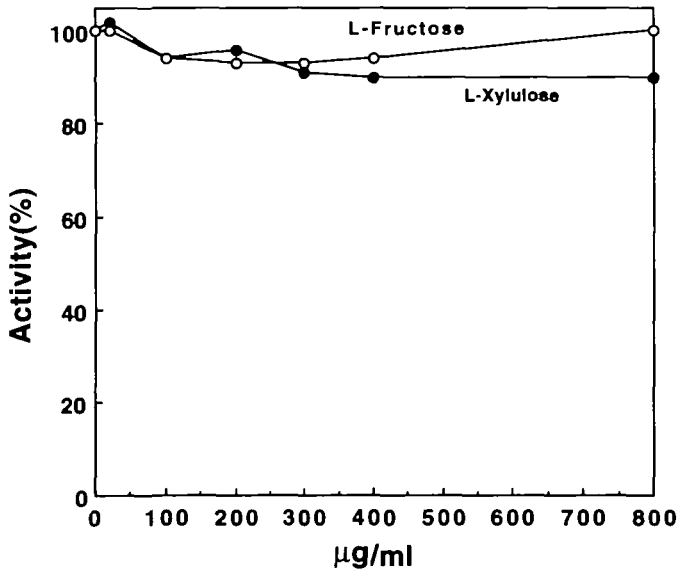


Fig. 2. Effect of concentration of L-xylulose and L-fructose on the *in vitro* enzymatic activity of purified soybean cell glucosidase II. Incubations were as described in the methods, except that various amounts of the inhibitors were added as shown in the figure. The amount of p-nitrophenol released from p-nitrophenyl-α-D-glucopyranoside was determined as a measure of glucosidase II activity.

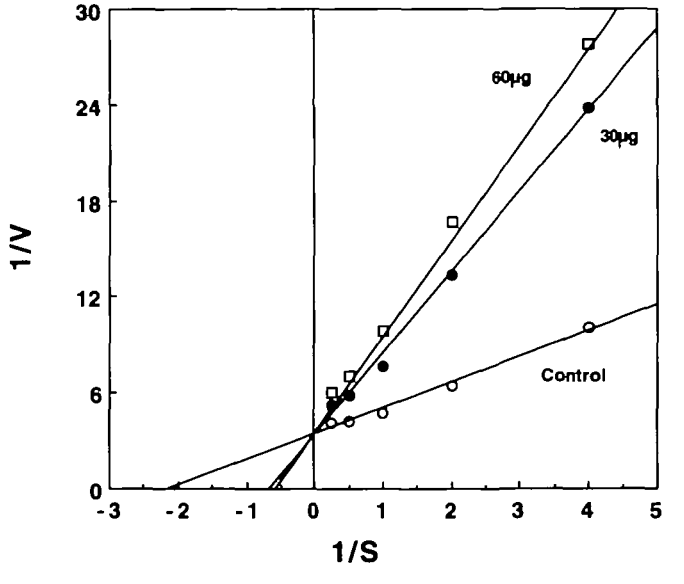


Fig. 4. Effect of substrate concentration on the inhibition by L-xylulose. Incubations for yeast α-glucosidase were as described in the methods except that various amounts of the substrate, p-nitrophenyl-α-glucopyranoside, were added as indicated. Several inhibitor concentrations were tested to determine whether the inhibition was of the competitive or noncompetitive type and the data was plotted according to the method of Lineweaver and Burk.

were then isolated and characterized by gel filtration on columns of Biogel P-4.

Figure 5 shows representative profiles of the radioactive glycopeptides from virus raised in the absence (Profile A), or in the presence of 1 (Profile B) and 5 (Profile C) mg/ml of L-xylulose. Since these shorter P-4 columns did not give complete resolution of the complex from the high mannose types of glycopeptides, the entire glycopeptide peak was pooled for each sample, digested with endoglucosaminidase H (Endo H) and rechromatographed on the same column. The elution profiles of the Endo H–treated glycopeptides are shown by the dashed lines in these figures.

The upper profile of Figure 5 shows that in the control incubations (i.e., without inhibitor) the radioactive glycopeptides emerged from the column in a single major peak that had a small shoulder, and treatment of this entire peak with Endo H resulted in the separation of the shoulder into a well-separated second peak that migrated slower than the original glycopeptide. However, most of the control glycopeptides were resistant to digestion by Endo H, and emerged in the same position as the original peak. This is consistent with the known oligosaccharide structures of the influenza virus, since most of the N-linked oligosaccharides on the viral glycoproteins are of the complex type, and only about 15–20% of the total oligosaccharides are of the high mannose structure and therefore sensitive to Endo H (Schwarz and Klenk, 1981; Wiley and Skehel, 1987).

On the other hand, when the virus was raised in the presence of 1 or 5 mg/ml of L-xylulose (see Profiles B and C), a much different glycopeptide profile was observed on the Biogel P-4 columns as compared to control cultures. Thus, as shown in profile B, 1 mg/ml of L-xylulose in the media caused the appearance of a new slower moving radioactive peak, and the amount of this peak increased with increasing amounts of L-xylulose in the medium (see Profile C). In addition, treatment with Endo H of the entire glycopeptide fraction from L-xylulose-treated cells demonstrated that this slower moving peak was completely susceptible to Endo H digestion, and this treatment caused a shift in the migration of this peak to a slower moving position. However, the first peak which is made up of the complex types of glycopeptides was still not susceptible to digestion by Endo H.

Figure 6 presents the results obtained using L-fructose as the inhibitor. Influenza virus was raised in the absence (Profile A) or in the presence of 1 (Profile B) or 5 (Profile C) mg/ml of L-fructose and the glycopeptides were chromatographed on the Biogel P-4 column. The profile of the control virus (Figure 6A) is essentially the same as that seen in Figure 5A, and again indicates that most of the oligosaccharide structures in the normal virus are of the complex types and are resistant to Endo H. L-Fructose also inhibited the formation of complex types of oligosaccharides as seen by the profiles in B and C, but it was considerably less effective than was L-xylulose. Thus, very little or no effect was seen at 1 mg/ml of this sugar whereas at 5 mg/ml, the slower moving peak became evident but it was only present to about the same extent as that seen with 1 mg/ml of L-xylulose. Thus, while L-fructose does inhibit the purified glucosidase I almost as effectively as does L-xylulose, it is clearly not as effective in cell culture. It is not clear whether this difference is due to differences in the uptake of these sugars by the cells, to the metabolism of one of these sugars to a less active or more active intermediate, or to other mechanisms of removal or detoxification of the inhibitor.

Characterization of the oligosaccharides produced in the presence of L-xylulose and L-fructose

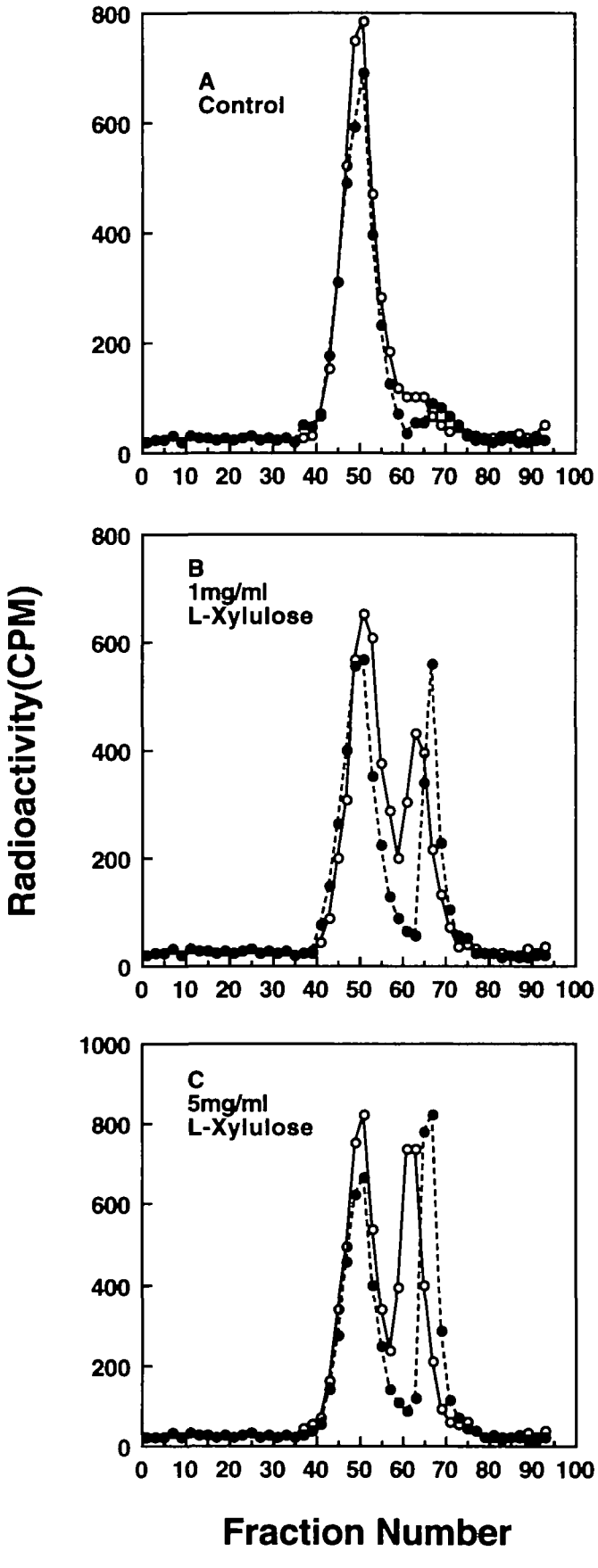
The Endo H–released oligosaccharides produced in the presence of L-xylulose or L-fructose were isolated from the Biogel columns as shown in Figures 5 and 6, and applied to a long (200 cm), calibrated column of Biogel P-4 in order to obtain an accurate determination of their size and the extent of homogeneity. Figure 7 (solid circles) shows that the radioactive oligosaccharide (produced in the presence of L-xylulose) eluted in a sharp, symmetrical peak suggesting that it was a single species, and it emerged in the area expected for a $Hexose_{12}GlcNAc$, as predicted for an oligosaccharide produced in the presence of a glucosidase I inhibitor.

This radioactive peak was pooled, and one part was treated with a purified glucosidase I preparation, while the second part was treated with a mixture of glucosidase I and glucosidase II. These digests were then applied to the same long calibrated column of Biogel P-4. These profiles are also shown in Figure 7, and are compared to the untreated oligosaccharide. It can be seen that treatment of the initial Endo H–released oligosaccharide with glucosidase I (open squares) resulted in a shift to a slower migrating oligosaccharide, indicating the loss of a single sugar residue in its structure. On the other hand, treatment with both glucosidase I and glucosidase II caused a greater shift in the mobility of the oligosaccharide, indicating the loss of three sugar residues. Similar results were obtained with the Endo H–released oligosaccharide produced in the presence of L-fructose. This data indicates that these L-sugars inhibit the formation of complex types of oligosaccharides by preventing the removal of the glucose residues, and this results in the accumulation of high-mannose oligosaccharides having mostly $Glc_3Man_9(GlcNAc)_2$ structures.

The oligosaccharide products, resulting from digestion with glucosidase I and the mixture of glucosidase I and glucosidase II, were treated with jack bean α-mannosidase and the resulting products were rechromatographed on the Biogel P-4 column. As shown in Figure 8 (Profile A), the untreated oligosaccharide gave two peaks after enzyme digestion. The faster migrating peak (Peak 1) was rather broad and emerged somewhat after the original oligosaccharide indicating the loss of two to three sugar residues. The second slower moving peak emerged in the monosaccharide area of the column and represents the free mannose released by α-mannosidase digestion. The ratio of radioactivity in these two peaks was about 6.5:2. Similar results were obtained with the glucosidase I–treated oligosaccharide except that the oligosaccharide produced by α-mannosidase treatment (Profile B) was slightly smaller in size than the one shown in Profile A, in keeping with it having one less glucose residue. Finally, as seen in Profile C, the oligosaccharide produced by treatment with the mixture of glucosidase I and glucosidase II was very susceptible to digestion by jack bean α-mannosidase, and this treatment resulted in the formation of two new radioactive peaks that corresponded to Man-GlcNAc and free mannose. The ratio of radioactivity in free mannose to that in the Man-β-GlcNAc was about 7.5:1. This data strongly supports the hypothesis that the major oligosaccharide produced in the presence of L-xylulose and L-fructose is indeed $Glc_3Man_9(GlcNAc)_2$.

Effect of L-xylulose and L-fructose on the formation of lipid-linked oligosaccharides and on protein synthesis

Since these two L-sugars were found to inhibit the processing of N-linked oligosaccharides in cell culture, it was important to



determine whether they also inhibited the formation of the lipid-linked oligosaccharide precursors, or protein synthesis. For these studies, uninfected MDCK cells were incubated with various amounts of L-xylulose or L-fructose, and then labeled with either [^{3}H]leucine (for determining protein synthesis), or with [^{3}H]mannose (for determining synthesis of lipid-linked oligosaccharides). Radioactivity incorporated into protein was measured after TCA precipitation (see Table IV), and radioactivity into lipid-linked saccharides was determined after extraction with organic solvents (see Table III) as described under *Materials and methods.*

Table III shows that these sugars had no effect on the biosynthesis of the N-linked intermediates, i.e., the lipid-linked saccharide derivatives. The data in the table shows that the synthesis of dolichyl-P-mannose, the precursor for the last four mannose residues of the $Glc_3Man_9(GlcNAc)_2$-PP-dolichol intermediate, was not inhibited even at concentrations of L-xylulose or L-fructose of 5 mg/ml. In addition, mannose incorporation into the lipid-linked oligosaccharides was not affected by these inhibitors. Thus, these compounds also do not decrease the biosynthesis of the N-linked oligosaccharide chains.

Neither L-xylulose nor L-fructose had any effect on the overall rate of protein synthesis when tested at various concentrations up to 5 mg/ml. These data are shown in Table IV. Because of limitations in the amount of material, higher concentrations could not be tested. Nevertheless, it is clear from these results that the amount of radioactive leucine incorporated into protein was essentially the same in control incubations as in those incubations containing various concentrations of the rare sugars. Thus, these compounds do not appear to affect the synthesis of proteins in cultured cells.

The studies described here demonstrate that certain unusual L-sugars, such as L-xylulose and L-fructose, are specific inhibitors of certain α-glucosidases, but do not inhibit various other glycosidases such as β-glucosidase or α- or β-galactosidases. In addition, these sugars also are specific inhibitors of the glycoprotein processing glycosidase, glucosidase I, but do not inhibit glucosidase II or other processing mannosidases. Thus, these sugars are among the few known inhibitors that are active on glucosidase I, without affecting glucosidase II. Another reported inhibitor of glucosidase I that is also inactive towards glucosidase II is australine, a pyrrolizidine alkaloid from the seeds of the Australian tree, *Castanospermum australe.* Interestingly enough, australine also inhibited the arylglucosidase, amyloglucosidase, which acts on p-nitrophenyl-α-D-glucopyranoside (Tropea *et al.,* 1989).

These sugar inhibitors also affected the *in vivo* processing of the N-linked oligosaccharide chains of the influenza viral hemagglutinin in cell cultures of MDCK cells. Since the major high-mannose type of oligosaccharides that were produced in

Fig. 5. Effect of L-xylulose on the oligosaccharide composition of the influenza viral glycoproteins. Infected MDCK cells were incubated for 1 h with 1 or 5 mg/ml of L-xylulose and then labeled with [2-^{3}H]mannose. Control flasks (A) were done in the absence of L-xylulose. After an incubation of 36 h, the virus particles were isolated from treated and untreated flasks by ultracentrifugation, and the viral pellet was digested exhaustively with pronase to produce glycopeptides. The glycopeptides were then separated on columns of Biogel P-4 as seen in this figure (○). The entire glycopeptide peak was then pooled, digested with Endo H, and reapplied to the same column. The Endo H-profile is shown by the dashed line (●). (B) shows results in the presence of 1 mg/ml of L-xylulose in the medium, and (C) that with 5 mg/ml of the sugar. Radioactivity was determined by liquid scintillation counting.

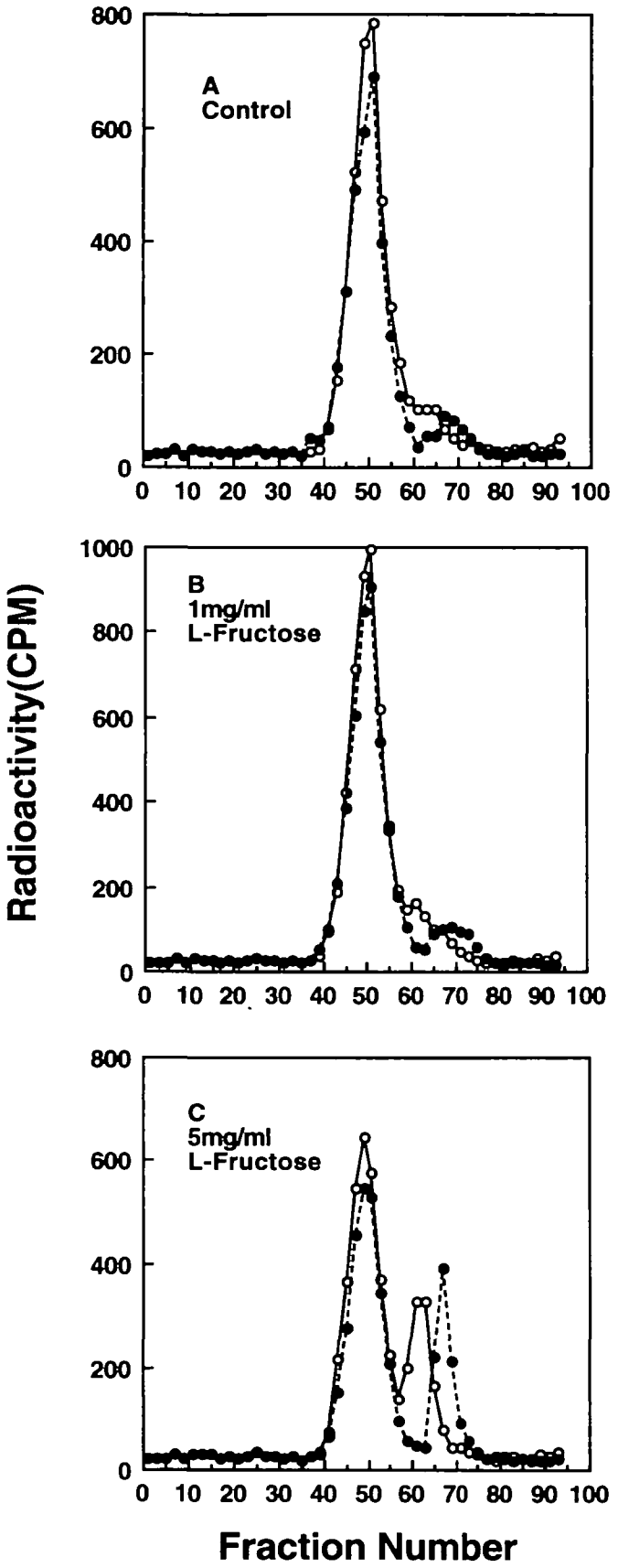


Fig. 6. Effect of L-fructose on the oligosaccharide composition of the influenza viral glycoproteins. The procedure was essentially the same as in Figure 5, except that L-fructose was used as the inhibitor instead of L-xylulose

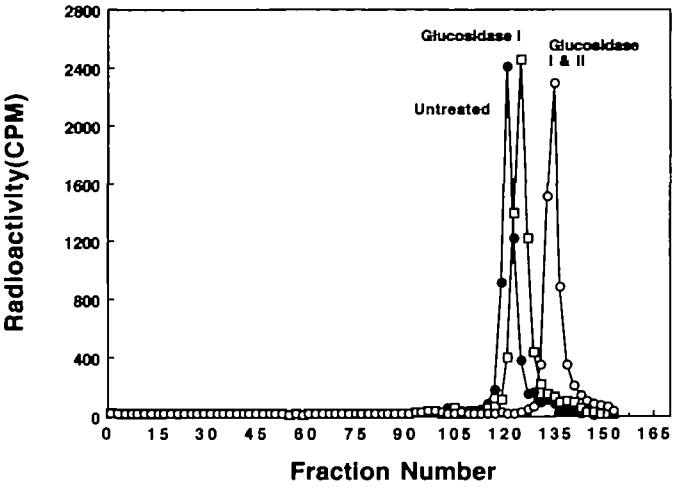


Fig. 7. Partial characterization of the glucose-containing oligosaccharides produced in the presence of L-xylulose (or L-fructose). The oligosaccharide released by Endo H (and seen in of Figure 5C) was chromatographed on a long calibrated column of Biogel P-4 (200–400 mesh; 200 cm column) This peak was then split into two fractions and one was treated with glucosidase I alone, whereas the other was treated with both glucosidase I and glucosidase II. Each of these digests was then run on the same Biogel P-4 column. Untreated oligosaccharide (●); Oligosaccharide treated with glucosidase I (□); oligosaccharide treated with glucosidase I and glucosidase II (○).

the presence of L-xylulose (or L-fructose) were $Glc_3Man_9(GlcNAc)_2$ structures, it seems most likely that the site of inhibition in cell culture is also glucosidase I. It is necessary to add these sugars in fairly high concentrations to the media in order to obtain a reasonable amount of inhibition. Thus, xylu-

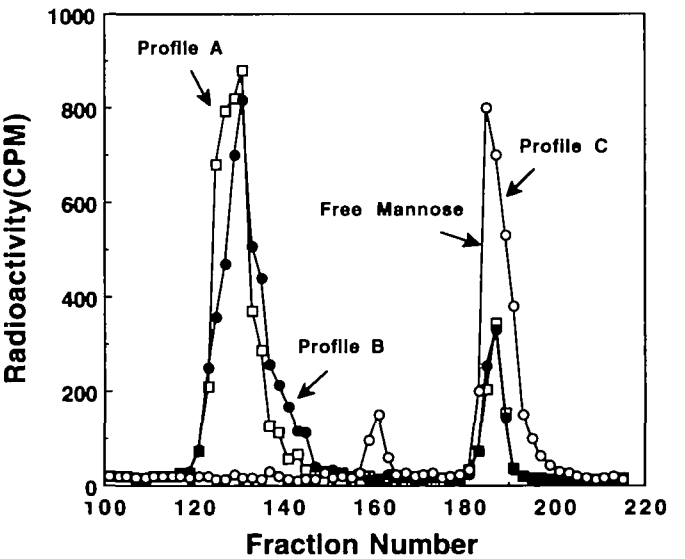


Fig. 8. Effect of jack bean α-mannosidase digestion on the structure of the Endo H–released oligosaccharides Oligosaccharides isolated from the column shown in Figure 7 were digested exhaustively with jack bean α-mannosidase, and the digestion products were chromatographed on the calibrated Biogel P-4 column. Profile A (□) shows the effect of this enzyme on the original Endo H-released oligosaccharide (i.e., no glucosidase treatment); Profile B (●) is digestion of the glucosidase I-treated oligosaccharide, Profile C (○) shows the effect of α-mannosidase digestion on the glucosidase I and glucosidase II–treated oligosaccharide

Table III. Effect of L-xylulose and L-fructose on the formation of lipid-linked saccharides

Inhibitor concentration (mg/ml)	Mannose (cpm) incorporated into: Lipid-linked monosaccharide 30 min	60 min	Lipid-linked oligosaccharide 30 min	60 min
L-Xylulose				
0	161	260	14,867	32,555
1	161	290	14,041	31,733
2	181	275	14,991	32,524
3	181	282	16,340	31,653
5	190	274	14,878	36,000
L-Fructose				
0	182	330	13,422	31,947
1	195	299	13,472	33,282
2	183	311	11,326	31,947
3	177	340	12,452	30,622
5	187	333	12,359	31,164

lose showed fairly good inhibition at about 5 mg/ml (40–50% inhibition of complex chain formation), but at this level, L-fructose gave only about 15–20% inhibition. However, these sugars appear to be nontoxic to cells and at 5 mg/ml in the medium, there was no inhibition of leucine incorporation into protein, nor of mannose incorporation into lipid-linked monosaccharides (i.e., dolichyl-P-mannose) or lipid-linked oligosaccharides. Therefore, they should be useful inhibitors to study glycoprotein processing.

These sugar inhibitors may also be valuable compounds to use to study the structural requirements for inhibition of glucosidase I. These two sugars, L-xylulose and L-fructose, probably exist in furanose ring structures in addition to being L-sugars. Furthermore, they do not have a nitrogen in the ring in place of the oxygen, as do the plant alkaloids that are glycosidase inhibitors. Most of the known glucosidase inhibitors such as castanospermine (Pan *et al.*, 1983) or deoxynojirimycin (Saunier, *et al.*, 1982) are structurally related to glucose and therefore exist in pyranoside types of rings with a nitrogen in the ring in place of oxygen. It will be of considerable interest to determine whether other types of L-sugars and/or furanosides have these kinds of biological activities, and if so, how effective and specific they are.

Table IV. Effect of L-xylulose and L-fructose on protein synthesis in MDCK cells

Inhibitor concentration (mg/ml)	Leucine (cpm) incorporated into protein 30 min	60 min
L-Xylulose		
0	22,690	32,926
1	26,180	29,690
2	25,600	31,280
3	30,080	35,250
5	27,560	36,000
L-Fructose		
0	27,210	32,744
1	29,426	37,440
2	28,800	31,560
3	25,796	35,280
5	24,522	32,500

Materials and methods

Materials

[2-^{3}H]Mannose (15 to 25 Ci/mmol) was purchased from American Radiolabeled Chemical Co., or from Du Pont-New England Nuclear [4,5-^{3}H]Leucine was obtained from ICN, pronase was from Calbiochem, and Endo H (i.e., endo-β-N-acetylglucosaminidase) was from Boehringer-Mannheim Co. *Aspergillus niger* amyloglucosidase and α-galactosidase, almond β-glucosidase, bovine liver β-galactosidase, jack bean α-mannosidase, all the p-nitrophenyl glycoside substrates and Concanavalin A–Sepharose were purchased from Sigma Chemical Co. Glucosidase I and glucosidase II were purified to homogeneity from suspension cultured soybean cells (Szumilo *et al.*, 1986; Kaushal *et al.*, 1993). [^{3}H]-glucose labeled oligosaccharides, i.e , $Glc_3Man_9GlcNAc$ and [^{3}H]$Glc_2Man_9GlcNAc$, were synthesized as previously described (Szumilo and Elbein, 1985; Kaushal *et al.*, 1993) and used as substrates for assaying glucosidase I and glucosidase II. Tissue culture materials were purchased from Flow Laboratories. Bio-Gel P2 and Bio-Gel P4 were obtained from Bio-Rad Laboratories. The inhibitory carbohydrates were synthesized biologically as described previously (Izumori *et al.*, 1990; Khan *et al.*, 1991; Muniruzzaman *et al.*, 1995).

Enzyme assays

Arylglycosidases. The enzymatic activities of amyloglucosidase, α-glucosidase, β-glucosidase, α- and β-galactosidase, and α- and β-mannosidase were determined colorimetrically by monitoring the release of p-nitrophenol from the appropriate p-nitrophenyl-glycoside substrates (Rudick and Elbein, 1975). All reaction mixtures contained 20 μmol of sodium acetate buffer, pH 5.0, 2 μmol of the p-nitrophenyl glycoside substrate, and enzyme in a final volume of 0.4 ml. Incubations were usually at 37°C for 15 min, and the reactions were stopped by the addition of 2.5 ml of 0.4 M glycine buffer, pH 10.4 The p-nitrophenol liberated in the reaction was measured at 410 nm in a Gilford spectrophotometer. Assays were done under conditions where the amount of p-nitrophenol released was linear with both time and protein concentration (Rudick and Elbein, 1975).

Assay of glucosidase I and glucosidase II. Glucosidase I activity was determined by measuring the release of [^{3}H]glucose from [^{3}H]$Glc_3Man_9GlcNAc$ as described previously (Szumilo *et al.*, 1986). Briefly, after incubation of purified enzyme with the radiolabeled substrate, the incubation mixture was applied to a column of Concanavalin A–Sepharose, and the liberated glucose was washed through the column with buffer. Oligosaccharides were then released from the Con A by washing the column with buffer containing 100 mM α-methylmannoside. Glucosidase II could be assayed either by following the release of radiolabeled glucose from [^{3}H]-$Glc_2Man_9GlcNAc$ as described above, or by measuring the formation of p-nitrophenol from p-nitrophenyl-α-glucoside. The incubation mixtures for both enzymes contained 50 mM MES buffer, pH 6 5, 0.1% Triton X-100, purified enzyme and substrate (about 25,000 c.p.m. of labeled substrate, or 2 μmol of the p-nitrophenyl-glycoside) in a final volume of 0 4 ml. A typical incubation was for 1 h at 37°C, and the reaction rate was linear with respect to both time and protein concentration.

Growth and labeling of influenza virus

The NWS strain of influenza virus was grown in MDCK cells as previously described (Pan *et al.*, 1983) MDCK cells were maintained in 75 cm^2 tissue culture flasks in modified Eagle's medium containing 10% fetal calf serum. At confluency, the cells were infected with influenza virus at a multiplicity of infection of about 75 PFU. One hour after infection, the inhibitors were added and flasks were incubated for another 2 h to allow the inhibitors to take effect before the radioactive sugar ([2-^{3}H]mannose) was added. The flasks were then incubated for 36–48 h until the cell monolayers had lysed and the mature virus was released into the media. At that time, the media was removed and saved, and any cells or cell debris remaining attached to the plastic were released by scraping with a plastic paddle. The medium and wash were pooled and subjected to low speed centrifugation to remove cell debris, and the virus was then sedimented from the supernatant liquid by ultracentrifugation at 100,000 × *g* for 18 h.

Preparation, isolation and analysis of glycopeptides

The mature influenza viral pellets, after isolation by ultracentrifugation, were suspended in 2 ml of 50 mM Tris buffer, pH 7.5 containing 1 mM $CaCl_2$, and 2 ml of a solution of 5 mg/ml of pronase in the same buffer were added. The mixtures were then incubated for 18 h at 37°C under a toluene atmosphere. At the end of this time a second 2 ml aliquot of the pronase solution was added and the viral pellets were incubated for another 18 h. Then, trichloroacetic acid (TCA) was added to the mixtures to a final concentration of 5% to precipitate

the protein, and the supernatant liquid was removed and extracted with ethyl ether five or six times to remove the TCA. The neutralized solutions were then subjected to gel filtration on columns of Biogel P-4 to isolate the glycopeptides.

Glycopeptides were separated on a 1.5 × 100 cm column of Biogel P-4 (100–200 mesh) equilibrated in 1% acetic acid. Aliquots of every other fraction were assayed for radioactivity to identify the positions of the glycopeptides and the free sugars. Since this column did not completely resolve the complex glycopeptides from the high mannose types, the entire glycopeptide fraction was pooled and treated with Endo H to cleave the high mannose oligosaccharides from their peptides. The Endo H digested mixture was then rechromatographed on the same Biogel column. In this second run, the complex glycopeptides emerged in the same position as in the first chromatography whereas the high-mannose oligosaccharides were now well separated from the complex structures. Thus, this method gave a reasonably rapid and quantitative assessment of the changes in oligosaccharide structure induced by the various inhibitors, and at the same time was useful for the separation and isolation of sufficient amounts of material for further characterization.

Characterization of the oligosaccharides

The structures of the Endo H sensitive oligosaccharides produced in the presence of the inhibitors was determined by a combination of chromatographic and enzymatic methods.

Chromatographic methods. The Endo H sensitive oligosaccharides were chromatographed on a calibrated 1 × 200 cm column of Biogel P-4 (200 to 400 mesh) which can separate oligosaccharides such as $Glc_3Man_9GlcNAc$ from $Glc_2Man_9GlcNAc$. The size of the oligosaccharide was then determined after various enzymatic treatments as indicated below.

Enzymatic treatments [^{3}H]mannose-labeled oligosaccharides were treated with purified glucosidase I or glucosidase II to determine their susceptibilities to these enzymes, and to aid in their structural characterization. After each treatment, the reaction mixtures were applied to the 200 cm column of Biogel P-4. Oligosaccharides were also treated with jack bean α-mannosidase, and the resulting products were characterized by gel filtration on calibrated columns of Biogel P-4.

Synthesis of lipid-linked oligosaccharides and protein

The effects of L-fructose and L-xylulose on the formation of lipid-linked saccharides and on protein synthesis were tested in uninfected MDCK cells. Confluent monolayers of MDCK cells in 6-well Limbro dishes were treated for 2 h with various concentrations of L-fructose or L-xylulose (1 to 5 mg/ml) in modified Eagle's medium containing 2% fetal calf serum. The cells were then incubated for 30 to 60 min with either [2-^{3}H]mannose (20 μCi/ml) to label the lipid-linked saccharides, or with [4,5-^{3}H]leucine (10 (Ci/ml) to label the cellular proteins. At the end of the labeling period, the medium was removed by aspiration, and the monolayers were washed three times with PBS. One milliliter of trypsin solution was added to each well, and the cells were incubated at room temperature for 5 min and then dislodged by scraping with a plastic paddle. The dislodged cells were then quantitatively transferred to test tubes and each well was washed with an additional 1 ml of PBS, and this wash was added to the tubes containing the initial cell suspension. Radioactivity incorporated into lipid-linked monosaccharides and oligosaccharides was determined after extraction with chloroform:methanol:water (1:1:1) and chloroform:methanol:water (10:10:3) as previously described (Tropea *et al.*, 1989). For the determination of radioactivity incorporated into protein, cells were extracted with 20% TCA in the presence of 200 μl of a pig kidney microsome preparation. Following an incubation of several hours at 4°C, precipitated protein was isolated by centrifugation. The precipitate was washed twice with 5% TCA and once with methanol. The precipitated proteins were then digested with pronase and the digests were subjected to scintillation counting to determine the amount of protein synthesized in the incubations.

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Abbreviations

ER, endoplasmic reticulum; TCA, trichloroacetic acid.

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