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Direct utilization of mannose for mammalian glycoprotein biosynthesis

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Direct utilization of mannose for glycoprotein biosynthesis has not been studied because cellular mannose is assumed to be derived entirely from glucose. However, animal sera contain sufficient mannose to force uptake through glucose-tolerant, mannose-specific transporters. Under physiological conditions this transport system provides 75% of the mannose for protein glycosylation in human hepatoma cells despite a 50- to 100-fold higher concentration of glucose. This suggests that direct use of mannose is more important than conversion from glucose. Consistent with this finding the liver is low in phosphomannose isomerase activity (fructose-6-P<–>mannose-6-P), the key enzyme for supplying glucose-derived mannose to the N-glycosylation pathway. [2–^{3}H] Mannose is rapidly absorbed from the intestine of anesthetized rats and cleared from the blood with a $t_{1/2}$ of 30 min. After a 30 min lag, label is incorporated into plasma glycoproteins, and into glycoproteins of all organs during the first hour. Most (87%) of the initial incorporation occurs in the liver, but this decreases as radiolabeled plasma glycoproteins increase. Radiolabel in glycoproteins also increases 2- to 6-fold in other organs between 1–8 h, especially in lung, skeletal muscle, and heart. These organs may take up hepatic-derived radiolabeled plasma glycoproteins. Significantly, the brain, which is not exposed to plasma glycoproteins, shows essentially no increase in radiolabel. These results suggest that mammals use mannose transporters to deliver mannose from blood to the liver and other organs for glycoprotein biosynthesis. Additionally, contrary to expectations, most of the mannose for glycoprotein biosynthesis in cultured hepatoma cells is derived from mannose, not glucose. Extracellular mannose may also make a significant contribution to glycoprotein biosynthesis in the intact organism.

Key words: mannose/glycosylation/mannose transporter/rat/plasma glycoprotein

Introduction

Mannose is required for N-glycosylation and glycophospholipid anchor synthesis. In eukaryotic cells mannose is assumed to be derived primarily or exclusively from glucose. Generating mannose requires phosphomannose isomerase (PMI) which converts fructose-6-phosphate (F6P) to mannose-6-phosphate (M6P) (Schwartz, 1992). This assumption is predicated on the ubiquitous distribution of PMI and the finding that deletion of PMI in yeast is lethal unless mannose is added to the medium (Gracy and Noltmann, 1968; Mendicino and Rao, 1975; Smith *et al.*, 1992). Recently two types of mannose-specific transporters were identified in mammalian cells. One is a sodium-dependent energy requiring transporter on the brush-border surface of Caco-2 cells and the other is a sodium-independent facilitated transporter on the basolateral surface of Caco-2 cells and in a variety of other cells (Ogier-Denis *et al.*, 1994; Panneerselvam and Freeze, 1996b). The K_{uptake} of the latter transporter is 35–70 μM, which is similar to the serum mannose concentrations in humans, rabbits, and flounders, the only species where mannose measurements have been reported (Pritchard *et al.*, 1982; Etchison and Freeze, 1997). Significantly, these transporters are relatively insensitive to the 100-fold higher physiological concentration of glucose in the blood (5 mM) suggesting that they could normally function to deliver mannose to cells for glycoprotein biosynthesis. In addition, the kidneys of dog and flounder have been shown to contain a sodium-dependent mannose transporter that is distinct from the glucose transporter (Silverman *et al.*, 1970; Pritchard *et al.*, 1982). At plasma concentrations of 50–100 μM mannose, the flounder reabsorbs up to 70% of the filtered sugar (Pritchard *et al.*, 1982).

Patients with carbohydrate-deficient glycoprotein syndrome type I (CDGS) underglycosylate many serum glycoproteins by failing to add entire N-linked oligosaccharide chains (Panneerselvam and Freeze, 1996a; McDowell and Gahl, 1997). The defect in the majority of these cases is a loss of phosphomannomutase (PMM) activity, which converts M6P to mannose-1-phosphate (M1P). Fibroblasts from these patients synthesize reduced amounts of a truncated lipid-linked oligosaccharide precursor and incorporate less [2–^{3}H]mannose into glycoproteins (Matthijs *et al.*, 1997a,b). Both underglycosylation and precursor truncation are corrected by supplementing the culture medium with mannose, but not with glucose, suggesting that direct mannose utilization is preferred over the contribution made by glucose (Panneerselvam and Freeze, 1996a).

There are very few studies exploring the occurrence, bioavailability, absorption, fate, or incorporation of mannose into glycoproteins of higher animals. One study in humans concluded that oral mannose was not efficiently absorbed, but that infused mannose was well tolerated and cleared normally (Wood and Cahill, 1963). A more recent study demonstrated that mannose was efficiently absorbed by humans (Alton *et al.*, 1997). It is well established that mannose can be transported by the intestinal epithelial cells of rodents (*ex vivo*) at ~15–20% of the rate for glucose (Cori, 1925; Deuel *et al.*, 1938; Wilson and Vincent, 1955). One study in rats concluded that mannose could increase liver glycogen (Deuel *et al.*, 1938), and another reported that mannose was not preferentially used over glucose for glycoprotein biosynthesis (Rambal *et al.*, 1995).

If extracellular mannose is directly used for glycoprotein biosynthesis, then (1) it should be observed routinely in the blood of mammals, (2) cells active in mannose absorption should have

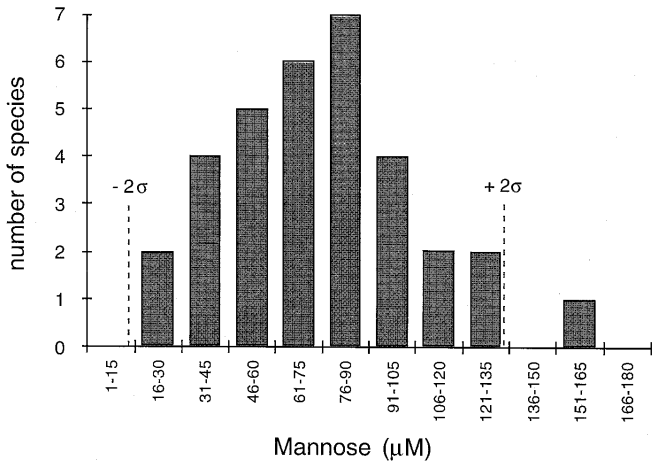

Fig. 1. Distribution of serum mannose concentrations in selected higher vertebrates. The average serum mannose concentration from 1–20 individuals of different species are given as a single value. Duplicate determinations for each sample were done as described in Etchison and Freeze, 1997. See Table I for actual values. This normal distribution pattern demonstrates a mean of 75 μM. Of the 35 species examined, only one has a mannose concentration greater than two standard deviations from the mean.

mannose-specific transporters that account for the mannose flux, (3) physiological concentrations of mannose should make a significant contribution to glycoprotein biosynthesis in the presence of normal levels of glucose, and (4) mannose should be efficiently absorbed through the gut and incorporated into tissue and plasma glycoproteins. This report provides initial evidence supporting these hypotheses and demonstrates that dietary and serum-derived mannose can contribute to protein N-glycosylation.

Results

Mannose is a ubiquitous component of mammalian sera

The identification of mannose-specific transporters in several mammalian cell types suggests that mannose may be important for glycoprotein biosynthesis. If true, then all animals should have sufficient blood mannose concentrations to drive uptake through the transporter. However, there is almost no information about mannose levels in the blood of animals. Therefore, we obtained frozen sera from 35 species of animals housed at the San Diego Zoo and San Diego Wild Animal Park and determined the mannose concentration. The results in Table I show that all have between 28 and 161 μM mannose in their blood. Bactrian camels have 161 μM, which appears to be outside the normal range seen for other animals. When the number of species is plotted against mannose concentration, a normal distribution is obtained with a mean of 75 μM (Figure 1). This is slightly higher than the average seen in humans (55 μM), but it is clearly in the K_{uptake} range measured for mannose transporters in fibroblasts (30–70 μM). Glucose concentrations are 50- to 150-fold higher than mannose (data not shown). The source of blood mannose is unknown, but some is probably derived from the diet. There is no published information on the bioavailability of mannose in various plants and animals. We found no obvious trend of serum mannose levels based on whether the animal is classified as a carnivore, herbivore, or omnivore. We conclude that mannose is a normal component of the blood of higher animals and that its concentration is sufficient to drive uptake through the mannose transporter.

Table I. Mammalian serum mannose concentrations

	Mammal	Serum mannose (μM)
Primates (simian)	Human (n = 23)	54
	East african drill (baboon)	75
	Sumatran orangutan	40
	Macaque (n = 2)	67
Primates (prosimian)	Lemur	87
Marsupials	Koala (n = 3)	88
Rodents	Rat (n = 4)	80
	Mouse (n = 2)	130
Lagomorphos	Rabbit (n = 2)	81
Carnivores (canine)	Spectacled bear	82
	Malayan sun bear (n = 2)	50
	Manchurian brown bear	62
	Alaskan brown bear	63
	Polar bear (n = 3)	76
	Domestic dog	65
	Arctic fox (n = 3)	92
	Styan's red panda	49
Carnivores (feline)	Domestic cat	135
	Fishing cat (n = 4)	97
	African leopard	90
	Persian leopard	94
Artiodactyls	Bighorn sheep (n = 2)	39
	Bantang	43
	Nubian ibex (n = 2)	116
	Pot-belly pig (n = 2)	114
	East Asian bongo (n = 2)	93
	Okapi	28
	Cow	60
	Bactrian camel (n = 3)	161
Perissodactyls	Sumatran rhino	40
	Indian rhino	56
Proboscids	Asian elephant	24
	African elephant (n=2)	73

Human hepatoma cells have a mannose-specific transporter

The liver is highly active in glycoprotein biosynthesis and therefore hepatocytes might also have a mannose transporter. To examine this, two human hepatoma cell lines, HepG2 and C3A, were incubated with increasing concentrations of unlabeled mannose and 14 μCi/ml (1 μM) of [2–^{3}H]mannose in the presence of 0.5 mM glucose, which is sufficient to maintain the cells during the experiment. An Eadie-Hofstee kinetic analysis shows a high-affinity transport process with a K_{uptake} of 75 μM and V_{max} of 33 nmol/h/mg for mannose in both cell types. A 5000-fold molar excess of glucose (5 mM) over [2–^{3}H]mannose inhibits uptake by only 35%, indicating that this kinetic system is glucose-tolerant (Table II). The uptake was highly specific for free mannose since a large variety of other monosaccharides and

mannose derivatives (e.g., α and β-methylmannoside, mannoheptose, mannitol, anhydromannose) or linkage isomers of mannobiose inhibited uptake by only 5–20% at 5 mM. Several examples are shown Table II. These data are comparable to the results of the mannose transporter characterized in fibroblasts (Panneerselvam and Freeze, 1996b). Deoxymannojirimycin, a well-known inhibitor of selected mammalian α-mannosidases (Daniel *et al.*, 1994; Moremen *et al.*, 1994), shows a slightly better inhibition than other competitors. Previous studies suggested that mannose uptake was partially inhibited by deoxymannojirimycin (Ogier-Denis *et al.*, 1994). The best inhibitor is ATB-BMPA, a 4-O azitrifluoroethylbenzoyl-substituted bismannose compound that has been used to specifically photoaffinity label the facilitated glucose transporters (Holman *et al.*, 1990; Jordan and Holman, 1992). These results show that mannose can be taken up by the mannose specific transporter on hepatoma cells but they do not show whether glycoprotein biosynthesis preferentially utilizes mannose or converts it from glucose via PMI.

Table II. Inhibition of cellular [2–^{3}H]mannose uptake

	Inhibitor	% Inhibition[a]
Control (0.5 mM d-glucose)		0
D-Glucose (mM)		
	1	15
	5	35
	10	70
D-Mannose (mM)		
	0.06	37
	0.25	68
	0.5	83
	1.0	91
Other sugars (5 mM)		
D-2-Deoxygalactose		25
D-Galactose		25
L-Rhamnose		15
D-Mannosamine		35
D-N-Acetyl-mannosamine		<5
D-6-Deoxymannose		<5
D-Altrose (3-epimer of mannose)		30
D-Talose (4-epimer of mannose)		35
Deoxymannojirimycin (4 mM)		55
ATB-BMPA (4 mM)		73

[a]Inhibition data for a 15 min uptake of [2–^{3}H]mannose (1 μM) by 70% confluent C3A hepatoma cells was obtained at 37°C. Cells were washed, scraped from the flask, and disrupted by ultrasonication, and an aliquot was taken for scintillation counting.

Utilization of [^{3}H]mannose by cultured human hepatoma cells

To directly estimate the mannose contribution to glycoprotein biosynthesis, actively growing hepatoma cells were labeled for various times with 130 μM [2–^{3}H]mannose (10 μCi/ml) in the presence of 5 mM glucose to mimic physiological conditions. The labeling medium was renewed every day so that the mannose concentration in the medium never fell below 70 μM. Cellular proteins were precipitated from the lysate and hydrolyzed, and the specific activity of mannose determined by high performance anion exchange-pulsed amperometric detection chromatography (HPAE-PAD) along with a series of [2–^{3}H]mannose standards of known specific activity. The specific activity of mannose in glycoproteins will increase as the cells grow. Using isotope dilution analysis extrapolated to infinite labeling time indicated that the specific activity of mannose in glycoproteins reaches at least 75% of the specific activity of mannose added to the medium (Figure 2). This means that exogenous transported mannose contributed 75% of the mannose found in hepatoma cell glycoproteins. Although we cannot be certain if this number includes [2–^{3}H]mannose salvaged from previously labeled glycoproteins the results show that direct use of mannose is highly preferred for glycosylation in culture. The remaining 25% is probably derived from glucose either directly or via gluconeogenesis. This is consistent with the results of short-term labelings using [2–^{3}H]mannose or [1, 5, 6–^{3}H]glucose at physiological concentrations. [2–^{3}H] Mannose contributed 70% of the total radioactivity found in PNGaseF released oligosaccharides and 30% comes from [1, 5, 6–^{3}H]glucose (results not shown). These results show that contrary to expectations, mannose not glucose, is the preferred source of mannose for glycoprotein biosynthesis. Nearly identical results are seen using human fibroblasts (Panneerselvam *et al.*, 1997).

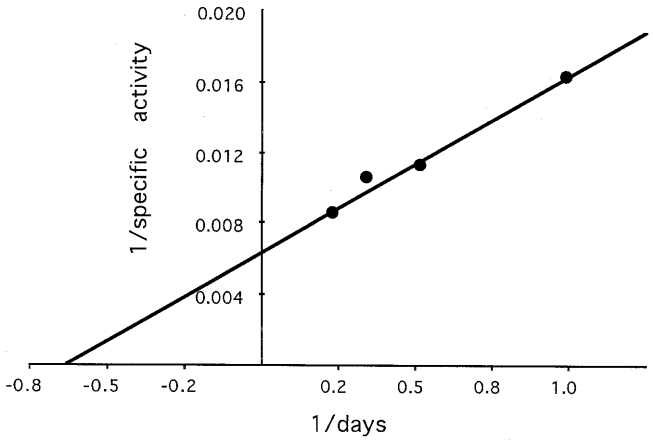

Fig. 2. Isotope dilution analysis of [2–^{3}H]mannose specific activity in C3A human hepatoma cell glycoproteins. C3A cells were grown in 3 ml of medium containing 130 μM mannose and [2–^{3}H]mannose at a specific activity of 170 d.p.m./pmol of mannose and 5 mM glucose. The medium was changed daily on each of 9 days, and the cells were harvested by scraping on days 5–9. The cells were lysed and the proteins precipitated with isopropanol, washed, and acid hydrolyzed to release the mannose. The specific activity of mannose in glycoproteins was measured at each time as described in *Materials and methods* and plotted as double reciprocal of specific activity vs. days. Extrapolation to infinite labeling time shows that the specific activity of mannose in glycoproteins reaches ~75% of that in the medium, indicating that 75% of the mannose had come from [2–^{3}H]mannose. Thus, only 25% could have come from other sources such as direct utilization of glucose.

Enzymatic activities of PMM and PMI in tissues

The preference of mannose over glucose for hepatoma cell glycoprotein biosynthesis suggest that the specific activity of PMI (F6P<–>M6P), the key enzyme used for endogenous mannose production from glucose, might be relatively low compared to other tissues, especially when normalized to liver glycoprotein

biosynthesis. To examine this, the specific activity of PMI and phosphomannomutase, PMM (Man6P<–>Man1P), a key intermediary enzyme for N-glycosylation were measured in various rat organs. As shown in Table III, PMM specific activity is highest in liver and intestine compared to other rat organs. Liver is low in PMI with 13 U/mg protein while it ranged between 21–40 U/mg protein for the other organs. When expressed as a PMI/PMM ratio, liver is the lowest of all organs. This is consistent with a preference for direct mannose utilization for liver glycoprotein biosynthesis.

Table III. Specific activity of PMI and PMM in rat organs

Organ	PMI	PMM	PMI / PMM
Heart	24	1.7	14
Spleen	24	2.0	12
Lung	21	1.6	13
Kidney	24	1.6	15
Brain	38	1.0	38
Muscle	32	1.3	25
Liver	13	3.8	3
Intestine	40	4.1	10

PMM and PMI activities are reported as units per mg protein (see *Materials and methods*).

Mannose is rapidly removed from the bloodstream

To examine the fate of mannose in the blood, anesthetized rats were given an intravenous injection of [2–^{3}H]mannose and the radiolabel was followed with time in the peripheral blood. After a rapid equilibration with interstitial fluid (Rang and Dale, 1987), [2–^{3}H]mannose is cleared from the blood with a half-life of ~30 min as shown in Figure 3. Incubation of [2–^{3}H]mannose with either serum, plasma, or whole blood showed no change in the label over several h indicating that it was not simply metabolized by blood components. Very little of the label was found in blood cells. After ~30 min, a portion of the [2–^{3}H]mannose began to appear in the blood as either labeled glycoproteins or as ^{3}HOH. At 4 h following the injection, radiolabeled plasma glycoproteins and ^{3}HOH production reached plateau values which then slowly decline over the next several days (data not shown). We could account for ~70% of the label injected at the beginning of the experiment. The production of ^{3}HOH indicates that some of the [2–^{3}H]mannose entered the glycolytic pathway through PMI. [2–^{3}H] Mannose was also incorporated into tissue glycoproteins and the distribution into various organs (data not shown) is virtually identical to that observed when [2–^{3}H]mannose is given as a gavage dose as discussed below.

Mannose is rapidly absorbed from the gut

The identification of mannose-specific transporters in Caco-2 cells suggested that mannose might also be actively absorbed from the intestine by transporters different from those used for glucose or fructose. Such a transporter could be important for delivery of dietary mannose to the organism. To examine this, we administered a gavage of [2–^{3}H]mannose to anesthetized rats and monitored the uptake into peripheral blood (Figure 4). [2–^{3}H]mannose is rapidly absorbed during the first hour when it peaks and then declines with approximately the same half-time of

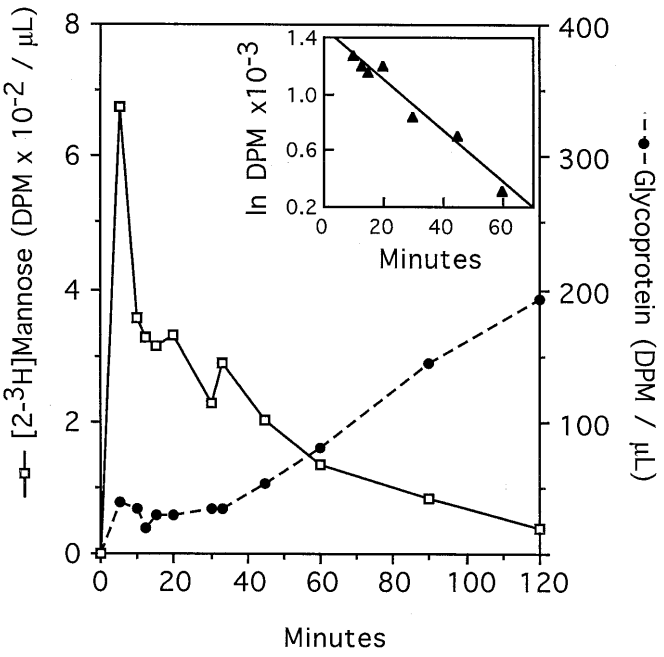

Fig. 3. Kinetics of [2–^{3}H]mannose appearance and clearance of radiolabeled glycoproteins in rat serum. [2–^{3}H] Mannose was injected into the circulation of an anesthetized rat and samples were withdrawn as described in the *Materials and methods*. After an initial equilibration with interstitial fluid, linear regression analysis of the semilogarithmic plot (inset) for the clearance of [2–^{3}H]mannose gives a half-time of 30 min.

30 min as an injected dose. The initial rate of uptake is not decreased by the presence of up to 2700-fold excess of fructose. In fact, the rate is about 2-fold greater in the presence fructose. These results suggest that mannose is carried by a transport system distinct from the facilitated fructose transporter, GLUT 5 (Olson and Pessin, 1996). A 100-fold excess of glucose does not affect the rate of [2–^{3}H]mannose uptake, but a 2700-fold excess decreases the initial uptake rate by 75%. Based on these results, we cannot be certain if the mannose transporter is distinct from sodium-dependent glucose transporters (Olson and Pessin, 1996). Less than 1% of the total radiolabel was found in the lumen of the intestine, feces or urine 4–8 h after the gavage demonstrating that uptake from the intestine was very efficient. These results also suggest that the kidney actively reabsorbs mannose. This probably occurs through efficient, previously identified mannose transporters in kidney tubules that are distinct from the kidney glucose transporter (Silverman *et al.*, 1970; Pritchard *et al.*, 1982).

Both radiolabeled serum glycoproteins and ^{3}HOH began to appear approximately 30 min after the gavage indicating that the [2–^{3}H]mannose was metabolized (PMI converts [2–^{3}H]M6P to ^{3}HOH and unlabeled F6P). Their appearance in the blood occurs at about the same time as that observed in the experiments using infused [2–^{3}H]mannose. This shows that transport from the intestine is not the rate limiting step for utilization of mannose. At any time point approximately 70% of the administered radiolabel can be accounted for as free [2–^{3}H]mannose, glycoprotein-associated [2–^{3}H]mannose, ^{3}HOH, or low molecular weight anionic compounds, which are presumably phosphorylated glycosylation intermediates. By 8 h ~95% of the radiolabel is converted into ^{3}HOH.

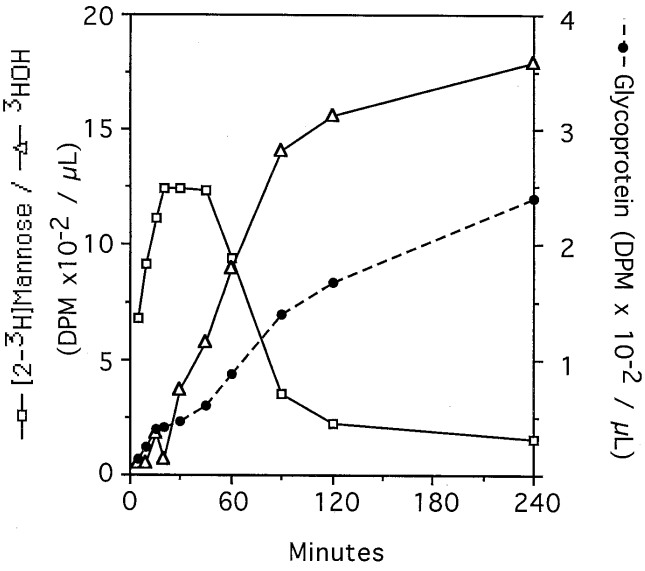

Fig. 4. Oral absorption of [2–^{3}H]mannose and the kinetics of appearance of radiolabeled glycoproteins and ^{3}HOH in female rats. Venous cannulated female rats were given a gavage dose of 500 μCi of [2–^{3}H]mannose and serum samples were monitored for the appearance of total radioactivity, [2–^{3}H]mannose, ^{3}HOH and TCA precipitable glycoproteins. At 2 h virtually no [2–^{3}H]mannose is left in the blood, but both ^{3}HOH and radioactive glycoproteins continue to accumulate. Results are shown as d.p.m./μl for [2–^{3}H]mannose and d.p.m./μl for glycoproteins.

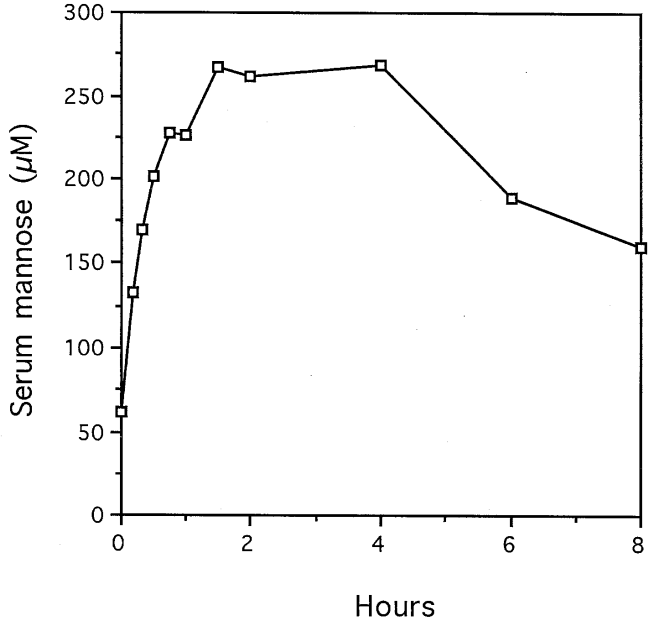

Fig. 5**.** Rat serum mannose concentration following a large oral dose of mannose. Female rats were given a gavage of dose of 0.1 g mannose per kg body weight, and the amount of mannose in the blood was monitored with time. Mannose continues to accumulate in the blood for several hours and reaches >3-fold higher than the basal level.

Metabolism of high doses of mannose

To determine if the intestinal transporter was capable of transporting a larger load of mannose, a gavage dose of 0.1 g mannose per kg body weight, including a small amount [2–^{3}H]mannose as a tracer, was administered to rats. Direct measurements of serum mannose concentrations and radiolabeled [2–^{3}H]mannose levels were coincidental for at least 6 h. The mean serum mannose level increased from 77 μM to 283 μM within 90 min as shown in Figure 5**.** There were no apparent side-effects such as osmotic diarrhea. These results indicate that relatively large loads of mannose can be transported efficiently.

Mannose is incorporated into tissue glycoproteins

Following a gavage dose with tracer amounts of [2–^{3}H]mannose, rats were sacrificed at 1, 4, and 8 h by whole body perfusion. The major organs were removed and analyzed for [2–^{3}H]mannose in glycoproteins by TCA precipitation. The identity of the radiolabel as >90% [2–^{3}H]mannose in glycoproteins was confirmed as described in *Materials and methods*, and at 1 h it accounted for ~1% of the administered label. Approximately 53% of the label incorporated into glycoproteins during the 1 h labeling is lost during the next 8 h of labeling. Since most of the [2–^{3}H]mannose in the blood has already been metabolized by 2 h, the loss of [2–^{3}H]mannose from proteins probably reflects a combination of oligosaccharide processing and glycoprotein degradation. The results in Figure 6 show the distribution of TCA precipitable material at 1 and 8 h, and the total amount of radiolabel at each time is defined as 100%. Figure 7 shows the specific activity at 1 and 8 h expressed as d.p.m./gm wet weight of tissue. Results obtained at 8 h resembled those at 4 h. It is clear that all of the major organs incorporated radiolabel, but that the liver is by far the predominant consumer both in total amount and specific activity of mannose uptake. At 1 h plasma contains a significant amount of labeled glycoproteins, and this increases about 2-fold during the next hour and then remains relatively stable between 4 and 8 h (not shown). Nearly 85% of the TCA precipitable radioactivity seen in the liver at 1 h is lost by 8 h, and 23% of this loss can be accounted for by an increase in labeled plasma glycoproteins. The remainder of label is probably lost through oligosaccharide processing within the liver and increases of labeled glycoproteins in other organs as seen in Figures 6 and 7.

The next major consumer of [2–^{3}H]mannose is the intestine. This high level is not simply the result of a higher localized concentration of the initial [2–^{3}H]mannose gavage, since the same distribution of radioactive glycoproteins is observed when [2–^{3}H]mannose is infused directly into the blood. The amount of [2–^{3}H]mannose in glycoproteins of the intestine decreases by 45% between 1 and 8 h. Like the liver, the decrease probably results from oligosaccharide processing and a high secretion rate of glycoproteins.

In contrast to liver and intestine, all of the organs examined, except the brain, show an approximate 2- to 6-fold increase in the amount of [2–^{3}H]mannose found in glycoproteins between 1 and 8 h (Figure 7). The lung shows nearly a 6-fold increase, skeletal muscle and heart show 5- and 4-fold increases, while kidney and spleen have <2-fold increase. Brain shows only a 20% increase between 1 and 4–8 h of labeling. Since all tissues are equally exposed to [2–^{3}H]mannose in the blood during the first hour and mannose cannot be stored within cells, the substantial increase in glycoprotein-associated radiolabel at 8 h may result from endocytosis of labeled plasma glycoproteins. Thus, organs differ in their direct uptake of mannose and its prompt incorporation into glycoproteins.

A gavage of [2–^{3}H]mannose was also given to pregnant rats. The label was incorporated into the placenta and fetus, including

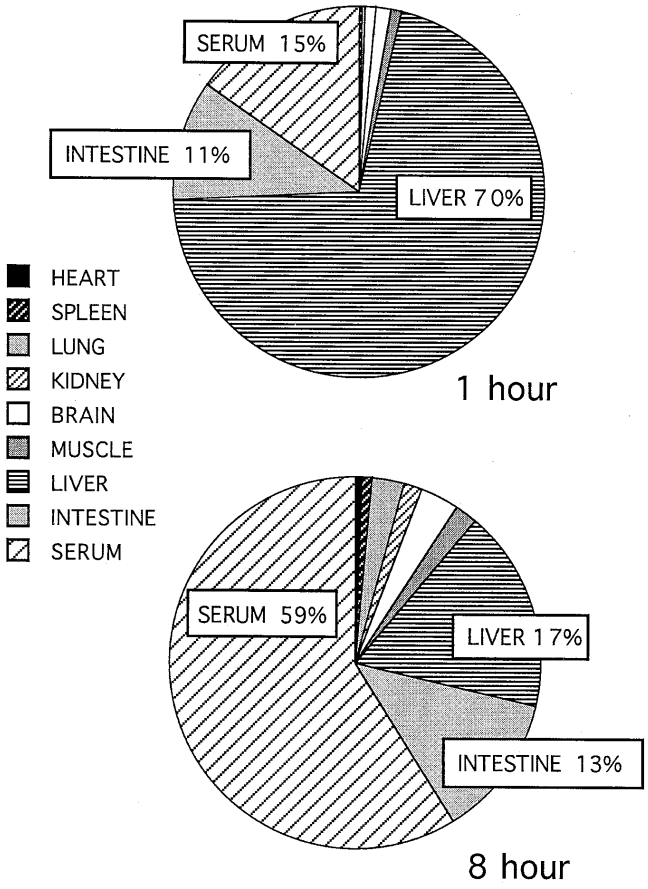

Fig. 6. Total radiolabeled glycoproteins in various rat tissues following a gavage of [2–^{3}H]mannose. Rats were given a 500 µCi dose of [2–^{3}H]mannose and the total amount of radiolabeled [2–^{3}H]mannose incorporated into all glycoproteins in the blood and in each organ are defined as 100%, at both 1 and 8 h. Total serum glycoprotein was estimated by using a blood volume of 18 ml for 250 g rats. The total amount of radiolabel in glycoproteins decreases by 53% between 1 and 8 h.

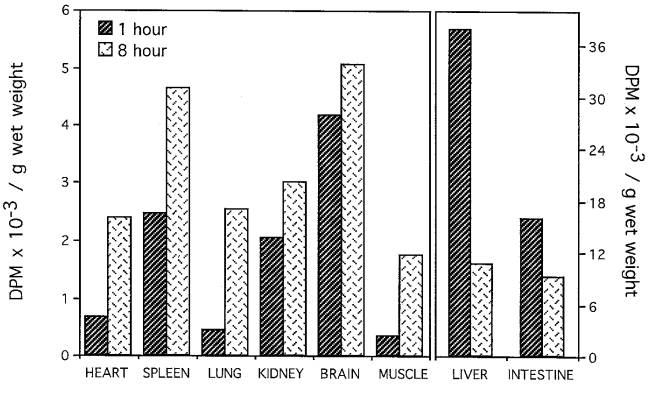

Fig. 7 Specific activity of radiolabeled glycoproteins following a 500 µCi gavage of [2–^{3}H]mannose. Comparison of the specific activity of [2–^{3}H]mannose in glycoproteins from each organ at 1 and 8 h post-gavage shows that only the liver and the intestine decrease whereas all other organs (except brain) show a 2- to 6-fold increase in specific activity. The dramatic decrease in the liver is partially due to serum glycoprotein secretion.

fetal brain. The specific incorporation of 48 d.p.m./mg protein in the fetus and in fetal brain is similar to that of the maternal brain (Table IV), demonstrating that [2–^{3}H]mannose crosses the placenta when available at normal physiological concentrations. Amniotic fluid of humans was previously shown to contain mannose comparable to its concentration in blood (Akazawa *et al.*, 1986).

Table IV. [2–^{3}H]mannose incorporation in rat organs and tissues

Organ	DPM/mg[a]	Mannose[b] (nmol/mg)	DPM/nmol
Heart	6	5	1.2
Spleen	16	10	1.6
Lung	5	12	0.4
Kidney	16	7	2.3
Brain	44	8	5.5
Muscle	3	4	0.8
Liver	220	3.5	62.9
Liver and plasma	264	3.8–4.1	64.4
Intestine	163	8	20.4
Fetus	48	nd[c]	nd

[a]Glycoprotein-associated [2–^{3}H]mannose was determined at 1 h following a 500 µCi gavage of [2–^{3}H]mannose.
[b]Glycoprotein-associated mannose was determined by TFA hydrolysis as described in *Materials and methods*.
[c]Not determined.

[2–^{3}H] Mannose incorporation into glycoproteins was also normalized to protein or mannose content of TCA precipitates from saline-perfused organs. These results should indicate which organs rely on the mannose transporter relative to their mannose requirement for *de novo* glycoprotein biosynthesis. The results are shown in Table IV. As expected the liver is much more active than all other organs whether the results are expressed for resident proteins alone or include the estimated amount of plasma glycoproteins synthesized and secreted during the 1 h labeling. The intestine is the next most active organ in mannose uptake and glycoprotein biosynthesis, and like the liver it probably secretes a significant amount of newly synthesized glycoproteins. Of the nonsecretory organs brain incorporates more mannose than other organs and also has a high mannose content per milligram of protein. The other organs examined all incorporate a similar amount of [2–^{3}H]mannose into glycoproteins whether normalized to total protein or to mannose content. They appear to be less reliant than the liver and intestine on direct incorporation of mannose for glycoprotein biosynthesis.

Discussion

The 28–161 µM mannose concentration found in mammalian sera is probably physiologically relevant, since the K_{uptake} of mannose by its specific transporter is 35–70 µM (Ogier-Denis *et al.*, 1994; Panneerselvam and Freeze, 1996b). The origin of mannose in the blood is unknown but the most likely sources are the diet, monosaccharide salvage and production from glucose. There are no data on the content or bioavailability of mannose in foods (Hardinge *et al.*, 1965; Shallenberger, 1974; Davis and Lewis, 1975; Bewley and Reid, 1985; Reid, 1985), and there are no animal studies on digestion of glycoproteins. Intestinal mucosal scrapings contain acidic pH α-mannosidases (Seetharam

and Radhakrishnan, 1972; Gossrau, 1973, 1976). These reports predate the discovery of N-linked oligosaccharide processing and an appreciation of the importance of neutral α-mannosidases in oligosaccharide degradation. The identification of α-mannosidase I and α-mannosidase II on the surface of enterocytes, on the brush border and in the secretory granules of pancreatic acinar cells is particularly significant since it suggests that they could function in glycoprotein digestion (Velasco *et al.*, 1993). Normal oligosaccharide processing as well as cytoplasmic and lysosomal degradation also liberate mannose (Cacan *et al.*, 1993; Daniel *et al.*, 1994; Moore and Spiro, 1994; Moremen *et al.*, 1994; Villers *et al.*, 1994; Saint-Pol *et al.*, 1997), but its fate is unexplored (Daniel *et al.*, 1994; Moremen *et al.*, 1994).

The mannose-specific transporter provides at least 75% of the mannose for glycoproteins of hepatoma cells and human fibroblasts (Panneerselvam *et al.*, 1997), clearly indicating that glucose is not the preferred source of mannose for these cells. This may also be true for glycoproteins synthesized by the liver. Although metabolic enzymes are not usually rate limiting, the high specific activity of PMM in the liver and intestine compared to other organs is probably required to meet the high glycoprotein synthetic demand. Two human PMM genes have recently been identified and Northern blots show high expression of both genes in liver (Matthijs *et al.*, 1997a,b). In contrast, PMI activity is surprisingly low in the liver (Table III), (Gracy and Noltmann, 1968; Mendicino and Rao, 1975; Proudfoot *et al.*, 1994). Since the only known function of PMI is to bridge glucose metabolism with mannose-6-P production, increased glycosylation using glucose-derived mannose might require higher PMI activity. The low PMI/PMM ratio in liver together with the high rate of [2–^{3}H]mannose incorporation further suggests that the liver preferentially uses exogenous mannose for glycoprotein biosynthesis.

The clearance rate of [2–^{3}H]mannose in rats ($t_{1/2}$ = 35 min) is very similar to that previously seen in humans (Wood and Cahill, 1963; Alton *et al.*, 1997). By 2 h most of the [2–^{3}H]mannose has been metabolized, regardless of whether [2–^{3}H]mannose is given intravenously or by stomach gavage. Absorption from the digestive system is very efficient, and <1% of total label is found in the feces or intestinal contents 8 h after a gavage dose. The absence of [2–^{3}H]mannose in the urine indicates that the kidney probably reabsorbs mannose using the mannose transporter that is distinct from the sodium-dependent glucose transporter (Silverman *et al.*, 1970; Pritchard *et al.*, 1982). The appearance of [2–^{3}H]mannose in the blood is very rapid suggesting that its uptake from the intestinal lumen is probably transporter-mediated. Uptake is not inhibited by fructose, so it is unlikely that mannose employs the GLUT5 fructose transporter (Olson and Pessin, 1996). A 100-fold excess of glucose does not inhibit tracer [2–^{3}H]mannose uptake, but a 2700-fold excess decreases the uptake rate by 75%. From these limited studies it is difficult to determine whether the same transporter normally carries glucose and mannose, but a mannose-specific active transporter has been identified in Caco-2 cells and human colon tumor cell line HT-29 (Ogier-Denis *et al.*, 1988, 1990, 1994).

Mannose appears to transcytose through the enterocytes since nearly all of the radioactivity first appearing in the blood is found as [2–^{3}H]mannose rather than as ^{3}HOH. After a 30–40 min delay [2–^{3}H]mannose is metabolized intracellularly by glycolysis to yield ^{3}HOH, or is incorporated into nascent glycoproteins. The great majority of [2–^{3}H]mannose is converted to ^{3}HOH over several hours, and since it is freely diffusable we cannot be certain which tissues carry out this conversion. However, fibroblasts and hepatoma cells labeled with [2–^{3}H]mannose for 1–3 h also convert ~85% of the label into ^{3}HOH (unpublished observations). We considered the possibility that the high proportion of label converted to ^{3}HOH was a labeling artifact specific for [2–^{3}H]mannose, but the same results were obtained using [1–^{14}C]mannose showing that they are metabolically equivalent precursors.

The liver and intestine are the major sites of [2–^{3}H]mannose utilization. Much of the label (53%) initially incorporated into glycoproteins by 1 h is turned over, probably due to oligosaccharide processing and glycoprotein secretion from both organs. In contrast, the amount of [2–^{3}H]mannose in plasma glycoproteins substantially increases between 1 and 8 h in lung, muscle, and heart. The most plausible explanation for this increase is that some of these cells endocytose a portion of newly labeled glycoproteins. Consistent with this view is the finding that brain, which is not in contact with plasma glycoproteins because of the blood-brain barrier, shows <20% increase during this time. Some of the [2–^{3}H]mannose released from degraded serum glycoproteins may be salvaged.

Monosaccharides are efficiently salvaged from endocytosed glycoproteins (Aronson and Docherty, 1983). In cell culture, the most dramatic example of this is the reutilization of at least 50% of the GalNAc and GlcNAc released from proteoglycans and glycoproteins endocytosed by fibroblasts (Rome and Hill, 1986). Salvage is also important in CHO*ldlD* cells that lack UDP-Gal/GalNAc-4-epimerase and require supplements of 20 μM galactose and 200 μM N-acetylgalactosamine for normal O-glycosylation (Kingsley *et al.*, 1986; Krieger *et al.*, 1989). Supplying 5–10% dialyzed fetal bovine serum or fetuin alone (Krieger *et al.*, 1989) also restores normal glycosylation. Finally, human glycoproteins do not contain N-glycolylneuraminic acid, but human cells growing on serum glycoproteins capped by N-glycolylneuraminic acid incorporate it into newly synthesized glycoproteins (Schauer, 1988). Thus, monosaccharide salvage seems to be ubiquitous.

Could the mannose transporter provide all of the mannose needed for liver glycoprotein production? It probably can. Hepatoma cells take up ~10 nmol mannose/mg/h under physiological conditions providing 1.5 nmol mannose/h/mg cell protein, assuming that 15% is available for glycosylation (not metabolized through glycolysis). The 1900 mg of protein in the liver of a 250 gm rat (Hellerstein and Munro, 1988a) synthesizes 10–20 mg of plasma proteins/h (10.5 μg plasma proteins/h/mg liver protein; Smith *et al.*, 1983; Hellerstein and Munro, 1988a,b). Plasma proteins contain about 20 nmol mannose per mg (Powell *et al.*, 1994), and since these glycoproteins are predominantly complex-type chains with three mannose residues, this would require synthesis of 0.3–0.6 nmol mannose /h/mg liver protein as the lipid-linked precursor. Thus, the mannose transporter (1.5 nmol mannose/h/mg protein) could supply the mannose required for liver glycoprotein biosynthesis (<0.6 nmol mannose/h/mg protein). Also, assuming that most of the mannose is cleared primarily by the liver (Figures 2, 3), the clearance rate of mannose from both blood and interstitial fluid is ~2 nmol mannose/h/mg liver protein (Guyton, 1981; Rang and Dale, 1987). Thus, both the mannose transport capacity of the liver and the calculated turnover rate of extracellular mannose is compatible with biosynthetic needs.

Could dietary mannose supply a significant amount of mannose used by rat liver? Calculations below suggest that it is unlikely to provide all of the mannose. Other sources, most likely glucose, must still make substantial contributions. The diet of a 250 gm rat (22.5 gm protein/kg/day) provides 11 mg of mannose,

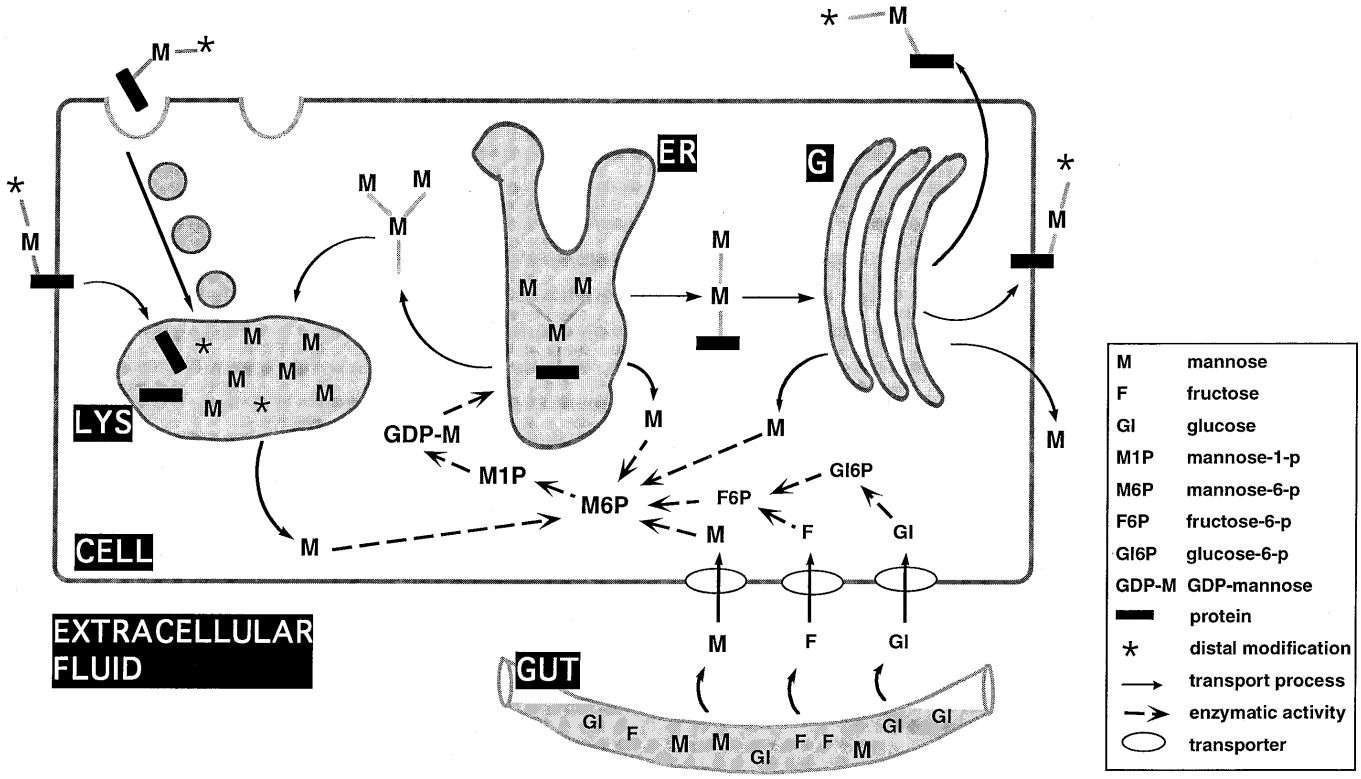

Fig. 8. Revised view of mannose metabolism in animals. In the traditional view, glucose (Gl) enters cells through the facilitated glucose transporters and a small portion of it is used for glycosylation by conversion of fructose-6-P (F6P) to mannose-6-P (M6P). Eventually, mannose derived from this pathway is incorporated into glycoproteins. In the proposed revised view, mannose derived from glucose makes a smaller contribution to glycoprotein biosynthesis and instead, *direct* mannose utilization is more important. Mannose is obtainable from the diet and from salvage pathways. Free serum mannose can be taken up directly by the mannose transporter, phosphorylated and used for glycoprotein biosynthesis. Mannose can be derived from dietary sources following digestion by pancreatic enzymes or by neutral mannosidases on the enterocyte cell surface and brush border (Velasco *et al.*, 1993). A mannose transporter (Ogier-Denis *et al.*, 1994) delivers mannose from the intestine (GUT) into the blood (EXTRACELLULAR FLUID) where it is taken up by facilitated mannose transporters on the surface of many types of cells (Ogier-Denis *et al.*, 1994), including hepatocytes. Mannose is incorporated into glycoproteins synthesized by the liver and they are secreted into the circulation. We suggest that these plasma proteins can be endocytosed, degraded and some of the released mannose reutilized for endogenous glycoprotein biosynthesis. Intact high-mannose chains cleaved from LLO or proteins within the endoplasmic reticulum (ER) are also degraded in the cytosol and lysosome (LY) providing additional mannose (Cacan *et al.*, 1993; Daniel *et al.*, 1994; Moore and Spiro, 1994; Moore *et al.*, 1995; Moremen *et al.*, 1994; Villers *et al.*, 1994; Saint-Pol *et al.*, 1997). Normal oligosaccharide processing of newly formed chains in the Golgi (G) releases mannose which may be secreted into the blood or into the cytoplasm for reutilization. Except for the preference of mannose by hepatoma cells demonstrated here, the relative contributions of each pathway are unknown. They are probably cell-type-dependent.

assuming mannose is 0.2% of the protein mass. Synthesis of precursor chains for plasma glycoproteins would require 2.2–4.5 mg mannose per day, assuming no mannose salvage. If 15% of this is available for glycoprotein biosynthesis, the liver would require 15–30 mg of mannose per day. Although there is a 35–200% shortfall, dietary mannose could still make an important and substantial contribution.

We suggest that the traditional view of mannose metabolism should be expanded (Figure 8) to include dietary sources and various mannose salvage pathways. The discovery of a myriad of intracellular neutral α-mannosidases (Daniel *et al.*, 1994; Moremen *et al.*, 1994), specific ER export and lysosome import pathways for high-mannose type oligosaccharides (Saint-Pol *et al.*, 1997; Moore *et al.*, 1995), and the distribution of neutral α-mannosidases on the surface of enterocytes and in pancreatic acinar cells (Velasco *et al.*, 1993) all suggest that many facets of mannose metabolism might be physiologically important. Discovering that mannose directly contributes to glycoprotein biosynthesis raises questions of mannose procurement, utilization, and salvage. It is also conceivable that malfunction of these processes in selected tissues might lead to localized pathologies affecting tissue or organ-specific glycosylation (Gahl, 1997; McDowell and Gahl, 1997).

Materials and methods

Materials

D-Mannose, D-glucose, D-fructose, ATP, PMI (P5153), hexokinase (H5625), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), and buffer salts were obtained from Sigma, St. Louis, MO. Hanks balanced salt solution, α-Minimal essential medium (α-MEM), and Dulbecco's modified essential medium (DMEM) were from GIBCO, Grand Island, NY. Antibiotics, glutamine, and trypsin were from Irvine Scientific, Santa Clara, CA. Fetal bovine serum was from Hyclone Laboratories, Logan, UT. [2–^{3}H]Mannose (15 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO.

Rats, cell lines, and culture conditions

Female Sprague–Dawley rats weighing 280 g were obtained from Zivik-Miller (Zelienople, PA) with a skull-mounted 20-gauge cannula subcutaneously connected to the jugular vein. The rats were handled in the Burnham Institute animal care facility. The animals were fed standard rat chow and water ad libitum. The human hepatoma cells HepG2 and C3A were from ATCC, Rockville, MD. They were grown in DMEM supplemented with dialyzed 10% heat inactivated fetal bovine serum (HepG2) or α-MEM supplemented with 20% heat inactivated fetal bovine serum (C3A), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere.

Measurement of [2–^{3}H]mannose uptake by cells

Nearly confluent cells in 35 mm multiwell plates were rinsed with DMEM containing 0.5 mM glucose (DMEM-g). Low glucose was used to maintain cells during the experiment and to prevent possible competition of mannose uptake. Preliminary experiments showed that [2–^{3}H]mannose uptake in the absence of glucose was nearly identical to that seen in 0.5 mM glucose. Uptake was initiated by the addition of labeling medium containing 20 μCi/ml of [2–^{3}H]mannose in DMEM-g and incubated at 37°C for 15 min. After removal of the radiolabeled medium, cells were washed five times with ice-cold phosphate-buffered saline (PBS), harvested by trypsinization, and solubilized in 0.1% SDS. An aliquot of the cell lysate was scintillation counted for radioactivity (Beckman LS 6000SC scintillation counter) and normalized to protein content (BCA protein assay, Pierce, Rockford, IL). The scintillation counter provided disintegrations per minute (DPM) via an internal quench correction calculation.

Gavage and injection of mannose, peripheral blood sampling, and whole body perfusion

Each experiment used a minimum of two rats, and all data reported are the mean values from at least two rats. No variation greater than 15% was observed in any physical determination on multiple rats. Injections and peripheral blood samplings were performed through the skull-mounted jugular vein cannula. All solutions were 0.2 μm sterile filtered (Puradisc 25AS, Whatman). Rats were lightly anesthetized with 5% isoflurane/O_2 prior to injection, gavage or blood sampling. Intravenous injections of 0.5 ml contained 50 μCi [2–^{3}H]mannose and 5 mM glucose in PBS and were prewarmed to 37°C. Gavage doses of 2 ml PBS containing 500 μCi [2–^{3}H]mannose, either in the absence or presence of 75 mM mannose (0.1 g mannose per kg body weight) were administered directly into the stomachs of lightly anesthetized rats. Between samplings or injections the rats were allowed to awaken from the anesthesia and consume water *ad libitum*. Aliquots of 0.5 ml of peripheral blood were removed from anesthetized rats, allowed to clot for 1 h, and centrifuged to give a clear supernatant serum. All analytical measurements were performed on serum.

Animals were sacrificed by whole body perfusion. Lightly anesthetized rats were injected with 45 mg/kg sodium pentobarbitol (Nembutal, Abbott Labs, Chicago, IL) interperitoneally. Depth of anesthesia was determined by lack of a toe-pinch reflex. The abdominal cavity was opened but the chest cavity was left closed. The organs were moved to one side to expose the aorta which was then clamped and a 20 gauge cannula was inserted into a partial cut in the aorta below the clamp. The cannula was clamped in place, the upper aortic clamp was removed, and 50 ml of prewarmed Hanks balanced salt solution was slowly injected by syringe. Simultaneously, a vein in the lower abdominal cavity was opened and the emerging fluid was monitored for loss of red color. Approximately 40 ml of Hanks solution provides a colorless (blood-free) perfusate indicating complete removal of blood from the major organs and tissues of the rat.

Determination of tritiated water, mannose, and [2–^{3}H]mannose

The amount of tritiated water (^{3}HOH) in a sample can be established by counting the total radioactivity before and after evaporation. Radioactive [2–^{3}H]mannose was determined by incubating 20 μl aliquots of serum (or TFA hydrolysates, see below) with 50 μl of 50 mM Tris–HCl, pH 7.2 containing 5 mM ATP, 18 U PMI, and 60 U hexokinase for 1 h at 37°C. The [2–^{3}H]mannose is converted to [2–^{3}H]mannose-6-phosphate by hexokinase. [2–^{3}H]Mannose-6-phosphate is converted to F6P by PMI which releases ^{3}HOH. After evaporation to remove the ^{3}HOH the difference of radioactivity remaining, relative to the control incubation (no PMI), provides a reliable determination of the original amount of [2–^{3}H]mannose in the serum. The actual molar concentration of mannose in serum was determined with a previously published spectrophotometric assay (Etchison and Freeze, 1997). Approximately 90–95% of the determined radioactivity in glycoproteins in either serum or TFA tissue hydrolysates is [2–^{3}H]mannose.

Determination of PMM and PMI enzyme activities in rat tissues

PMM and PMI were obtained by homogenization of freshly perfused rat organs in 50 mM Tris–HCl, pH 7.5, and collecting the supernatant from a 100,000 × *g* centrifugation (1 h). PMM and PMI enzymatic activities were assayed fluorometrically with a NADPH-linked coupled enzyme system as described previously (Sa-Correia *et al.*, 1987).

Quantitation of glycoprotein-associated mannose and [2–^{3}H]mannose

Radioactivity incorporated into glycoproteins was determined by adding an equal volume of 10% TCA to an aliquot of the tissue homogenate (1 g per 10 ml PBS) or serum. After vortexing and standing 10 min on ice the precipitated protein was collected by centrifugation. The pellet was washed twice with 10% TCA and resuspended with 0.85 M NH_4OH, transferred to a scintillation vial and counted for radioactivity.

To identify the glycoprotein-associated radioactivity as [2–^{3}H]mannose a 0.5 ml aliquot of each tissue homogenate or serum sample was precipitated with TCA. The washed pellets were heated at 100°C in 1.5 ml 2 M TFA for 4 h. The solutions were evaporated under flowing N_2 (g) at 80°C, and then lyophilized from 3 ml water. The freeze-dried samples were resuspended in 0.3 ml of 300 mM Tris–HCl, pH 7.2 and the [2–^{3}H]mannose determined by the hexokinase/PMI assay as described above. The mannose content of TFA hydrolyzed tissue was determined by the spectrophotometric mannose assay described above. Quantitative estimates of the recovery of mannose in each TFA hydrolysate were judged by the yield of PMI-convertible [2–^{3}H]mannose internal standards (see above).

Determination of [2–^{3}H]mannose specific activity in N-linked glycoprotein oligosaccharides

C3A hepatoma cells were grown in α-MEM as described above and with an additional supplement of 130 μM mannose and 10 μCi/ml [2–^{3}H]mannose for 9 days with new media added each day. Based on preliminary experiments, this concentration of mannose is sufficient to maintain mannose at >70μM throughout the day. Cells were washed as described above, scraped from the tissue culture plate, and disrupted by ultrasonication in 1 ml PBS. Glycoproteins were precipitated at -20°C overnight with 8 volumes of isopropyl alcohol. Following centrifugation the pellet was washed and hydrolyzed in TFA as described above. The resultant material was analyzed for monosaccharide composition by Dionex HPAE-PAD. Mannose peaks eluting from the column were collected and radioactivity was determined by scintillation counting. The radioactive mannose content per picomole of mannose (d.p.m./pmol) of the culture media was compared to the TFA hydrolysates from each day of labeling.

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