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Use of compounds naturally labeled with stable isotopes for the study of the metabolism of glycoprotein neutral sugars by gas–liquid chromatography–isotope-ratio mass spectrometry. Technical validation in the rat *

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ABSTRACT

In order to develop an alternative method to radioactive labeling for the study of the glycoprotein sugar metabolism in man, the possible use of stable isotopes provided by naturally, ^{13}C-enriched dietary compounds has been explored in rat intestine and serum. Rats were fed a semisynthetic diet containing 67% wheat starch (containing 1.08692 ^{13}C atom/100 carbon atoms) for a week, and then the same diet containing corn starch (1.10042% ^{13}C) for a week. Neutral sugars were prepared from delipidated, trichloroacetic acid-precipitable material from 200–400 mg of intestinal mucosa or 1 mL of serum, separated, and analyzed as alditol acetates by gas–liquid chromatography coupled to isotope-ratio mass spectrometry. This technique allowed the determination, in a single experiment, of the amount and ^{13}C abundance of six neutral sugars (including xylose used as internal standard). Despite the low abundance of ^{13}C in natural products, the sensitivity of the technique was found to be sufficient to detect isotopic enrichment as low as 0.001% with good accuracy and reproducibility in 2 μg of each glycoprotein neutral sugar. As an example, the pattern of labeling by dietary D-glucose from corn starch appears to be very different for fucose, ribose, mannose, galactose, and glucose of intestine or serum macromolecules.

INTRODUCTION

Radioactive labeling of complex carbohydrates is of general use in animal cells in vitro for metabolic or structural studies[1,2]. The in vivo metabolism of the glycan sugars of glycoproteins is commonly studied after injection of radioactively-labeled

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sugars to the animals. The radioactivity incorporated into glycoproteins can be monitored by autoradiography in histological studies[3], or radioactivity counting in subcellular fractions or partially purified proteins[4]. Less frequently, oral administration of radioactively-labeled sugars has been used[5]. In healthy humans, the use of radioactive labeling is limited by ethical and legal, reasons, especially for metabolic or nutritional studies without direct benefit to the patient.

The use of the stable isotope of carbon (^{13}C) represents an interesting alternative to radioactivity for the labeling of glycoproteins in metabolic studies. Until now, the use of ^{13}C in glycan biochemistry has been restricted to structural using NMR spectroscopy[6,7]. For metabolic studies, the coupling of gas–liquid chromatography to isotope-ratio mass spectrometry (GLC–IRMS) provides a powerful tool to determine, in a single step, both the amount of glycan sugars and their abundance in stable isotopes. This technique has been validated in the man for the metabolic study of soluble serum molecules, such as glucose[8] or lactic acid [9].

On the other hand, previous works from this laboratory and others (see review in ref. 10) demonstrated the existence of a nutritional regulation of glycoprotein metabolism either for glycosyltransferase activities or for glycan composition. A possible mechanism for the dietary regulation of glycosylation lies in the availability of specific sugars provided by the diet, though, theoretically, new sugars for glycoproteins can all be obtained from D-glucose. The existence of sugar recycling, of distinct subcellular sugar pools, or of a channeling of reactions involved in substrate availability may be also important physiological factors in the whole organism. This report describes the possibility of applying GLC–IRMS to follow the incorporation of ^{13}C from glucose, naturally present in the diet, into glycoproteins or macromolecules in the intestine or the serum of the rat.

EXPERIMENTAL

Animals and diets.—Sprague–Dawley rats (IFFA–CREDO, L'Arbresle, France) were used, and weighed 140–160 g at the beginning of the experiment. They were housed under controlled conditions of temperature (21°) and lighting (daily cycle of 12 h light/12 h darkness) in cages of six animals. They were fed for a week on a semisynthetic diet (Table I) containing 67% wheat starch as the sole source of D-glucose. Then they received the same diet containing 67% of corn starch. The analysis of corn and wheat starch composition (by capillary column GLC after hydrolysis) indicated that there was no contamination by other sugars. Three animals were killed by decapitation either just before the diet change (basal) or at various times after diet change (12, 40, 68, 85, and 158 h). Weight gain, food, and water intakes were regularly monitored.

Sampling.—The small intestine of each rat was removed, thoroughly washed with cold 0.9% NaCl, weighed, and opened. The mucosa was harvested by scraping and homogenized in water (5 mL/g wet tissue) with a Potter–Elvejhem homogenizer (10 strokes). The homogenate of each mucosa was immediately frozen and

TABLE I

Composition and ^{13}C natural abundance of diets and components [a]

Components and diet	Percent in diet	δ‰	Atom%
Corn starch	67	−9.84±0.04	1.10042±0.00005
Wheat starch	67	−22.12±0.01	1.08692±0.00002
Casein	20	−25.32±0.27	1.08340±0.00029
Cellulose	2	−23.17±0.10	1.08576±0.00011
Mineral mix	4	−21.24±0.31	1.08789±0.00034
Vitamins	2	−23.93±0.26	1.08492±0.00029
Methionine	0.2	−24.99±0.16	1.08376±0.00018
Oil mix	4.8	−27.35±0.11	1.08116±0.00012
Total corn starch diet		−17.52±0.64	1.09197±0.00071
Total wheat starch diet		−23.67±0.05	1.08520±0.00005
Standard commercial diet		−21.73±1.14	1.08734±0.00125

[a] Diet components (0.4 mg; ICN, U.S.A.) or total diet powder (0.4 mg; Souriffarat, IFFA-CREDO, France) were submitted to flash combustion in a Carlo Erba ANA 1500 apparatus. The generated CO_2 was analyzed in line by IRMS. Results are expressed as means±SD for three determinations.

stored at −20° until used. Aliquots (2 mL) of the homogenate were lyophilized to constant weight for the determination of mucosal dry weight.

Macromolecules in 2 mL of each homogenate were precipitated with 2 mL of 40% cold trichloroacetic acid, and then centrifuged off at 5000*g* for 10 min. The pellet was resuspended in the same acid by a brief sonication and centrifuged off under the same conditions. Then the pellet was delipidated by two successive extractions with 4 mL of chloroform–methanol (2:1, v:v) according to Folch et al.[11].

Preparation of neutral sugars for GLC–IRMS.—The final pellet was resuspended in M HCl and hydrolyzed at 100° for 2 h. After cooling, 100 μg of D-xylose was added as an internal standard. After centrifugation, the supernatant was diluted with water and purified by ion-exchange chromatography, according to Montreuil and Spik[12], on 4 mL of Dowex 50X2 (50–100 mesh) and 4 mL of Dowex 1X2 (50–100 mesh). Column effluents and washings were concentrated by lyophilization. The sample was diluted in 100 μL water and treated according to Blakeney et al.[13] and Henry et al.[14]: sugar reduction was achieved in 1 mL of Me_2SO with 2% $NaBH_4$ for 90 min at 40° and stopped with the addition of 100 μL of glacial acetic acid. Acetylation was performed with 2 mL of acetic anhydride in the presence of 200 μL of *N*-methylimidazole. After 10 min of reaction, the excess of acetic anhydride was removed by addition of 5 mL of water. The acetylated polyols were extracted with 1 mL of dichloromethane. The extract was diluted to 10 mL with dichloromethane and washed five times with 2 mL of water. The final solution was evaporated to dryness under an N_2 stream and stored at −20°C until analysis.

Standards of various sugars were acetylated under the same conditions as the samples, either individually or in a mixed solution. In order to control isotopic

composition, chemicals from the same batches were used throughout the experiment; they were from Merck (acetic anhydride and dichloromethane) or Sigma (for the other chemicals and standard sugars).

GLC–IRMS.—GLC was performed on a capillary column (SP-2380 from Supelco; 0.32 mm × 30 m) with an He flow of 2 mL/min in a HP5890 gas chromatograph (Hewlett–Packard, Evry, France). Temperature gradient was 13 min at 235°C, and then 20°C/min up to 270°C and 7 min at this temperature. At the end of the column, a heart-cut valve directed the He flow either to a flame-ionization detector or to a quartz tube containing CuO and heated at 800°C where CO_2 was generated from acetylated sugars. The ions at m/z 44, 45, and 46 were separated in an isotope-ratio mass spectrometer (SIRA 12, VG ISOGAS, Middlewich, United Kingdom). The heart-cut valve was controlled by a computer (HP9816), which also calculated and reported the surface of the peak of the main ion (m/z 44) and the ratios of 45:44 and 46:44 ions. Before and after the peaks arising from acetylated sugars, a reference CO_2 sample of known enrichment, calibrated against the international standard (PDB), was automatically injected in the spectrometer. Results were expressed as δ ^{13}C ‰, after correction for ^{17}O content[15], according to equation:

$$\delta\ ^{13}C‰ = \frac{[(^{13}C/^{12}C)\text{sample} - (^{13}C/^{12}C)\text{standard}] \times 10^3}{(^{13}C/^{12}C)\text{standard}} \quad (1)$$

Determinations were performed in duplicate on each sample.

Calculations.—The derivatization of reduced sugars by acetic anhydride induced an isotopic dilution of the natural label, which is not the same for the hexaacetyl derivatives (from mannose, galactose, and glucose) and the pentaacetyl derivatives (from fucose and ribose). Thus, in order to eliminate the isototopic dilution due to the naturally labeled carbon of acetic anhydride, the results were expressed in atom per cent excess (APE ^{13}C):

$$\text{APE } ^{13}C = \text{Atom\% sample at time } t - \text{Atom\% basal mean value} \quad (2)$$

with

$$\text{Atom\%} = [100R(0.001\ \delta‰ + 1)]/[1 + R(0.001\ \delta‰ + 1)] \quad (3)$$

where R is the $^{13}C/^{12}C$ of the international standard (PDB) ($R = 0.0112372$).

Table I gives the values of δ‰ and Atom% for the components of the diets. The values of δ‰ for standard sugars were the same for a sugar studied alone or in the standard mixture, indicating that chromatographic conditions did not induce cross-contamination between sugars which could alter their isotopic composition. The comparison of values for underivatized or derivatized standard sugars indicated that there was no isotopic fractionation or artificial shift in δ‰ values during the sample acetylation procedure. In the figures or tables, results are expressed as means ± SD. The statistical significance of the values were analyzed by one-way variance analysis. When the F test was significant, the Newman–Keuls

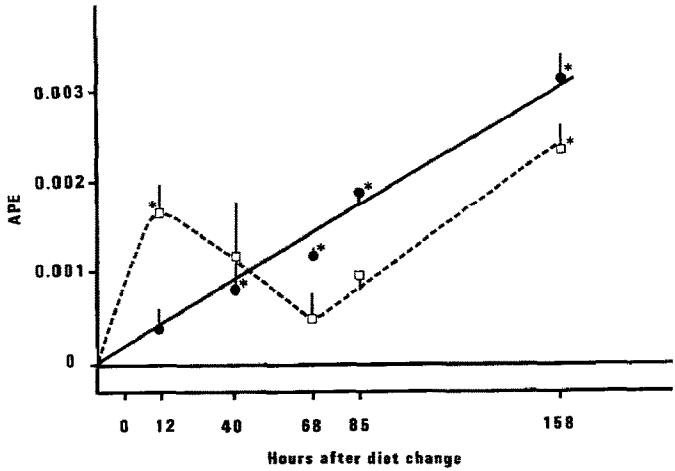

Fig. 1. ^{13}C enrichment (in atom per cent excess, APE) in glucose (□) and ribose (●) bound to delipidated trichloroactic acid–precipitable material of individual intestinal mucosae, obtained from groups of three rats nourished during one week with a corn starch diet after one week of wheat starch diet. One-way variance analysis and Newmann–Keuls test were performed on measured δ‰ (and not APE). Asterisks indicate mean values significantly different (at $p < 0.05$) from basal mean value.

test was used with $p < 0.05$ for the comparison of means between the different groups.

RESULTS

Figs. 1 and 2 show the stable-isotopic enrichments determined for five sugars bound to trichloroacetic acid–precipitable macromolecules in the intestinal mucosa. The theoretical, highest atom per cent excess was 0.0135, calculated from the difference in the atom per cent between wheat starch and corn starch (Table I). This level of enrichment was never observed for any sugar after one week of corn starch diet. The labeling of glucose (Fig. 1) displayed a significant peak at 12 h, then decreased from 40 to 85 h; it increased again to reach 19% of the maximum theoretical APE at 158 h. The labeling of ribose was approximately linear during this week (Fig. 1). The patterns for the labeling of glycoprotein sugars (Fig. 2) were different. For fucose and galactose, there was a rapid and significant increase at time 12 h to reach a plateau level between 40 and 85 h; then the label increased at 158 h to reach 27% of the maximum theoretical APE for fucose and 30% for galactose. For mannose, there was no significant enrichment until 40 h, and then the label increased slowly to reach 18% of the maximum APE at day 7 (158 h).

The enrichment of serum glycoproteins was expected to be lower than that of intestinal glycoproteins; thus, only the longer time was studied. Results are reported in Table II. The labeling was in the same order of magnitude as in the

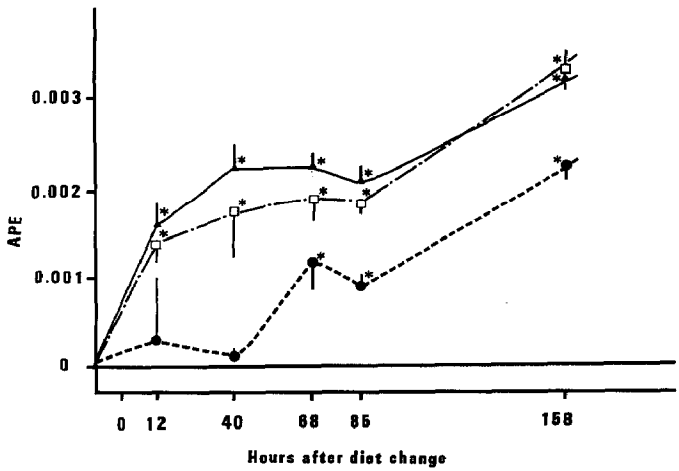

Fig. 2. ^{13}C enrichment (APE) in glycoprotein neutral sugars of rat intestinal mucosa under the same conditions as described in legend to Fig. 1: (□) Galactose, (▲) mannose, and (□) fucose.

TABLE II

^{13}C enrichment in serum and intestine glycoprotein sugars after one week of corn starch diet [a]

Sugar	Serum	Intestine
Fucose	2.04 ± 0.14	3.99 ± 0.01
Mannose	2.95 ± 0.33	2.48 ± 0.18
Galactose	3.04 ± 0.30	3.66 ± 0.23

[a] Results are given in atom per cent excess (APE $\times 10^3$) and are means of duplicate determinations in three rats.

intestine for mannose and galactose, whereas it was about one half of that of the intestine for fucose.

DISCUSSION

The use of radioactivity for metabolic experiments, without direct benefit or therapeutic purpose, must be considered as very controversial in the healthy human. Thus, it is important to test the possibility of using stable isotopes to trace glycoprotein sugar metabolism. Before applying this technique to man, the technical validation in the animal is a useful prerequisite. We have chosen to use stable isotopes naturally present in some nutrients and to study the intestinal model in the rat, because many data are available on the developmental and nutritional regulation of glycosylation in this organ[10]. We also tested the possible labeling of serum glycoproteins in the same experiment. From the results reported herein, several points can be discussed, i.e., the technical characteristics of the methodol-

ogy, the use of nutriments naturally enriched in stable isotopes, and the possible physiological significance of the results.

These demonstrated that the stable-isotope technology can be applied to the study of glycoprotein sugars. For intestinal tissue, 200–400 mg of wet tissue were found to be sufficient to give accurate results. The intestine is particularly rich in glycoproteins, owing to the presence of mucins. However, the label pattern of mannose, which is absent from mucins, showed significant variations. In serum glycoproteins, results have been obtained from 1 mL of serum, which corresponds approximately to 30–40 mg of glycoproteins. At least, 2 μg of each sugar were injected, corresponding to 75 nmol of carbon dioxide. Owing to the presence of a split at the injection port and an open split after the reactor quartz tube, the actual quantity of carbon dioxide in the source of the mass spectrometer was $\sim$2–4 nmol, which is the minimal amount to give reliable results. Obviously, better results would be obtained with larger quantities. The combined GLC–IRMS allowed to determine isotopic enrichment[8] of 0.001% with a precision of 3% for the measured δ‰. The invariance of internal standard isotopic enrichment realizes an internal control for the spectrometer performance. In the intestinal samples, xylose was preferred as an internal standard in the place of inositol, because the glucose peak was high and wide so that there was a frequent overlap between the glucose and inositol peaks. The chromatographic conditions are somewhat different from those used for GLC alone. The coupling of IRMS to GLC makes necessary that peak retention times should be separated by at least a 1-min interval.

As compared to organic MS, IRMS does not allow measurements of the isotopic enrichment on specific C atoms of the sugars; this is compensated by a greater sensitivity as coupling GLC or organic MS has a detection threshold of $\sim$0.5% enrichment, vs. 0.001% for IRMS. Organic MS after GLC[16] or capilary supercritical fluid chromatography[17] is more useful for the separation and analysis of complex glycan mixtures. Thus, the IRMS method can be applied to trace sugars of membrane-bound or soluble glycoproteins with satisfactory sensitivity, accuracy, and precision.

The level of stable isotope enrichment in some natural products (from plants known to have a C_4 cycle for CO_2 fixation) is very low (0.0135% difference between wheat starch and corn starch used in this study). But this difference is sufficient to give significant results. The results obtained for glycoproteins sugars in this study are of the same order of magnitude as those reported for the clearly significant enrichment of serum D-glucose in man after ingestion of products containing naturally enriched corn starch[18]. Thus, correct results would also be obtained from the use of more enriched sugars obtained by chemical synthesis[19]. By contrast to radioactivity, where the natural label is negligible, the control of isotopic enrichment of ingesta represents a crucial point. In the animal, this problem is easily overcome by the use of controlled, semisynthetic diets, in which starch or a specific sugar is the sole source of ^{13}C excess (Table I). In man, this

might represent a problem for long term studies which requires careful attention.

The physiological significance of the results reported herein is now difficult to assess, since the experiment was essentially designed to test the practicality of the method. However, some suggestions can be pointed out. The use of naturally labeled compounds allows a continuous labeling of the diet. It is noteworthy that the maximum enrichment level is not reached after a week for any sugar. Thus, the duration of the wheat starch diet was too short to obtain a complete equilibration at the minimum level of ^{13}C before changing to corn starch diet. However, the standard diet, with which rats were fed before the beginning of the experiment, had a low abundance of natural ^{13}C (Table I). In cow fed an exclusive corn diet, the maximum enrichment in milk lactose was not obtained before 6 weeks[20]. Moreover, it must be kept in mind that epithelial cells of intestinal mucosa are continuously renewed within a period of 2–3 days for the rodent. During the experimental period, up to 80% of the mucosa had been completely changed. Therefore, the incomplete labeling of glycoprotein sugars after one week suggests that dietary glucose is not the sole source for glycan sugars in the intestine.

D-Glucose is rapidly incorporated into macromolecular structures, likely glycogen. Surprisingly, glucose labeling did not appear to be more rapid or more important than for other sugars. This observation might also suggest that glycogen was not directly derived from dietary D-glucose. Moreover, the essential fuel for the intestine is not glucose, but glutamine[21]. By contrast, the labeling of ribose appeared to be linear for 7 days, indicating a continuous renewal of RNA, the ribose unit of which could be derived from nutritional glucose through the pentose cycle.

Similarly, despite a continuous flow of labeled glucose and a rapid renewal of intestinal mucosa, the labeling of glycoprotein sugars appeared to be delayed and very low for mannose. For fucose and galactose, after a rapid increase, a plateau level was reached and maintained for at least 45 h. This result suggested that some metabolic controls are involved either by glycoprotein sugar recycling or by sugar availability from other sources than dietary glucose. From the work of Reutter and assoc.[22,23], recycling of glycoprotein sugars occurs independently from metabolism of the protein component and the turnover of fucose is more rapid than that of mannose.

For fucose and galactose, it must be pointed out that the enrichment is significant at time 12 h, after a single feeding period with corn starch. Thus, nutritional pulse-chase experiments may be conceivable. The sensitivity of the technique also offers the possibility to test the effect of specific dietary sugars, either poorly labeled in ^{13}C and incorporated in a corn starch diet or specifically enriched in ^{13}C and incorporated in a wheat starch diet.

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