A $^{13}CO_2$ breath test

for

liver glycogen oxidation

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A $^{13}CO_2$ BREATH TEST FOR LIVER GLYCOGEN OXYDATION

EEN $^{13}CO_2$ ADEMTEST TER BEPALING VAN LEVERGLYCOGEEN OXIDATIE

PROEFSCHRIFT

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Prof. dr. J.M.J. Lamers

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Our little systems have their day
They have their day and cease to be
They are but broken lights of Thee
And Thou o Lord art more than they

Alfred Tennyson (1809-1883)

Contents

Chapter 1

1.1 The storage of nutrients

Eating is an episodic phenomenon in man. The majority of the human beings consumes two or three meals during the day and goes without food for a period of eight to twelve hours during the night.
In contrast to the intermittent intake, the utilisation of food by the body is relatively constant for most of the body organs. In some organs, like skeletal and cardiac muscle, the energy expenditure can fluctuate substantially, but such fluctuations are usually independent of the time of eating. This entails the need to store energy and nutrients to be utilised during the fasting periods.

In other words the capacity of the human body to store energy and nutrients makes it possible to eat periodically.

Of the three major foodstuffs – fat, carbohydrate and protein – only the first two have an identified storage place. Fat is stored in adipose tissue and carbohydrates are stored as glycogen in liver and skeletal muscle. The amino acids derived from protein intake are “stored” as proteins in the body cells.
An excess of carbohydrate or protein will be converted to triglyceride and stored as such.

Under normal circumstances the healthy human body is able to handle a wide variety of foodstuffs in which the proportion of fat, protein and carbohydrate may vary substantially.
For instance a diet based almost entirely on unpolished rice contains about 85% of the total energy intake as carbohydrate, 10% as protein, and 5% as fat; while a meat diet contains less than 5% as carbohydrate, about 40 % as protein and 55% as fat. Both these diets are eaten habitually in some parts of the globe and both can be adequate. [1]
However the ability of man to thrive under different dietary circumstances does not mean that the use of the different nutrients is equally efficient.
If there is a imbalance between the intake and the consumption of protein, fat and carbohydrate, the body is often able to compensate by converting for instance protein and glycerol into carbohydrate, (gluconeogenesis). However these converting processes are inefficient and limited. [1,2]

1.2 Impaired liver glycogen storage in cirrhosis?

In 1984 and 1989 Swart et al. postulated that an impaired glycogen storage capacity in the liver could cause an early shortage of liver glycogen and as a result an enhanced gluconeogenesis in patients with cirrhosis. This concept was based on the observation that the nitrogen balance in patients with cirrhosis of the liver improved when meal frequency increased. In these experiments the total amount of dietary input and the composition of the diet did not change. They postulated that when there is a long period between two meals, the body usually draws on the glycogen stored in the liver for its carbohydrate supply. If there is a shortage of liver glycogen, glucose will be formed through gluconeogenesis. The main substrate of gluconeogenesis is protein. An increased gluconeogenesis entails increased proteolysis and thus a more negative nitrogen balance during fasting. Frequent

periods of proteolysis could result in muscle wasting. Muscle wasting is often observed in patients with cirrhosis. An increased meal frequency provides a more even dietary carbohydrate supply from the gut. This reduces the need for glycogenolysis from liver glycogen and if there is a shortage of liver glycogen, less demand for gluconeogenesis. A reduced gluconeogenesis will in turn results in less protein utilisation. Indeed the nitrogen balance was found to be less negative in cirrhotics while on an increased meal frequency. [3-5]
These findings were confirmed by an experiment that showed that nocturnal oral glucose supplementation improved nitrogen balance in patients with cirrhosis, while it had no effect in healthy volunteers. [6]

Following this concept, guidelines were proposed to prevent muscle wasting in patients with cirrhosis. Food intake should be spread over the day and a late evening meal is advised. The protein intake of patients with cirrhosis should be about 1 gram per kilogram bodyweight per day. [4,5,7,8]

In a recent study Peterson et al provided confirmatory evidence for the above hypothesis. They combined ^{13}C nuclear magnetic resonance spectroscopy (NMR) and stable isotope [6,6-$^{2}H_2$]glucose infusion techniques to determine liver glycogen content, liver glycogen depletion and glucose production over a period of five to thirteen hours postprandially. In cirrhosis the postprandial hepatic glycogen concentrations were 34% lower than those in healthy controls. Gluconeogenesis as percentage of glucose production was markedly increased in cirrhosis, 87% vs 60% in controls. [9]

1.3 The aim of the study

In order to obtain a better insight into the postulated early glycogen depletion and increased gluconeogenesis, we wanted to study glycogen depletion of the liver in more detail during the early postprandial phase. A better understanding of the pathophysiology should provide a basis for a more individual advice regarding diet and nutritional intervention. Development of an easily applicable bedside test could help to select patients who would benefit from dietary treatment. This in turn could reduce morbidity by preventing muscle wasting and possibly by improving the immune status of patients with cirrhosis of the liver.

However, no easily accessible method to study liver glycogen depletion was available. In the past, glycogen concentrations have been measured in serial liver biopsies. [10-12] More recently, various catheterisation techniques, indirect calorimetry and nuclear magnetic resonance (NMR) techniques, sometimes combined with isotopic tracers, have been used to study liver glycogen, hepatic glucose output and substrate oxidation. [13-24]

The research described in this thesis was aimed at exploring the liver glycogen depletion by a new method based on a selective labelling of the liver glycogen pool by a natural label, the ^{13}C-isotope. [25,26] The intention was to develop a reliable, reproducible and relatively, easily applicable non-invasive test.

The theoretical background and the design of the method are presented in chapter 2.
Various aspects of the concept on which the model is based were tested and are described in chapter 3. They include studies on the distribution of the ^{13}C isotope in exhales CO_2 of a control population and the short and long term reproducibility.
Chapter 4 deals with different aspects of the model. The course of ^{13}C enrichment in breath CO_2 after a ^{13}C-enriched diet was studied. The influence of a higher ^{13}C-enrichment of the muscle glycogen

was measured and analysed. The test was carried out inversely in a population used to a ^{13}C-enriched diet. The model was tested on its reproducibility and a 1-day labelling period was compared with a 3-day period of labelling with a naturally ^{13}C-enriched diet. Results of initial tests of the model used in patients with cirrhosis are presented in chapter 5. Chapter 6 ends this thesis with a general discussion and conclusions.

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Chapter 2

The model

2.1 glycogen metabolism
2.2 the use of ^{13}C in metabolic studies
2.3 the design of the experimental model

2.1 Glycogen metabolism

As more than 99 % of carbohydrate absorbed by the human body is used for energy production, a considerable part of the absorbed carbohydrate needs to be stored in the form of glycogen for later use. The formation of the polysaccharide glycogen occurs in small amounts in practically all body tissues. However substantial glycogen stores are only found in liver and muscle tissue.
A normal human adult with a body weight of 70 kg will have about 72 gr. of glucose stored as glycogen in the liver (4% of a liver of 1800 gram), 245 gr. of glucose in skeletal muscle (0.7% of 35 kg muscle mass) and 10 gr. of extracellular glucose (0.1% of 10 litre). The human liver may contain up to 6% of its wet weight as glycogen when analysed shortly after a carbohydrate rich meal. Muscle tissue contains rarely more than 1% of its wet weight as glycogen. The function of the muscle glycogen is to be an energy source for the muscle itself. In contrast, the liver glycogen pool is used to maintain an adequate supply of glucose to different organs during the postabsorptive period, mainly during the night. [1-3]

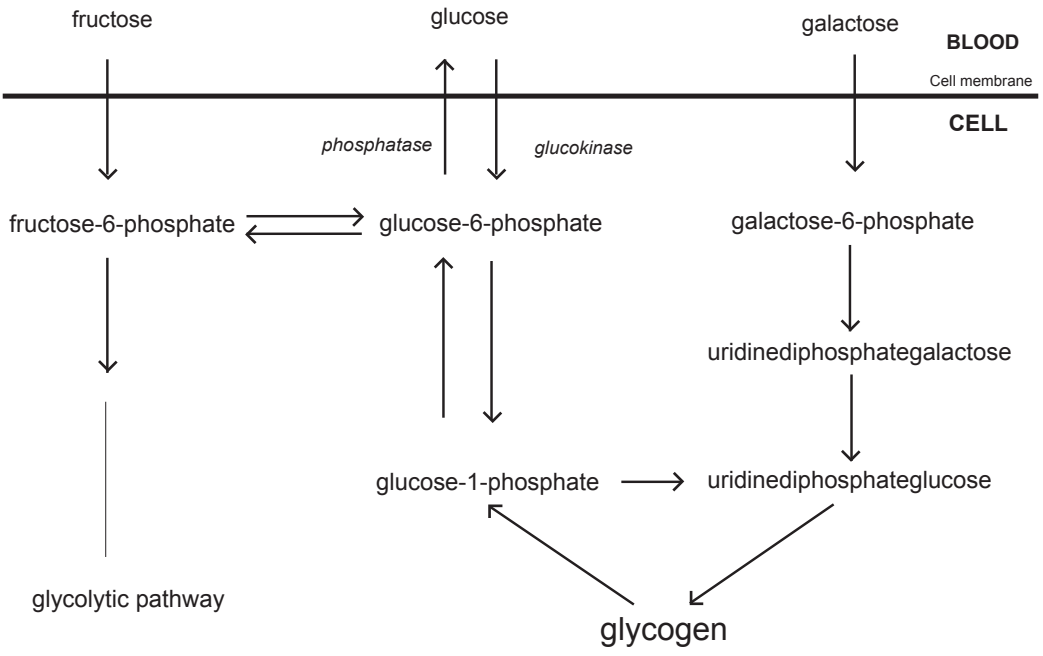

Figure. 2.1 the interconcersion of the three major monosaccharides, the glycogenesis and glycogenolysis in the liver cell

Transcellular membrane monosaccharide transport

The final products of carbohydrate digestion within the alimentary tract are almost entirely the three monosaccharides glucose, fructose and galactose. Of these three, glucose represents about 80 percent under most conditions.
During the process of absorption and transport through the epithelial cells into the portal blood most of the fructose is also converted to glucose. The remaining fructose is then, along with galactose, converted into glucose by the liver. And so the final destination of almost all digested carbohydrate is glucose, as shown in figure 2:1.

In the epithelial cells of the alimentary tract the monosaccharides are taken up by active sodium co-transport.
The same process occurs in glucose transport through renal tubular cells.
For all other cell membranes the monosaccharide trans-membrane transport is by a process of a facilitated diffusion. Monosaccharides are only transported from higher concentration toward lower concentration by the special binding properties of the glucose carrier.
The rate of the trans-membrane transport of monosaccharides is greatly increased by the presence of insulin. The only exception to this is the liver, which is able to take up glucose more easily than other tissues. [4,5]

Upon entering the cell glucose is transformed to glucose-6-phosphate. This reaction is facilitated by the enzyme glucokinase.
In the liver cell galactose and fructose are transformed to galactose-6-phosphate and fructose-6-phosphate. See fig 2:1.
This phosphorylation of monosaccharides is almost completely irreversible. This means that once a monosaccharide-6-phosphate molecule is formed the monosaccharide molecule is “trapped” in the cell to be used as fuel within that cell.
Three exceptions to this rule are the liver cells, the epithelial cells of the renal tubules and the intestinal cells. These cells contain the enzyme glucose phosphatase, which makes it possible to reverse the process and to make glucose available to recross the cell membrane. [1,2]

Glycogenesis

The formation of a glycogen molecule begins with the formation of a primer molecule, glycogenin.
Glycogenin undergoes a self-glycosylation to generate an oligosaccharide primer. The enzyme glycogen synthetase then enables the reaction to elongate the polysaccharide chain, leading ultimately to the formation of glycogen. [6,7] The glycosylation of glycogenin is probably determined by the balance between the self-glycosylation of glycogenin and the opposing action of the enzyme glycogen phosphorylase. [8]
An intermediate in the glycogen pathway is proglycogen. Proglycogen is the low molecular mass form of glycogen that serves as a stable intermediate in the pathways to and from depot glycogen.
It has been suggested that glycogen oscillates, according to glucose supply and demand, between the macroglycogen and the proglycogen, but usually not the glycogenin, forms. The glycogenin mass is 37kDa, the proglycogen mass is approximately 400 kDa and the (macro)glycogen has a mass of about 10^7 kDa The proportion of proglycogen to total glycogen varies in different organs from 3% in liver, 15% in skeletal muscle to 50% in heart tissue. [9]
The actual amount of glycogen stored in the liver and muscle cells depends on the amount of glycogenin, the type of cell and the previous demand. Less glycogenin means less glycogen formation, at least in skeletal muscle. [10]

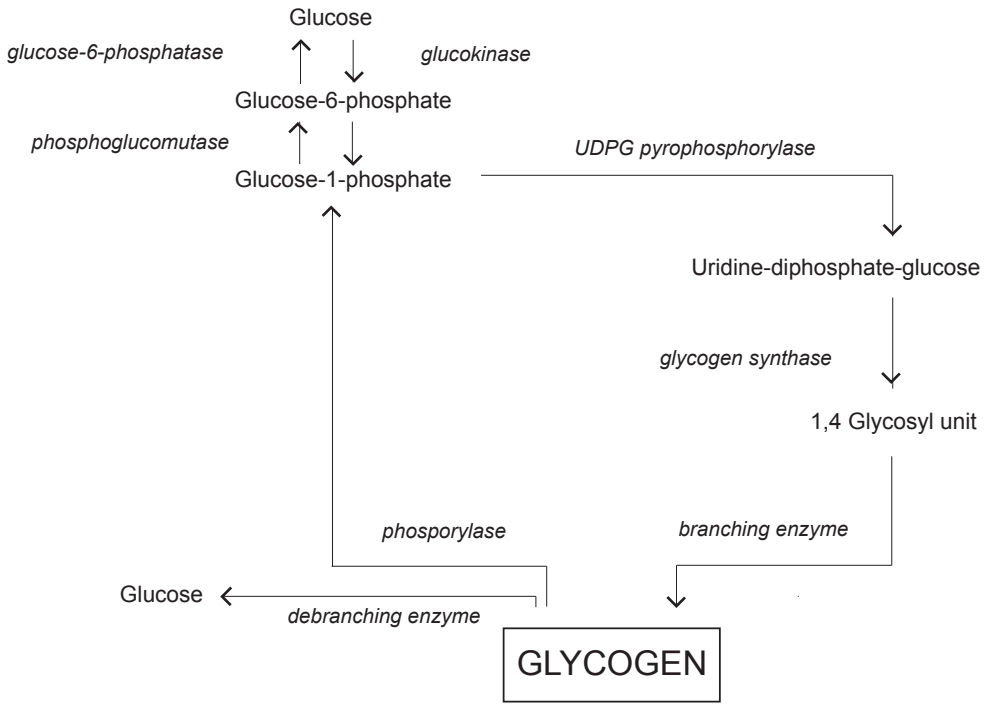

Figure 2.2 Pathway of glycogenesis and glycogenolysis in the liver.

The liver cell needs less glycogenin to form the same amount of glycogen compared with muscle glycogen. This can be explained by the formation of larger glycogen molecules or by the presence of still unknown glycogenin-like proteins. The latter could be the case since recently a second human gene, CYG2, encoding a liver specific isoform of glycogenin, glycogenin-2 was reported. [11,12]
It has been reported that after rigorous exercise more glycogen is formed in skeletal muscle than without such an exercise. This process is dependent on the production of primer proteins. [10]
In spite of extensive research the exact mechanisms controlling glycogen biogenesis are still poorly understood. [10] There is evidence that glycogenin plays a role as regulator and initiator. Other factors influencing the glycogenesis are insulin, glucagon, glucose, lactate, adrenaline [13], islet amyloid polypeptide (or amylin) [14], lipids [15,16], cortisol [17] and glutamine. [18]

The glycogenesis pathway is illustrated in figure 2:2.
It is obvious that any compound that can be converted into glucose or glucose-6-phosphate can enter into these reactions. Examples are the monosaccharides galactose and fructose, but also lactic acid, glycerol, pyruvate and some de-aminated amino acids.

The enzyme glycogen synthetase exists in an inactive form (b form) and an active form (a form).
Cyclic AMP and compounds stimulating the formation of cyclic AMP, like adrenaline, insulin and thyroid hormone initiate the transformation of the enzyme from its inactive to its active form. Through this pathway glycogen formation is stimulated.

Glycogen formation takes place by the process of linking the C1 of the glucose moiety of the uridine

diphosphate glucose (UDPG) to the C4 of a terminal glucose of the glycogen molecule, through the action of glycogen synthetase (or glycosyltransferase).
After the addition of between 6 and 11 glucose residues, the branching enzyme amylo-1,4à 1,6-transglucosidase acts on the molecule. Through this process a large branched glycogen molecule can be created which is, due to its open structure, very accessible for quick and efficient mobilisation of glucose when required. “Mobilising” enzymes have a good access even within the molecule. Figure 2:4.

During glycogen synthesis large granules are formed in the cytoplasm. Such granules have a diameter of 100-400 Å and consist of a number of polysaccharide molecules, B-particles that are covalently linked to form larger A-particles.

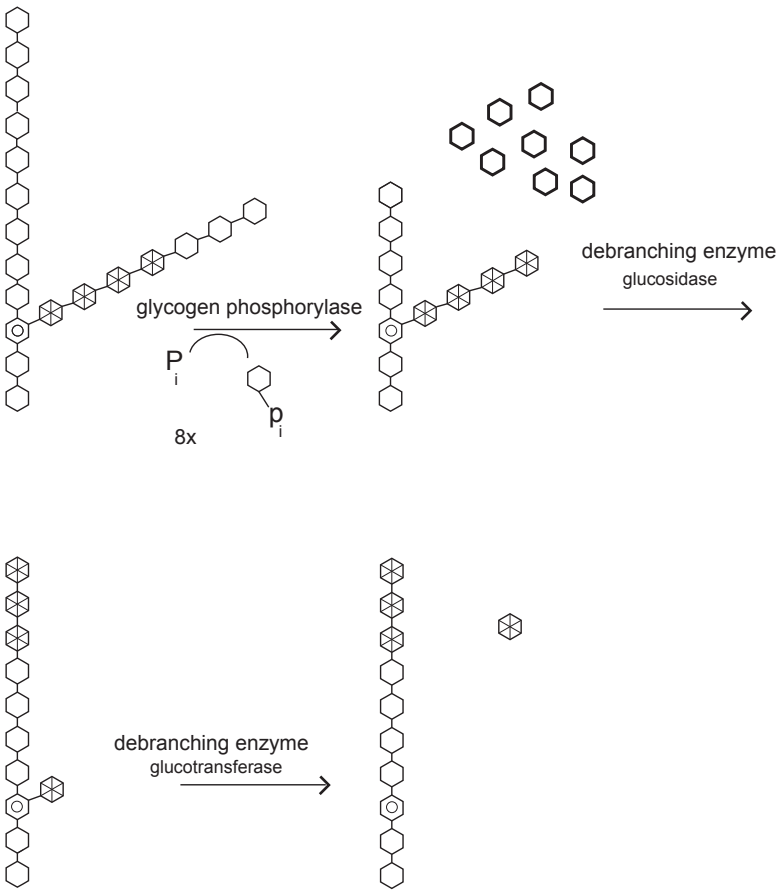

Figure 2.3 The debranching of the glycogen molecule. Through the glycogen phosphorylase glucose-1-phosphate is formed. However glycogen phosphorylase is unable to remove the remaining 4 glucose residues. Debranching enzyme glucotransferase moves three of those residues to a free C-4 end of a second branch. The last residue is than removed by the debranching enzyme glucosidase.

Proteins are also present in the granules, as well as enzymes participating in the synthesis and breakdown of polysaccharides.
The branched structure of the glycogen molecule is very well suited for its metabolic function. This structure makes it possible to store a large amount of glucose in a small volume [hardly affecting the osmotic pressure in the cell] and at the same time allows a very rapid release of substantial amounts of glucose when needed.

Glycogenolysis

The process of mobilising the glucose molecule from glycogen, glycogenolysis, is not simply the reversal of the glycogenesis pathway. Glycogenesis and glycogenolysis are catalysed by a different set of enzymes.[19-21] This makes it possible to facilitate both glycogenesis and glycogenolysis at the same time. Such a construction enables a quick response of both formation and utilisation of glycogen to a changing demand.

The glycogenolysis pathway is illustrated in figure 2:2, with the debranching process shown in figure 2:3.

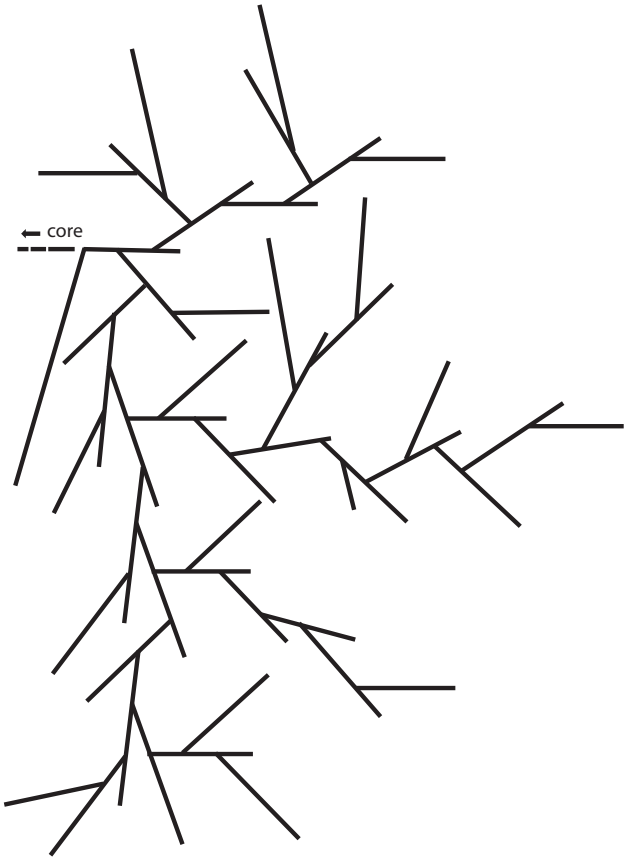

Figure 2.4 A cartoon of the branched structure of the glycogen molecule.

Gluconeogenesis

Gluconeogenesis is the process through which the body is able to convert noncarbohydrate precursors into glucose. The glucose generated through this pathway can be used for oxidation or to restore the depleted glycogen stores. The principle organs in the human body responsible for gluconeogenesis are the liver and the kidney. These two organs are equipped with a set of enzymes to facilitate the process of gluconeogenesis.
The compounds used for this process fall into two categories.
In the first category there is a conversion of compounds to glucose without significant recycling. Examples of these compounds are some amino acids and propionate.
In the second category there are the products of the partial metabolism of glucose in certain tissues that are resynthesized to glucose after they have been conveyed to the liver and kidney. Examples of these compounds are the lactic acid cycle (Cori cycle), the glucose – alanine cycle (the required energy is derived from fatty acid oxidation) and the cycle in which glucose is transported from the liver and kidney to adipose tissue and glycerol is returned to be synthesized into glucose by the liver and kidney.
In healthy subjects gluconeogenesis accounts for 50% to 60% of the glucose production after an overnight fast. [22-24]

Cycling of glucose carbon atoms

Simultaneous synthesis and breakdown of a substance is called cycling. Such cycling is possible when there are 2 separate metabolic pathways for synthesis and breakdown or when the same pathway is used in the opposite direction at a different location.
The central molecule in the cycling of glucose and glycogen is glucose-6-phosphate. Re-cycling in the context of glycogen-metabolism means the recycling of carbon atoms from glycogen or glucose via other molecules back to glucose and glycogen. Because of the uncertainty about the extent of (re)cycling and the complexity of the processes involved, cycling has been a matter of concern and of debate over the past years. Landau discussed this in a recent paper. He mentioned that even on the point of the definition of cycling there is no unity. The different methods used to study the glycogen metabolism are based on different assumptions. This does not make it easy to compare the results from different studies. [25]
Lactate and alanine are the predominant substrates by which glucose carbons can recycle back to the liver for production of new glucose. [26]
Combining the results of recent studies it can be concluded that cycling is undetectable in healthy humans after an overnight fast. However such cycling may be of importance in diabetes and the response to severe stress. Certain hyperinsulinemic and hyperglucagonemic conditions could play a role. It is therefore possible that the hyperglucagonemic conditions in cirrhotic subjects promote cycling. [23,25-27]

Last in – first out?

When (liver) glycogen metabolism is studied with the aid of tracers or labelled substrate, the question arises if the glycosyl units of the glycogen molecule that have been most recently synthesized are the first to be removed. There is some experimental evidence supporting the hypothesis that the last incorporated glycosyl units are the first to be removed. When radioactive galactose was infused in rats and glycogen was isolated from the rat liver and degraded, the radioactive units were liberated first. [28]

In another experiment radioactive galactose was infused into dogs. Following administration of glucagon, the labelled glucose appeared first in the circulation. [29]
On the other hand, there is evidence that the last-deposited-first-removed hypothesis is at least far from absolute. Magnusson labelled liver glycogen with [1-^{13}C]glycosyl units. After the labelling he infused a substantial amount of unlabelled glucose. Despite the administration of large amounts of unlabelled glucose after the ^{13}C-glucose, the ^{13}C-label disappeared from the liver at an early stage. [30]
Incorporation of label at different sites of the glycogen granules could be an explanation for the phenomenon of the last-deposited not being first removed. Synthesis and breakdown in different areas of the liver lobules could also be an explanation. Thus, the order of release of glycosyl units of glycogen in relationship to the time of their deposition remains uncertain. In most studies, using isotopes, NMR or both, it is assumed that the last-in-first-out hypothesis does not hold. [25]

2.2 The use of the ^{13}C isotope in metabolic studies

^{13}C is one of the eight known carbon isotopes. ^{12}C and ^{13}C are stable isotopes, while the other six are radioactive. The half-life time of the radio-active isotopes is short with the exception of the ^{14}C-isotope, which has a half-life decay of over 5700 years. (See Table 2:1)

Table 2:1 – Carbon isotopes

Type	^{9}C	^{10}C	^{11}C	^{12}C	^{13}C	^{14}C	^{15}C	^{16}C
Half-life	127 (ms)	19.5 (s)	20.3 (min)	stable	stable	5730 (years)	2.45 (s)	0.2 (s)
Natural abundance (%)				±98.9	±1.1	10^{-10}		

ms = milliseconds; s = seconds; min = minutes

Rudolf Schönheimer (1898-1941) introduced the concept of using stable isotopes for labelling organic compounds in order to assess their metabolic fate. [31]
Only fairly recently, when the potential biological hazards and the special problems of the long half-life time of the ^{14}C isotope came into general perception, did the use of the stable ^{13}C isotope become more popular for metabolic studies. The main reason for this delay was the technical difficulty and complex methodology for measuring the stable isotopes as compared with the ^{14}C technology. [31-33]
After oxidation carbon atoms end up in the breath as CO_2. Therefore *C-enrichment of breath CO_2 can be used in metabolic studies. [32-38]

In addition to the ^{14}C-isotope being radioactive and the ^{13}C-isotope being stable, there is another major difference between the two. ^{13}C is present in relatively high concentrations the human body (approximately 1,08%) while ^{14}C forms less than 0.0001% of the total body carbon.
This, for instance, makes the use of the ^{13}C-glycocholic not applicable as a diagnostic breath test.

The amount of labelled substrate required to induce a measurable increase of the $^{13}CO_2/^{12}CO_2$ ratio in breath is in the range of 800 mg. This amount is no longer a tracer dose (as with the ^{14}C-labelled substrate) but a pharmacological dose causing diarrhoea.[31] Because of this difference in isotopic concentration not all the results produced by ^{14}C-studies can automatically be transposed to ^{13}C-studies.

$^{13}CO_2$ tests are based on the fact that the oxidation of the administered labelled substrate can be measured by an increase in $^{13}CO_2/^{12}CO_2$ ratio in breath. A fundamental prerequisite for this is that the excretion of the labelled substrate, which is reflected by the ratio of breath $^{13}CO_2/^{12}CO_2$ over time, can be used to assess a defined rate-limiting step in the overall metabolic process.
The specificity of the $^{13}CO_2$ test is thus defined by the respective substrates and by the test conditions. [31,32]

A third difference between the ^{14}C and ^{13}C isotopes is that the ^{13}C abundance is not completely steady in biologic materials. This variation is caused by so-called isotope fractionation as described by Hatch and Slack. There are two pathways in which carbohydrates are synthesised through photosynthesis, the C3 and the C4 pathway. Both pathways lead to the production of carbohydrates, but the carbohydrate produced has a slight difference in ^{13}C abundance. Plants, that use the C3 pathway (Calvin-Benson cycle), produce carbohydrate with a relative low ^{13}C-abundance of 1.082%. Plants, using the C4 pathway (Hatch-Slack pathway), are relatively ^{13}C enriched, 1.096%. [39]
This small but consistent difference can be exploited in breath tests using naturally ^{13}C-enriched carbohydrate derived from C4 plants.
Most plants used for human consumption use the C3 photosynthesis pathway. Examples of C4 plants are corn, sugar cane, millet and pine-apple. [33]

Table 2:2 - Conditions for a simple breath test

1. There should be a steady baseline label production
2. Of the pathway that is studied one process is the rate limiting step
3. The amount of label should be large enough to produce a significant signal but should be small enough not to interfere with the studied (patho)-physiological process.
4. The CO_2 production should be steady or should be measured.
5. There should be no cycling of the label or loss of label by other pathways

2.3 The design of the experimental model

We came to the following model:

1. A person used to a C3-plant diet has a low ^{13}C-abundance of all tissues.
2. If such a person consumes (naturally) ^{13}C-enriched carbohydrate, the glycogen pools will become ^{13}C-enriched. The fat and protein pool will stay at basal ^{13}C-abundance, un less the label is consumed for a long period. Since during a sedentary fasting period the muscle glycogen is hardly used, the ^{13}C-enriched liver glycogen pool is the sole source

of ^{13}C-enriched glucose during those conditions. The oxidation of such glycogen would increase the $^{13}C/^{12}C$ ratio in breath CO_2.
3. If the CO_2 production is known it will be possible to calculate the amount of liver glyco gen that is oxidized.

The model is not a classic tracer study as such. Liver glycogen is both label and substrate. This has an advantage of no interference due to administration of a large amount of labelled tracer during the test. [26]

The following assumptions were made in developing the model:

1. The CO_2 production would be constant during the observation period. This assumption was also tested.
2. A one-compartment model for the CO_2 pool, which is usually assumed in metabolic studies using breath CO_2 enrichment, would be applicable in this investigation.
3. Cycling of labelled carbohydrate plays a negligible role during the observation period.
4. Part of the C-atoms can be “lost” to other pathways. The model provides no tools to indicate the proportion of such a loss. Since the label is measured at the end of the oxidation pathway, only the C-atoms that reached this endpoint are accounted for. This limits the results to that part of the liver glycogen, which is fully oxidized. Following Gay we applied a correction factor of 0.8 [40] Re-cycling of enriched C-atoms will not be meas ured by this method, since such atoms do not reach the breath as CO_2.
5. The substrates for gluconeogenesis can be very diverse, but all those substrates will be enriched with ^{13}C at the basal level. Oxidation of such newly formed glucose and glyco gen will not be detected by our model. The only exception will be lactate derived from muscle glycogen, which could have a higher ^{13}C-enrichment. Since the study is carried out under sedentary conditions the lactate production will be very low. Zillekens found lactate levels of 1.0 mmol/l after an overnight fast in both healthy subjects and patients with cirrhosis of the liver. [41]
6. The ^{13}C-enriched carbohydrate is evenly distributed throughout the liver glycogen granules.
7. Muscle glycogen is hardly utilised during a sedentary fast. In combination with the fact that the non-enriched muscle glycogen pool is large the contribution of oxidised ^{13}C-en riched muscle glycogen is likely to be negligible. This assumption was tested.
8. The intestinal absorption of carbohydrate is complete at four to six hours after the meal.
9. In the general population the $^{13}C/^{12}C$ ratio in exhaled CO_2 is relatively constant and within anarrow range. This assumption was tested.

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Chapter 3

Orientation studies

3.1 The distribution of basel ^{13}C-enrichment in breath CO_2

In order to use the ^{13}C-enrichment in breath CO_2 for comparative studies the basal ^{13}C-enrichment of the studied population should preferably follow a Normal distribution. [1]
We studied the basal ^{13}C-abundance in breath CO_2 of 244 persons attending our laboratory to undergo a Helicobacter pylori breath test. We considered this group to represent the healthy Dutch population, like also our study population. Their particulars are presented in Table 3.1

TABLE 3.1 - Particulars of 244 consecutive persons attending the laboratory for a ^{13}C Helicobacter pylori breath test.

Age in yr.	Number	Male	Female
00-09	2	1	1
10-19	5	4	1
20-29	23	10	13
30-39	29	17	12
40-49	59	29	30
50-59	58	24	34
60-69	37	16	21
70-79	26	13	13
80-89	4	1	3
90-99	1	1	0
Total	244	115	129

Breath samples were collected at the start of the breath test, after an overnight fast.
The mean value of the ^{13}C-abundance in breath CO_2 was 1.0828 At% and the standard deviation 0.00082.
The data follow a normal distribution as is shown in figure 3.1.
There were only 6 (2.4%) outlying data. The ethnic origin of the attendants could not be traced. Taking into account the multi-racial population of Rotterdam, which might mean different life-styles and different eating habits, there is a surprisingly small standard deviation. Others reported a ^{13}C abundance of 1.0827 on the European continent. This in contrast to corn (^{13}C-enriched) eating Americans, whom had a basal ^{13}C abundance of 1.0882 At%. [2]

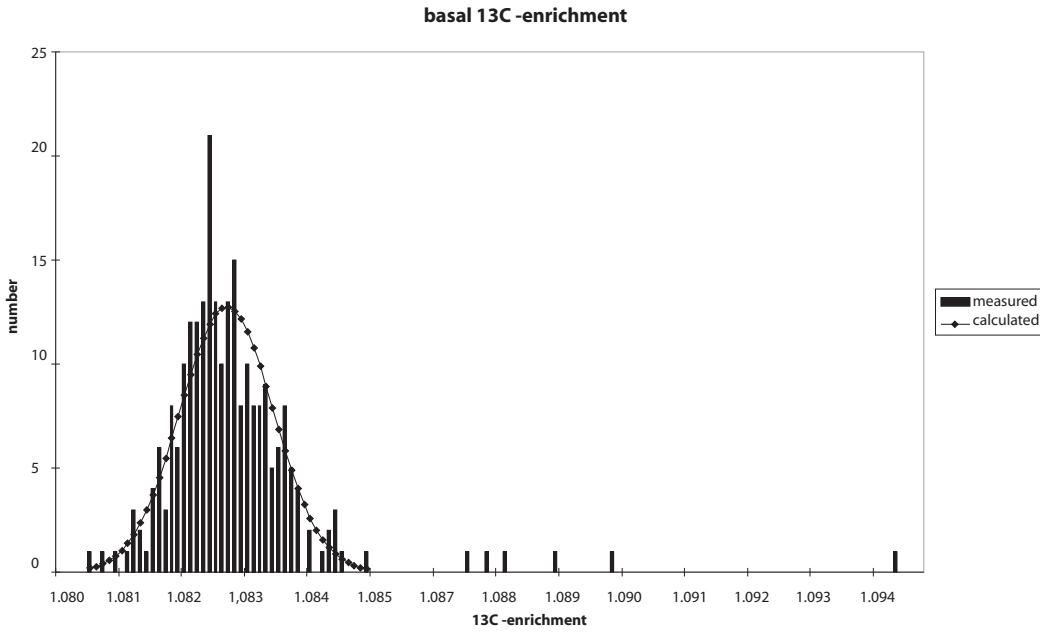

Figure 3.1 The distribution of the $^{13}CO_2$ abundance in 244 persons attending the laboratory for a helicobacter breath test together with the calculated normal distribution.

3.2 Intra individual variation in basal ^{13}C-abundance

Of the 244 persons attending for the ^{13}C-urea breath test, 20 repeated the test after some time. The mean interval between the two measurements was 102 days, SD 34.
No significant difference was observed between the two basal values, as shown in figure 3.2.1 and 3.2.2. The mean difference in ^{13}C-abundance was –0.00026 At% with a standard deviation of the differences of 0.00089. This gives a 95% limits of agreement of 0.00152 and – 0.00204. As shown in figure 3.2.2 there was no trend in deviation over time. Very often Helicobacter pylori infection is initially diagnosed by means of an endoscopic procedure. The 13C-urease breath test is mainly used to check the effect of Helicobacter eradication therapy. This explains the large proportion of single measurements in the study group.

3.3 Day to day variation of basel 13C-abundance

The day to day variation of ^{13}C abundance in breath CO_2 was studied in ten healthy volunteers, 6 female and 4 male. All participants collected two breath samples before breakfast during five consecutive days. There were no dietary restrictions during those days. The averages of the 2 daily measurements were calculated and the difference between the two measurements was plotted against

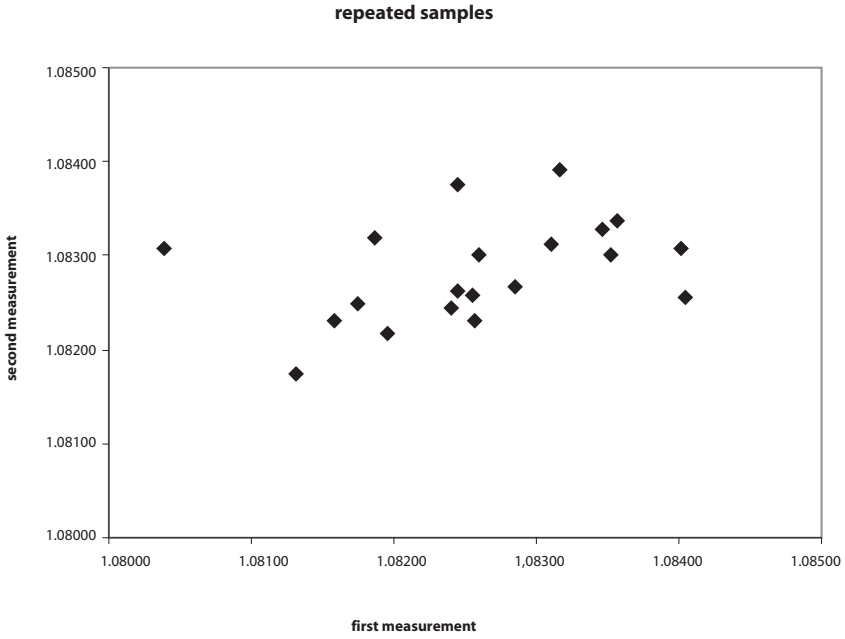

Figure 3.2.1 The basal $^{13}CO_2$ enrichment in two breath samples taken at two different days. The interval between the two samples varied between 50 to 180 days.

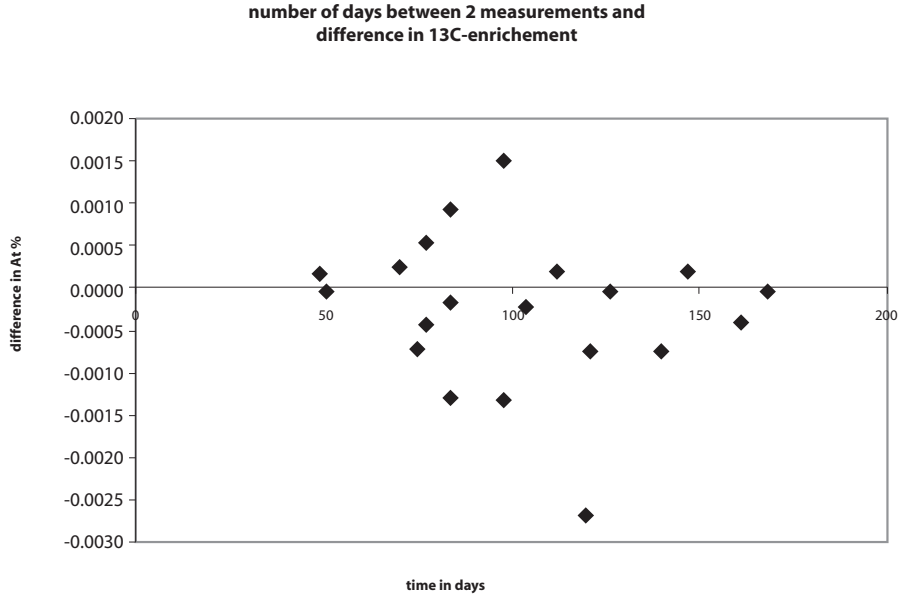

Figure 3.2.2 The difference in $^{13}CO_2$ abundance between the first and the second sample in relation with the time between the two measurements.

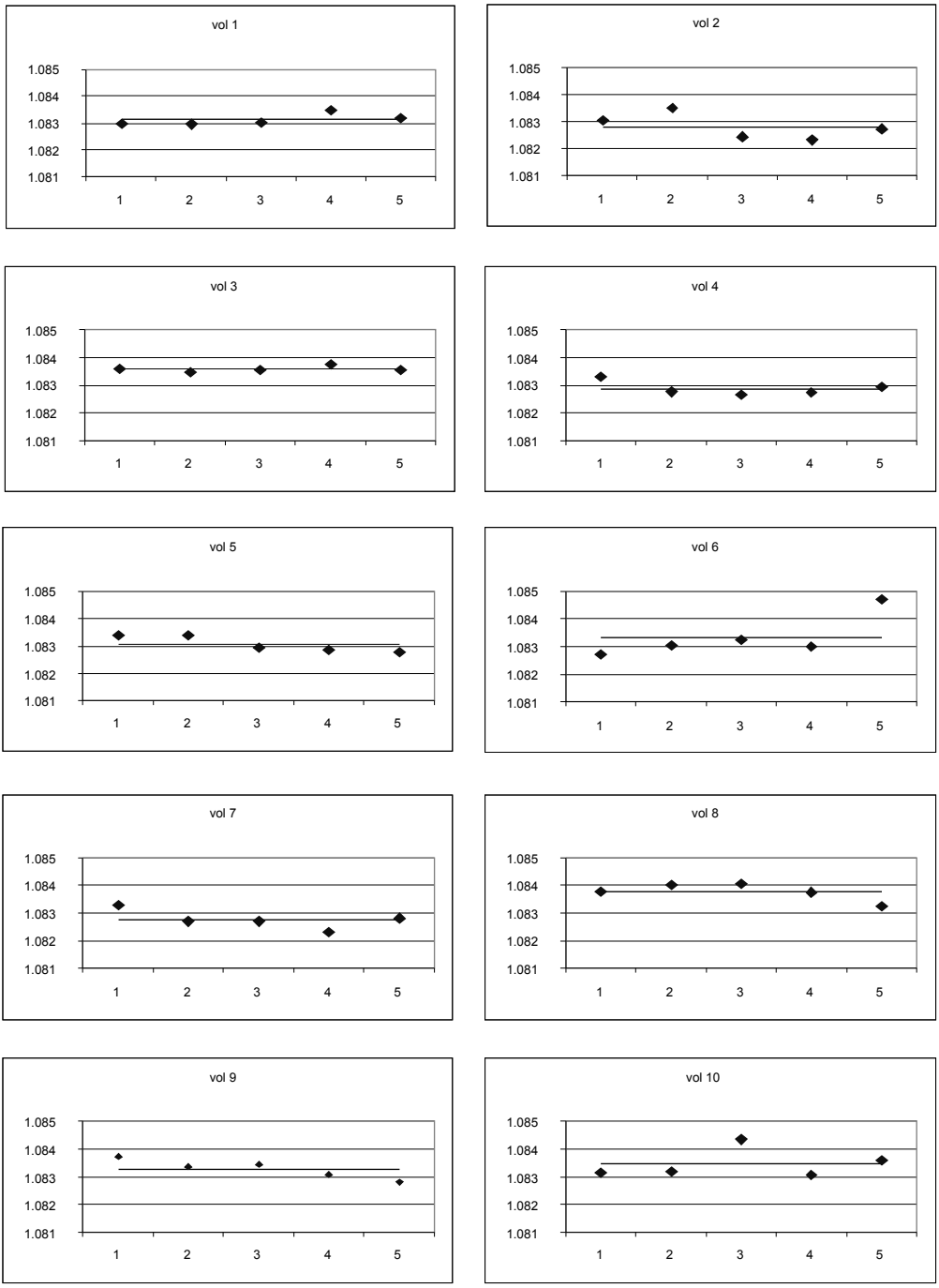

Fig. 3.3.1 The daily variation in ^{13}C abundance of exhaled CO_2.

the average. The standard deviation of the differences was 0.00035. Since the standard deviation of duplicate analyses of one breath sample is 0.0004 [personal communication JLD Wattimena] a standard deviation of the differences of 0.00035 of 2 samples taken the same morning before breakfast is acceptable.

The daily variation of the ^{13}C abundance was also analysed (Fig. 3.3.1). The mean of the five days was calculated for each individual.
The difference from the mean was plotted against the mean. The standard deviation of the differences from the mean was 0.00037. This shows that the daily variation of the ^{13}C abundance of breath CO_2 is small and acceptable since the variation falls within 1.96 times the standard deviation for a duplicate analysis of one breath sample.

3.4 Conclusions

The observations described above show that the basal ^{13}C-abundance in breath CO_2 is distributed according to a Normal distribution. The study group had a reasonable size (n=244) and consisted of patients recruited from Rotterdam. No selection was made on ethnicity or habitual diet. Since the population of the city of Rotterdam is multi-cultural, different diets might be consumed in this group. In spite of this the distribution follows the normal distribution pattern with a mean in the expected range.
This led us to conclude that the diet as used in the Rotterdam region does not cause too wide fluctuations in the basal ^{13}C-abundance.
The observations also show that there is hardly any variation in the individual basal ^{13}C-abundance in breath CO_2. This is true both for the day to day variation and for the long term variation, although the long-term observations showed a greater variance as compared with the variation within a week.
From this information we concluded that the basal ^{13}C abundance could be used in the calculation of the planned studies and that the variation is within an acceptable range.

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Chapter 4

The model tested

4.1 Human liver glycogen metabolism assessed with a ^{13}C-enriched diet and a $^{13}CO_2$ breath test

4.2 Muscle glycogen does not interfere with a $^{13}CO_2$ breath test to monitor liver glycogen oxidation

4.3 Influence of the ^{13}C-enrichment of the habitual diet on a $^{13}CO_2$ breath test used as an index of liver glycogen oxidation: a validation study in Western Europe and Africa

4.4 The $^{13}CO_2$ breath test to monitor liver glycogen oxidation in man. A study of its reproducibility

4.5 The $^{13}CO_2$ breath test for liver glycogen oxidation after a three-day labelling of the liver with a naturally ^{13}C-enriched diet

4.1

Human liver glycogen metabolism assessed with a ^{13}C-enriched diet and a $^{13}CO_2$ breath test

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European Journal of Clinical Investigation 1998;28:466 - 474

Abstract

Background Adequate liver glycogen stores to maintain hepatic glucose output by glycogenolysis in the post-absorptive state are essential to prevent protein loss through gluconeogenesis. There are no simple techniques to monitor liver glycogen use.

Methods In this study, we labelled liver glycogen with naturally ^{13}C-enriched carbo hydrate and measured the pattern of $^{13}CO_2$ excretion and the postprandial time during which oxidation of ^{13}C-labelled liver glycogen was demonstrable by $^{13}CO_2$ enrichment in breath. Two experiments were performed in 24 healthy volunteers.

Results In the first experiment we observed that breath $^{13}CO_2$ enrichment returned to baseline values at 20.3 (SD 2.3, n=12) hours postprandially, indicating exhaustion of the ^{13}C-labelled liver glycogen at that time. In a second experiment, breath $^{13}CO_2$ enrichment in the early hours of the post-prandial phase was studied. After a steep decline, which started 2 - 4 h after the last meal, the $^{13}CO_2$ enrichment reached a plateau phase at 6 hours post-prandially. This plateau phase lasted for about 6 - 8 h, suggesting steady-state glycogenolysis during this period. The plateau phase was followed by a further decline in $^{13}CO_2$ excretion, suggesting a gradually diminishing contribution of ^{13}C-labelled liver glycogen to substrate oxidation.

Conclusion It is possible to label liver glycogen with a diet of naturally ^{13}C-enriched carbohydrate. The oxidation of the labelled liver glycogen can be monitored by measuring ^{13}C-enrichment in breath CO_2.

Keywords ^{13}C-enriched carbohydrate, $^{13}CO_2$ breath test, hepatic glucose output, glycogenolysis, liver cirrhosis, liver glycogen, stable isotopes.

Introduction

Liver glycogen is a readily available source of glucose for the maintenance of stable blood glucose levels between meals and during an overnight fast.

Impaired liver glycogen storage will result in a reduced contribution from glycogenolysis to the hepatic glucose production during fasting. More energy will be supplied by free fatty acids. However, since adequate blood glucose levels are to be maintained, a reduced contribution from glycogen will result in an early-onset increase in gluconeogenesis and hence in increased proteinolysis. Recurrent proteinolysis for gluconeogenesis could result in muscle wasting and weight loss. This could be the case in patients with cirrhosis of the liver, especially if the protein intake is limited.[1-3].

Recent studies have shown that patients with cirrhosis of the liver need ample protein intake to meet their higher protein requirements.[2] It has also been shown that a late evening meal has a positive effect on the nitrogen balance.[4] Nocturnal oral carbohydrate supplementation improves the nitrogen balance in patients with a cirrhosis of the liver. This phenomenon is not observed in healthy subjects.[5] This suggests that during an overnight fast in cirrhotic patients, with presumably a small liver glycogen content, the liver glycogen is exhausted to such an extent that gluconeogenesis is enhanced. Nocturnal glucose supplementation could prevent this and reduce the proteinolysis.

It would therefore be valuable to be able to measure the liver glycogen stores and to study glycogen kinetics in patients who are at risk for an early depletion of their liver glycogen. With such a method, the effects of nutritional intervention schemes could also be evaluated. However, at present, an easy and clinically applicable test to measure liver glycogen is not available.

In the past, glycogen concentrations have been measured in serial liver biopsies.[6-8] Various catheterization techniques and indirect calorimetry have been used to study hepatic glucose output and substrate oxidation.[9-13] Recently, nuclear magnetic resonance (NMR), sometimes combined with isotopic

tracers, has been applied to study liver glycogen and liver glycogen kinetics.[14-21]
These different techniques all have drawbacks for clinical application. Therefore we decided to study a model using ^{13}C-enriched carbohydrate to label the liver glycogen pool and breath $^{13}CO_2$ to monitor the oxidation of labelled carbohydrate. In this model the liver glycogen is (partly) depleted by a day of hypocaloric feeding and an overnight fast. Then, liver glycogen stores are refilled with ^{13}C-enriched carbohydrate. Subsequently, during fasting, the appearance of $^{13}CO_2$ in breath is measured to monitor the utilization of the ^{13}C-enriched liver glycogen. The aim of this study was to test the feasibility of this approach and to describe the pattern of the $^{13}CO_2$ appearance in breath over time.

Materials and methods

Naturally ^{13}C-labelled carbohydrate

In nature, the background abundance of the stable isotope ^{13}C is about 1.1% of all carbon atoms. One would expect to find this abundance reflected in all carbon-containing macromolecules in plants too. However, this is not the case. There are two pathways in the photosynthesis of carbohydrates in plants, the C-3 and the C-4 pathway, leading to the production of carbohydrates with a different abundance of ^{13}C. The difference in abundance is the result of a different degree of isotopic fractionation in the two pathways.[22] Carbohydrates formed via the C-3 pathway (Calvin-Benson cycle) have a relative low ^{13}C abundance of 1.082%, whereas carbohydrates synthesized via the C-4 pathway (Hatch-Slack pathway) are relatively enriched in ^{13}C with an abundance of 1.096%. In the human body, no isotopic fractionation takes place. This difference in natural ^{13}C enrichment of carbohydrates can be exploited to study human glucose metabolism.
Most plants used for human consumption are C3 plants. A few exceptions are corn, sugar cane, millet and pineapple, which use the Hatch-Slack pathway. In the European diet the main sources of carbohydrate are C3 plants. As a result, the $^{13}CO_2$ abundance in breath in Europeans is relatively low. When such dietary C3 plant- derived carbohydrates are replaced by ^{13}C-enriched carbohydrate, the body glycogen stores will become labelled with ^{13}C-enriched glycogen. Fat and protein will not significantly become enriched on a C4 carbohydrate diet, which is given for five days or less.[13] In short-term studies, therefore, ^{13}C enrichment will only be found in the glycogen stores (muscle and liver). In other words, in the post-absorptive state the oxidation of ^{13}C-enriched glycogen will be the source of ^{13}C enrichment in CO_2. Breath $^{13}CO_2$ excretion will than reflect the oxidation of ^{13}C-enriched glycogen.
The oxidation of muscle glycogen might interfere with a test in which the focus of attention is on the contribution of liver glycogen to glucose production.
However, during a short period of fasting, less than 24 h, muscle glycogen is hardly used in individuals who are at rest or performing light exercises.[23-26]
The combination of the selective ^{13}C enrichment of the glycogen pools and the minimal use of muscle glycogen during an early sedentary fast establishes the conditions under which the ^{13}C enrichment of exhaled breath reflects the use of ^{13}C-enriched liver glycogen. We based our studies on this concept.

Study groups

Twenty-four healthy volunteers participated in the study and followed a 3-day study protocol. For practical reasons the group of 24 healthy volunteers was split in 2 groups of 12 subjects, group 1 and group 2. All participants habitually consumed a European, low ^{13}C-abundance, diet. They did

not use any medication. All gave informed consent. The protocol was approved by the local ethical committee.

Group 1

For each individual, the daily energy requirement was calculated using the Harris-Benedict formula. During day 1 of the study the subjects received 80% of their calculated energy requirement while they used their habitual low-^{13}C diet. The figure of 80% was chosen assuming that glycogen would then not be fully replenished. A depletion of three-quarters of the pool is to be expected.[27] Thus the liver glycogen pool would be sufficiently depleted during a subsequent overnight fast.
During day 2, the subjects consumed 120% of energy requirements as a naturally ^{13}C-enriched carbohydrate diet, to ensure an ample refill of the liver glycogen pool with ^{13}C-enriched carbohydrate. The composition of the diet is described below.
The diet was consumed between 08.00 h and 22.00 h. This was followed by an overnight fast.
During all 3 days, breath samples for analysis of $^{13}CO_2$ enrichment were collected at the times indicated in figure 1. No breath sampling took place during the night.

Group 2

This group followed the same study protocol. However, there were a few differences. During day 2, the period of food intake was from 08.00 h till 18.00 h; mealtimes were strictly standardised. Breath samples were collected postprandially on day 2 and throughout the subsequent night till 09.00 h on day 3.

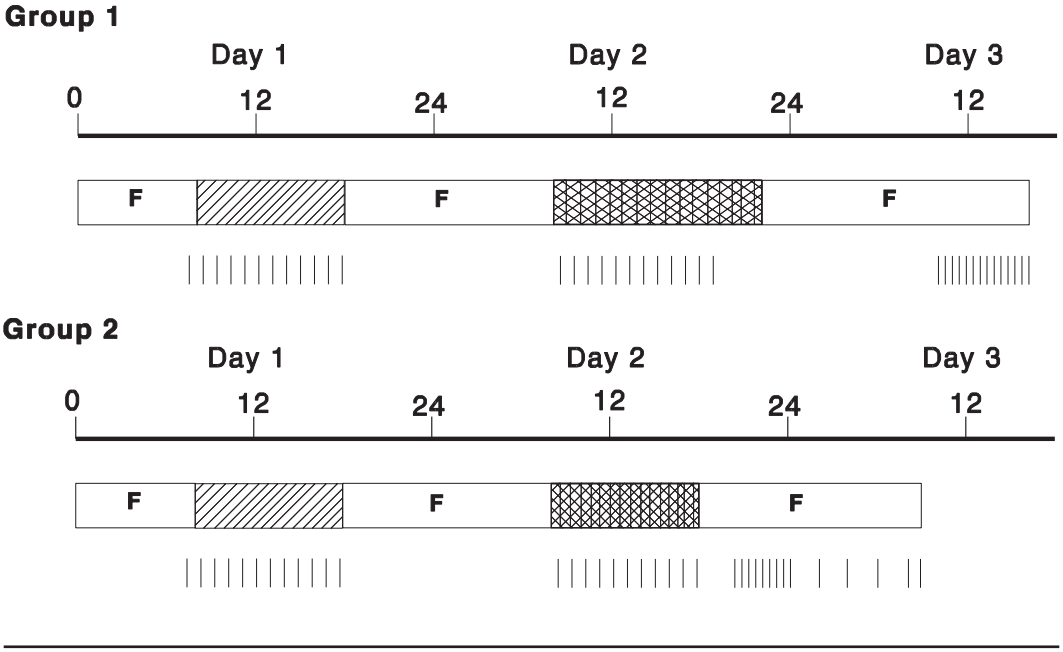

Fig. 1. Study protocol used for group 1, subject 1 - 12, and group 2, subject 13 - 24.
The empty boxes with an F represents the period of fasting. The striped boxes are the period during which each individual consumed 80% of their habitual diet. The crossed boxes are the period during which 120% of the naturally ^{13}C-enriched diet was consumed. The | sign indicates the time when breath samples for $^{13}CO_2$ analysis were collected.

The ^{13}C-enriched diet

The standard diet used during day 2 of the study consisted of 60% (w/w) carbohydrate, 27% (w/w) fat and 13% (w/w) protein. Eighty percent of the carbohydrate came from corn and sugar cane. The overall enrichment of the carbohydrate part of the diet was 1.0960 At% (atom per cent).
For breakfast, lunch and the late evening meal a pudding was consumed. This pudding was prepared from custard, a corn starch product (APJ bv, Koog a/d Zaan, The Netherlands), cane sugar, milk and cream. Dinner consisted of cooked polenta (milled corn, De Francescani, s.p.a. Monfalcone, Italy) with meat and vegetables. As “in-between”, cornflakes with milk and cane sugar were served.

Breath sampling

To obtain breath samples, alveolar air was collected in 20 mL vacutainer tubes. The rubber plug of the tube was removed and a drinking straw was inserted. After a normal inhalation, the subject quietly exhaled through the straw. The straw was removed and the tube was quickly closed.

Analytical procedures

$^{13}CO_2$ enrichment in breath CO_2 was measured using an Isotope Ratio Mass Spectrometer (SIRA-10, Series II, VG Isogas, Middlewhich, UK) fitted with an autosampler for the vacutainer tubes. Breath CO_2 was isolated by condensation in a liquid nitrogen trap. Subsequently, the CO_2 was released and led through the mass spectrometer. The measured isotope ratio was reported as percentage $^{13}CO_2$, using a reference gas that was calibrated against the international PDB (Belemnitella americana) standard of 1.1235 Atom % ^{13}C.

Calculations and statistics

For each subject, the baseline $^{13}CO_2$ enrichment was calculated as the mean of the 12 individual observations on day one and expressed as At %. The increase in $^{13}CO_2$ enrichment measured during day 2 and 3 was expressed in At% excess above baseline (APE).

Group 1
For group one (subjects 1-12) the maximal enrichment at 08.00 h on day 3 was compared with the baseline enrichment. Then, for each individual, these data at day 3 were plotted over time and subsequently a linear regression analysis was performed. The period between the time of last meal and the time the regression line reached baseline value was calculated. This “return to baseline time” is considered to reflect the time it will take before the liver glycogen pool is depleted of its ^{13}C-labelled glycogen, i.e. the time after which no more net hepatic glucose production from ^{13}C-labelled glycogen will take place.

Group 2
For group two (subjects 13-24) the pattern of breath $^{13}CO_2$ enrichment was studied in more detail during day 2 and the subsequent night. This included the time of maximal enrichment, and the decline in enrichment occurring in the early postprandial hours and during the night.
The naturally ^{13}C-enriched carbohydrates used for the diet of day 2 are easily absorbed by the gut.[28]

Therefore one may presume that the absorption of carbohydrate will be completed within 4 h after the last meal. As muscle glycogen has a minimal contribution to glucose production and oxidation during sedentary conditions, the only source of enriched $^{13}CO_2$ will be liver glycogen from 4 h postprandially onwards.
To determine the contribution to glucose production from the liver ^{13}C glycogen pool, we corrected our data for the ^{13}C enrichment of the CO_2-pool at 4 h post-prandially. When no further production of $^{13}CO_2$ could take place, the $^{13}CO_2$ enrichment would decline as described by the $^{13}CO_2$ elimination curve. This curve was applied to the 4-h post-prandial data. The calculated elimination curve was subtracted from the measured data curve. The resultant curve reflects the $^{13}CO_2$ actually being produced and entering the CO_2-pool from 4 hours postprandially onwards. Under the given conditions, this $^{13}CO_2$ originates from the ^{13}C-enriched liver glycogen pool.
For this purpose, the CO_2 pool was considered to be one compartment, and a half-life time of the label in the CO_2 pool of 60 minutes was assumed (k=0.0115).[13,29] An elimination curve was then constructed using the equation

$$APE_{t2} = APE_{t1} \times \mathbf{e}^{-0.0115 \times (t2-t1)}$$

In this equation t is the time expressed in minutes and t1 is 4 h after eating.
When applicable the Student's t-test was used for paired or un-paired data. A $P \leq 0.05$ was considered to be significant.

Results

The characteristics of the 24 participants are shown in Table 1.

Group 1

The mean basal breath $^{13}CO_2$ enrichment at day 1 for group 1 was 1.0827 At% (SD 0.0004). The ^{13}C-enrichment (Table 2) increased with 0.0041 APE (SD 0.0006) during day 2 ($P < 0.001$). At day 3 the mean 08.00 h value was 0.0022 APE (SD 0.0006) which differed from the basal value ($P < 0.001$), as well as from the maximal APE observed during day 2 ($P < 0.001$).
A linear decline in ^{13}C-enrichment was observed during day 3. The mean time of return of breath ^{13}C-enrichment to baseline values was 20.3 h after the last meal (SD 3.2 ; $r^2 = 0.98$, Fig.2) with a range from 16.5 till 27.0 h (Table 2).

Group 2

For group 2, the mean basal $^{13}CO_2$ enrichment was 1.0820 At% (SD 0.0006). This value did not differ from the fasting value at day 2 (at 08.00 h), which was 1.0821 At% (SD 0.0004). During day 2, while the naturally ^{13}C-enriched carbohydrate diet was consumed, the ^{13}C-enrichment of breath CO_2 increased to a maximum of 0.0048 APE (SD 0.0010). This maximum was reached at a mean of 160 minutes (SD 59) after the last meal. (Fig. 3)
The influence of the 3 main meals is clearly visible in the $^{13}CO_2$ enrichment profile.
The measured individual postprandial data are shown in Fig. 4. This graph also shows the theoretical $^{13}CO_2$ wash-out curve 4 hours after the last meal and onwards, as well as the constructed curve describing breath $^{13}CO_2$ excretion from ^{13}C-glycogen-derived and subsequently oxidised glucose. The resultant curve indicates a plateau phase of $^{13}CO_2$ appearance during the night. This plateau is followed by a decline resembling the pattern of excretion of $^{13}CO_2$ found in the group 1.

Table 1. Particulars of the study group.

Group 1					Group 2				
sex	age	weight in kg.	height in cm.	BMI kg/m^{-2}	sex	age	weight in kg.	height in cm.	BMI kg/m^{-2}
1 F	20	60	173	20.0	13 M	48	78	178	24.6
2 F	20	69	167	24.7	14 F	44	72	172	24.3
3 F	47	58	165	21.3	15 M	36	83	191	22.8
4 F	20	60	175	19.6	16 F	35	73	167	26.2
5 F	44	61	176	19.7	17 M	29	75	197	29.3
6 F	19	60	178	18.9	18 F	30	69	172	23.3
7 F	59	66	167	23.7	19 M	33	80	178	25.3
8 F	22	73	185	21.3	20 F	32	63	167	22.6
9 M	20	74	183	22.1	21 F	42	63	175	20.6
10 M	20	80	180	24.7	22 M	42	96	189	26.9
11 M	51	90	189	25.2	23 M	33	88	186	25.4
12 M	53	70	175	22.9	24 F	32	67	171	22.9
mean	32.9	68.4	176.1	22.0	mean	36.3	75.6	178.6	23.7
sd	16.2	9.7	7.5	2.2	sd	6.1	10.0	9.9	2.2

Table 2 Group 1, basal $^{13}CO_2$ abundance, maximum $^{13}CO_2$ enrichment, $^{13}CO_2$ enrichment 10 hours postprandial, the postprandial time period before baseline values are reached again and the r^2 of the individual regression analysis of the return to baseline time.

Subject	day1 baseline	day2 maximum	day3 8.00am	return to baseline	regression line
	abundance AP	enrichment APE	enrichment APE	time (h)	($r^{2)}$)
1	1.0833	0.0041	0.0017	19.0	0.78
2	1.0827	0.0048	0.0025	18.8	0.96
3	1.0825	0.0043	0.0015	27.0	0.65
4	1.0824	0.0041	0.0015	18.7	0.88
5	1.0825	0.0045	0.0029	19.8	0.92
6	1.0832	0.0045	0.0017	26.7	0.76
7	1.0827	0.0046	0.0022	19.4	0.82
8	1.0829	NA	0.0016	20.6	0.80
9	1.0827	0.0032	0.0022	19.2	0.97
10	1.0823	0.0044	0.0035	19.0	0.78
11	1.0828	0.0030	0.0021	19.3	0.89
12	1.0831	0.0039	0.0027	16.5	0.91
mean	1.0828	0.0041[a]	0.0029[b]	20.3	
SD	0.00045	0.00057	0.00063	3.19	

Basal abundance expressed in atom percent (AP), enrichment in atom percent excess relative to baseline values (APE) and return to baseline time in hours. [a] Significantly different from baseline abundance ($p<0.001$) [b] Significantly different from baseline abundance ($p<0.001$) and from maxi-

mum enrichment day 2 ($p<0.001$). NA = not available

The average enrichment during the plateau phase was 0.0020 APE (SD 0.0007). The end of the plateau phase was estimated for each individual and a mean of at least 13.5 h after the last meal was observed. (n=10) In two subjects the end of the plateau was not reached within the observation period.

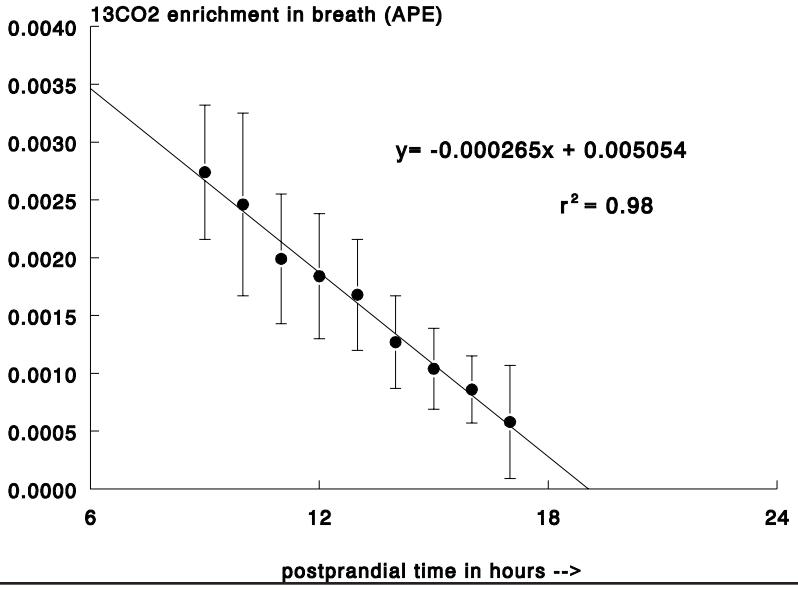

Fig. 2. The hourly mean (±SD) and the regression line of the 13CO2 enrichment in breath of group 1 in the postprandial phase during day 3 of the experiment.

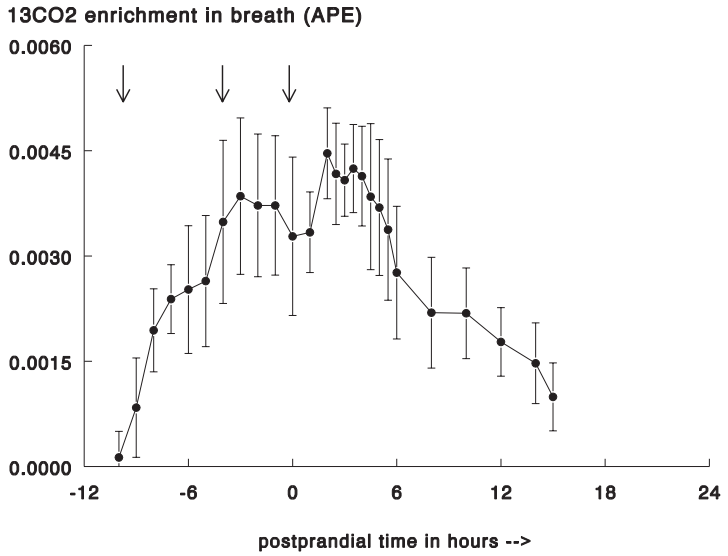

Fig. 3. The mean $^{13}CO_2$ enrichment (±SD) in breath samples of group 2 during day 2 and 3 of the experiment. During day 2 a 13C-enriched diet was consumed. Breakfast, lunch and dinner are indicated by an arrow,↓. From 6 pm onwards all subjects were fasting till 9 am on day 3. Time zero, 6 pm day 2, is the time when the last meal was consumed.

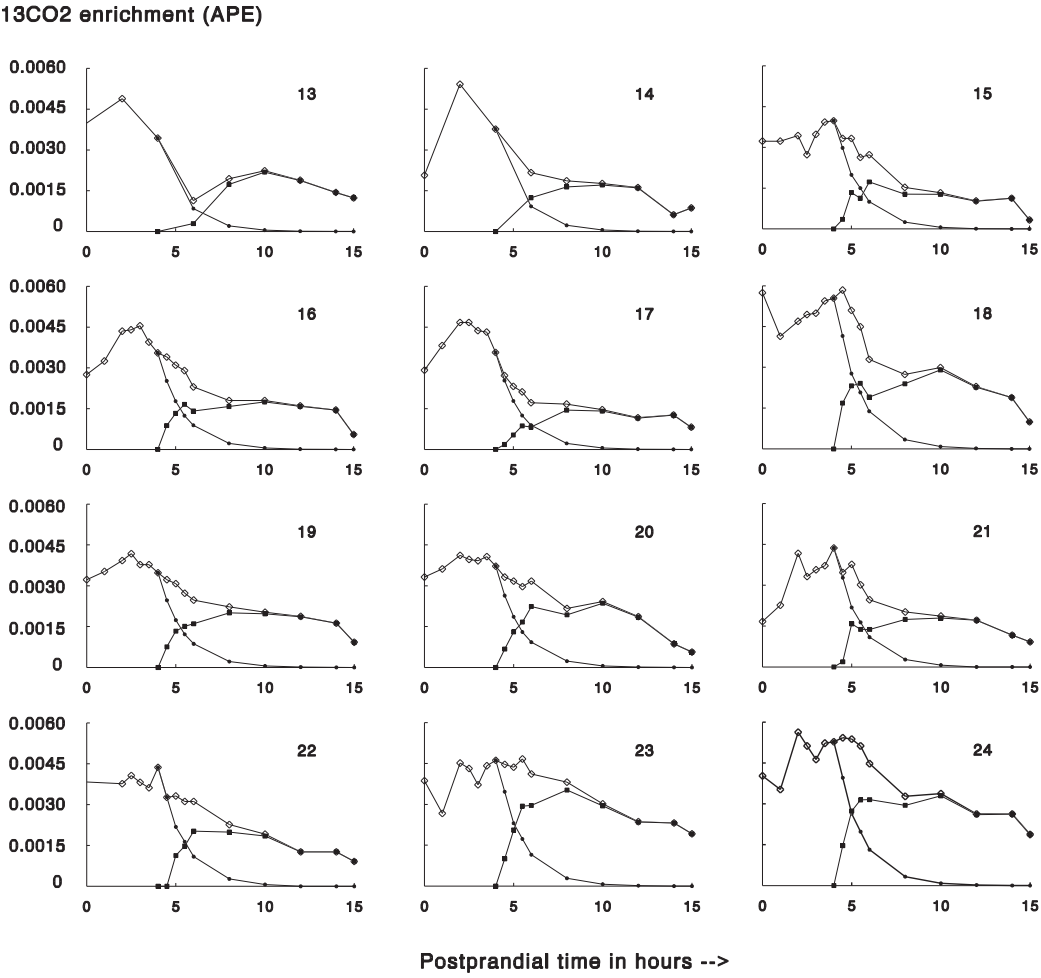

Fig. 4. The 12 individual curves of the participants of group 2 showing the 13CO2 enrichment from the moment the last meal at day 2 till the end of the experiment at 9 am on day 3. The continuous lines with the open square markers (◊) show the measured data. The dotted lines with the filled dots (•) are the calculated $^{13}CO_2$ elimination curves from 4 hours postprandial. The interrupted lines with the filled square markers (■) are the resultants of the subtraction of the elimination curves from the measured data, reflecting the oxidation of carbohydrates derived from ^{13}C-enriched glycogen.

Discussion

The ^{13}C-enrichment of carbohydrates formed by C4-plants, such as corn and sugar cane, is slightly but consistently higher than in other plants. This can be exploited by using their carbohydrate in clinical research.

In 1973, Lacroix et al.[30] reported on the use of such naturally ^{13}C-enriched glucose in combination with $^{13}CO_2$ breath analysis. They demonstrated that a glucose tolerance test carried out with naturally ^{13}C-enriched glucose produces reproducible ^{13}C-enrichment in breath CO_2. Since that time this approach has been used to study different aspects of the glucose metabolism in man.[28,31-34]

Recently, a naturally ^{13}C-enriched carbohydrate diet was used to label the liver glycogen pool in a study with the aim of determining the contribution of gluconeogenesis to total endogenous glucose production and hepatic glycogen renewal.[13] In that study, $^{13}CO_2$ breath analysis and plasma ^{13}C-glucose levels were measured under post-absorptive conditions. In our study, we used a similar approach to label the liver glycogen pool with naturally ^{13}C-enriched carbohydrate. We, however, focussed specifically on the possibility of monitoring glycogenolysis in the post-prandial state. To this end the ^{13}C enrichment of breath CO_2 was used as a parameter for the oxidation of ^{13}C-labelled liver glycogen-derived glucose.

The concept of our study is based on two assumptions. First, fat and protein should not be ^{13}C enriched but should have the same ^{13}C abundance as the baseline ^{13}C abundance of glycogen. Moreover this abundance in fat and protein should not change during the use of the ^{13}C-enriched carbohydrate diet. A study comparing the ^{13}C-abundance of fat (VLDL) and protein (plasma protein) before and after a 5-day diet with naturally ^{13}C-enriched carbohydrate showed no increase in ^{13}C-enrichment of fat and protein over this period.[13] However, when a ^{13}C-enriched carbohydrate diet is given for a much longer period or is continuously used as a standard diet, the large pools of fat and protein will become ^{13}C-enriched too. Rats fed only with ^{13}C-enriched carbohydrate for 35 days showed a $^{13}CO_2$ enrichment which equalled the ^{13}C-enrichment of the consumed carbohydrate.[31]

Owing to the short duration of our protocol, no interference of labelled proteins or lipids is expected.

The second assumption is that there should not be a significant contribution of muscle glycogen to glucose oxidation during the post-absorptive resting state.

It has been established that at rest and during conditions of low-level exercise the main energy substrates oxidized by muscle cells are free fatty acids and blood glucose.[24-26,35] They are used in preference to the available glycogen in muscle glycogen.[23-25,35]

Studies on the influence of diet on muscle glycogen stores, using muscle biopsies, showed that total starvation over a period of 1 day had very little effect on the glycogen stores, provided the subjects were at rest.[23,37] In the resting muscle, glycogenolytic activity is extremely low[38] and muscle glycogen is only to be used when more than light exertion is demanded from the muscle.[23,24,35]

During low-intensity exercise while fasting, muscle energy comes from blood glucose and free fatty acids but not from muscle glycogen.[24,35]

Although repletion of the depleted muscle glycogen stores occurs at a very low rate during starvation, rapid synthesis of glycogen takes place on a carbohydrate-rich diet. The latter process is restricted to depleted muscles only.[39] Thus during a sedentary fast, the muscle glycogen pool is not very active metabolically.

These observations lead us to conclude that the contribution of glucose from muscle glycogen to substrate oxidation is very low during a sedentary fast.

Recently, we demonstrated that a higher muscle glycogen ^{13}C-enrichment does not interfere with the determination of liver glycogen depletion.[40]

We conclude that during a sedentary fast the ^{13}C-enrichment in breath CO_2 is a parameter of the oxidation of ^{13}C-labelled liver glycogen.

In our study the basal $^{13}CO_2$ abundance in breath confirmed the low ^{13}C abundance of the habitual (Western-European) diet used by the volunteers.[30,32] There was no difference between the $^{13}CO_2$

abundance in breath measured at different times during a whole day (day 1) and the 08.00 h (fasting) values measured on the second study day.

Cahill and Owen[27] calculated that the liver glycogen stores would be exhausted after 18 - 24 hours of fasting. Serial liver biopsies in men showed a decline from 232 to 42 mmol glycosyl units per kilogram wet liver tissue between 12 and 36 h of fasting. The maximum postprandial concentration in that study was 503 mmol glycosyl units per kg wet liver. During a longer period of fasting, up to 10 days, the glycogen concentration of the liver remained at this low level.[7] NMR studies showed a decrease of liver glycogen to 16% of the initial content after 40 h of fasting. During fasting, the liver decreases in size. Taking this into account, 16% could even be an overestimate.[14] These data show that liver glycogen content decreases substantially during fasting. Moreover, they indicate that liver glycogen is not totally exhausted even during prolonged fasting, but that low concentrations remain. We observed that breath $^{13}CO_2$ enrichment returned to baseline values at approximately 20 h after the last meal. This indicates that the contribution of the ^{13}C-labelled liver glycogen to substrate oxidation had diminished to an undetectable level.

Presuming an evenly mixed distribution of ^{13}C-enriched glycogen throughout the whole liver, this implies that the whole liver glycogen pool is reduced to a minimal level and that the net hepatic glucose production from hepatic glycogen is low. We believe this to be a realistic result, because the time period of 20 hours in which the breath $^{13}CO_2$ enrichment reaches baseline values is rather congruent with the findings in the literature. Indeed, after a period of about 22 h, gluconeogenesis accounts for 70% of the glucose production.[14,41,42]

After we found that it is possible to use the naturally ^{13}C enriched diet to label liver glycogen and to come to a plausible “liver glycogen depletion time” in the first experiment (group 1), we studied the pattern of $^{13}CO_2$ enrichment in breath in more detail in a second group of volunteers (group 2). In this group, a steep rise in breath $^{13}CO_2$ enrichment was observed after breakfast followed by a more gradual increase. Lunch and dinner were reflected by an accelerated increase in breath $^{13}CO_2$ output (Fig. 3).

In our study, no information could be obtained about the net hepatic glucose production in the first 4 hours postprandially when gut-derived glucose was readily available for oxidation. However, after 4 hours, when gut glucose absorption has been completed, glucose supply comes from hepatic glycogenolysis and gluconeogenesis. Therefore, we constructed our theoretical $^{13}CO_2$ wash-out curve starting at 4 h post-prandially. Taylor et al.[20] reported a down-regulation of the hepatic glucose output to almost zero during the first hours postprandially. This down regulation was followed by a gradual increase of hepatic glucose output until a plateau was reached at 6 hours postprandially.

We observed a plateau in breath $^{13}CO_2$ enrichment that started at 6 hours after the last meal and continued until 12 - 14 h postprandially in 10 of the 12 subjects. In the other two, the plateau lasted beyond the observation time of 15 hours. This plateau phase indicates a steady state in ^{13}C-labelled carbohydrate oxidation during this period. This is interpreted as a constant rate of liver glycogen consumption. Such a linear use of liver glycogen was found by NMR studies as well.[14,20]

The decline in breath $^{13}CO_2$ observed at the end of the plateau phase reflects a reduced contribution of ^{13}C-labelled carbohydrate to oxidation. In other words, at this time a change in energy sources used for oxidation seems to occur. We speculate that at this time the process of gluconeogenesis is accelerating.

Because in our second series of experiments the observation time was limited to 15 hours, and the breath $^{13}CO_2$ enrichment did not return to baseline values in that time frame, insufficient data were available to calculate for each individual a time of return to baseline $^{13}CO_2$ values. Therefore we applied the formula derived in our first study group to the collective data of the second group. The result was a mean postprandial time of return to baseline ^{13}C-enrichment of 19.0 (SD 1.79, n=10) h. This is in line with the results that were obtained in the first group.

After we observed the plateau phase in the second experiment (group 2), we re-examined the group one data to see whether retrospectively a plateau phase in $^{13}CO_2$ excretion might also be discernible.

Indeed in subject 3 and 6 the $^{13}CO_2$ enrichment curve is initially flat; this could indicate the end of a plateau phase. For those two individuals a regression line was calculated starting at the end of the plateau phase. As a result we found a return to baseline in 21.9 hours (r^2=0.97) in subject 3 and in 21.3 hours (r^2=0.98) in subject 6 instead of the earlier calculated 27.0 hours (r^2=0.65) and 26.7 hours (r^2=0.76). These results are more in line with the data of the other 10 subjects of group 1. (Table 2)
Gay et al.[13] showed that the maximal ^{13}C-enrichment in glycogen was reached after 3 days of using the ^{13}C-enriched diet. In our protocol with only one day of ^{13}C-labelling of the liver glycogen pool it is unlikely that a maximal ^{13}C-enrichment in glycogen was achieved. However, as long as the ^{13}C-label is evenly mixed throughout the liver glycogen pool, the lower degree of ^{13}C-enrichment will not interfere with the outcome of our study.
The issue of an even distribution of ^{13}C-label is of importance in the interpretation of the data. One might wonder whether newly synthesised glycogen will be deposited at the periphery of the glycogen macromolecule and will be mobilized in preference to already existing glycogen. In such a situation, the ^{13}C-enrichment will not be representative for all glycogen. This would lead to a false estimation of exhaustion of the liver glycogen pool in our study design.
We think that a more even distribution of label will take place. The glycogen molecule is spherical and consists of concentric tiers of branched and unbranched chains. The configuration of the molecule is such that the amount of glucose directly available to be released by phosphorylase is about 35% of the total molecule.[43,44] Owing to the concurrent processes of glycogen synthesis and glycogenolysis and the inherent exchange of labelled and unlabelled molecules during this process, a fair mix of ^{13}C-enriched glycosyl units throughout the macromolecule can be expected, especially if a substantial part (more than three-quarters of the total) of the glycogen granules has been used before the enriched diet started. To achieve this, the subjects in our study used a hypocaloric diet during the first day of the experiment. Altogether, it seems reasonable to assume a fair distribution of the ^{13}C-enriched glycosyl units throughout all glycogen particles.
In conclusion, after the liver glycogen pool is labelled with naturally ^{13}C-enriched carbohydrate, it is possible to obtain a degree of enrichment that is sufficient for it to be used in a test where liver glycogen consumption is measured through breath testing for $^{13}CO_2$ excretion. During an overnight fast under sedentary conditions a steady state exists in glycogenolysis for about 10 h, starting from 4 h post-prandially. Subsequently, a period of gradual decrease in breath $^{13}CO_2$ enrichment follows this plateau phase. This is compatible with a shift from glycogenolysis to gluconeogenesis during that time.
This easily applicable, non-invasive method could be a tool to study liver glycogen mobilisation in healthy subjects and in patients with liver disease. However, further studies are needed first to validate the method.

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4.2

Muscle glycogen does not interfere with a $^{13}CO_2$ breath test to monitor liver glycogen oxidation

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Summary

Naturally ^{13}C-enriched carbohydrate has been used to label the liver glycogen pool for metabolic studies. The utilisation of this glycogen was then monitored by the appearance of $^{13}CO_2$ in breath. Using this method, it is assumed that during sedentary fasting the contribution of muscle glycogen towards oxidation is negligible. We investigated the influence of a different level of ^{13}C enrichment of muscle glycogen on the ^{13}C enrichment of breath CO^2 while the breath test was carried out. In six healthy volunteers the muscle glycogen stores were grossly depleted by a cycling exercise prior to the consumption of the ^{13}C-enriched diet which was given over a 10 h period. The oxidation of liver glycogen was measured during an 18 h sedentary fast. The results were compared with a control group who had not depleted their muscle glycogen before labelling. A higher ^{13}C enrichment of muscle glycogen did not interfere with two parameters of liver glycogen oxidation i.e. the duration of the plateau phase of $^{13}CO_2$ and the return to baseline time. It was also shown that the ^{13}C-labelled muscle glycogen was still available after the 18 h fast because a strenuous exercise led to a rapid $^{13}CO_2$ enrichment. It is concluded that muscle glycogen ^{13}C enrichment does not invalidate a $^{13}CO_2$ breath test to measure liver glycogen oxidation during a sedentary fast.

Keywords

$^{13}CO_2$ breath test, fasting, muscle exercise, naturally ^{13}C-enriched carbohydrate, stable isotopes.

Introduction

Recently we described a new non-invasive method to monitor liver glycogen oxidation during fasting. The purpose of the used technique was to design a method, which would be easily applicable in the clinic. With such a tool, patients at risk for early depletion of their liver glycogen stores could be identified and effects of nutritional intervention could be evaluated.

In this method, body glycogen stores are labelled by means of a diet containing naturally ^{13}C-enriched carbohydrate derived from corn and cane sugar. Part of the ^{13}C-enriched carbohydrate will be used for glycogen synthesis in liver and skeletal muscle.

In the postprandial phase, this ^{13}C-labelled glycogen is mobilized and oxidized, the ^{13}C enrichment of the CO_2 pool will increase. This increase can be measured in breath CO_2. Since during fasting under sedentary conditions muscle glycogen is hardly oxidized, the measured increase in ^{13}C enrichment of CO_2 under those conditions derives from liver glycogen oxidation (Tanis et al. 1998).

The assumption that during fasting under sedentary conditions muscle glycogen is hardly oxidized is based on various biopsy and isotopic tracer studies. (Andres et al. 1956; Hultman & Bergstrom, 1967; Romijn et al. 1993; Hultman et al. 1994; Coyle, 1995; Felber & Golay, 1995). Other studies showed that as long as subjects are at rest, a one-day total starvation has little effect on muscle glycogen stores (Andres et al. 1956; Loy et al. 1986) and glycogenolytic activity is very low (Chasiotis, 1983). Under those circumstances skeletal muscles use free fatty acids and blood glucose as substrates (Romijn et al. 1993; Hultman et al. 1994; Coyle, 1995; Felber & Golay, 1995).

Gay et al. (1994) reported that the ^{13}C enrichment of body fat and protein does not increase during a few days diet with naturally ^{13}C-enriched carbohydrate.

Combining these findings, we concluded that the increase in ^{13}C enrichment of breath CO_2 is a parameter for the oxidation of glucose derived from liver glycogen and not of muscle glycogen, provided the subject is sedentary during a 24-hour period of fasting.

We decided to test this assumption. This was done by comparing the $^{13}CO_2$ breath test in persons

with a low and a high ^{13}C enrichment of their skeletal muscle glycogen. A high ^{13}C enrichment of skeletal muscle glycogen was achieved by the consumption of a naturally ^{13}C-enriched carbohydrate diet shortly after the study subjects grossly depleted their skeletal muscle glycogen stores through a short period of intensive exercise.
The control group followed the same protocol but did not exercise before the ^{13}C-enriched diet and hence had a lower ^{13}C enrichment of the skeletal muscle glycogen.
The aim of this study was to test the hypothesis that the degree of ^{13}C enrichment of muscle glycogen will not interfere with the measurement of mobilisation of liver glycogen during a period of a sedentary fast.

Methods

Twelve healthy volunteers participated in the study. Their usual diet was of a low ^{13}C abundance, the typical West-European diet, with carbohydrates derived from C3-plants (Lefèbvre, 1985).
All subjects followed a three-day study protocol as shown in Fig.1. Details of this protocol have been described previously (Tanis et al. 1998). In short, all participants used their habitual diet during day 1. The caloric intake was 80% of the calculated caloric requirements. (Harris-Benedict, light duties) This hypocaloric intake was chosen to ensure a sufficient depletion of the liver glycogen stores on the morning of day 2 after an overnight fast.
On day 2, breath samples were taken to determine basal ^{13}C abundance in breath CO_2.
In six subjects (the cycling group), depletion of the muscle glycogen pool was attained by a 30 min cycling exercise at 80% of the calculated maximal exercise capacity. Vital signs (blood pressure, heart rate, respiration rate and blood O_2 saturation) were monitored during this period. After the exercise, breath samples were collected and a diet containing naturally ^{13}C-enriched carbohydrate was consumed. This diet provided 120% of calculated caloric requirements. Subsequently the subjects fasted for 18 h under sedentary conditions.
Six subjects served as control group. They followed the same protocol, but they did not exercise and they fasted for 24 h.
During the entire observation period of eating and fasting, breath samples for $^{13}CO_2$ enrichment analysis were taken in both groups at regular intervals.
At the end of the sedentary fasting period, a second cycling exercise, identical to the first one, was performed in the cycling group.

The naturally ^{13}C-enriched carbohydrate diet

The ^{13}C-enriched diet used during day 2 of the study consisted of 60% (w/w) carbohydrate, 27% fat and 13% protein. Eighty percent of the carbohydrate came from corn and sugar cane. The ^{13}C enrichment of the carbohydrate in the diet was 1.096 At%.
For breakfast and lunch, a pudding was consumed, prepared from custard. (A corn starch product, APJ bv, Koog a/d Zaan, The Netherlands), cane sugar, milk and cream. Dinner consisted of cooked polenta (milled corn, De Francescani, s.p.a. Monfalcone, Italy) with meat and vegetables. In between, cornflakes with milk and cane sugar were consumed.

Breath sampling

Alveolar air samples were collected in 20ml vacutainer tubes (Becton Dickinson Vacutainer Systems Europe, Plymouth, UK). The rubber plug of the tube was removed and a drinking straw was inserted. After a normal inhalation, the subject quietly exhaled through the straw. The straw was then removed and the tube was quickly closed.
Analytical procedures
$^{13}CO_2$ enrichment in breath CO_2 was measured using an Isotope Ratio Mass Spectrometer (SIRA-10,

Series II, VG Isogas, Middlewhich, UK) fitted with an autosampler for the vacutainer tubes. Breath CO_2 was harvested by condensation in a liquid nitrogen trap. Subsequently the CO_2 was released and led into the mass spectrometer. The measured isotope ratio was reported as percentage $^{13}CO_2$. As reference was used a calibrated CO_2 gas, that was standardised against the Pee Dee Belemate limestone value of 1.1235 Atom %.

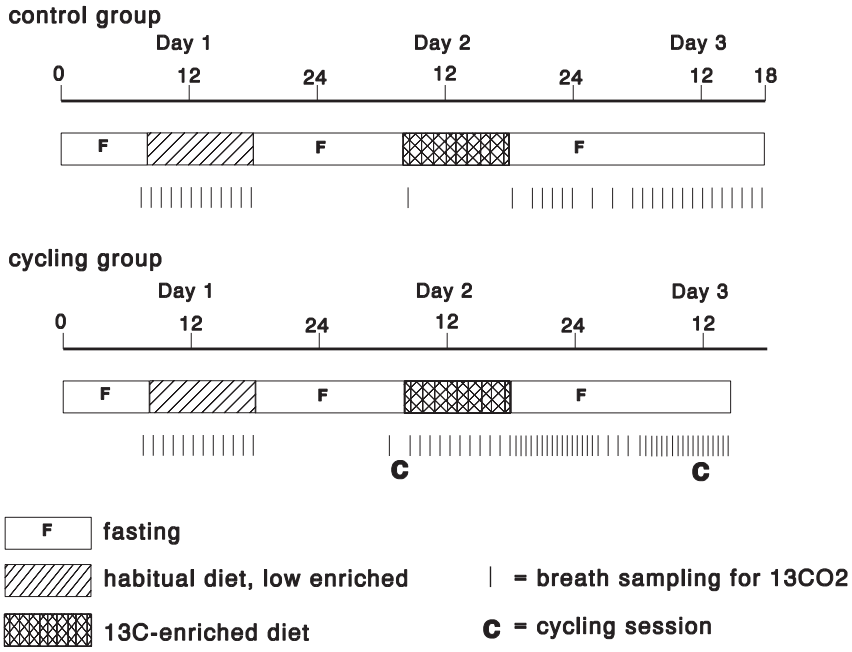


Figure 1. Study protocol used for the control group and the cycling group. The empty boxes with an F represent the period of fasting. The striped boxes are the period during which each individual consumed their habitual diet. The crossed boxes are the period during which the naturally ^{13}C-enriched diet was consumed. Breath samples for $^{13}CO_2$ analysis were collected at the indicated times. The cycling group performed a 30 minutes cycling exercise at the time indicated by a C.

Calculations and statistics

For each subject, the baseline $^{13}CO_2$ abundance on day 2 was calculated as the mean of two fasting observations at the start of that day. The increase in $^{13}CO_2$ enrichment measured during day 2 and 3 was then expressed in atom percentage excess ^{13}C above baseline (APE).

The breath $^{13}CO_2$ enrichment is the result of the two simultaneous processes of production and elimination. The elimination of $^{13}CO_2$ follows the half-life time of the CO_2 pool. Since the half-life time is 60 min (Meineke et al. 1993), the first-order rate constant related to the half-life of the process (k) is 0.0115 (time in min^{-1}). Using this constant, an elimination curve can be constructed by application of the equation

$$APE_{t2} = APE_{t1} \times \mathbf{e}^{-0.0115 \times (t2-t1)}$$

Assuming that carbohydrate absorption from the gut is completed 4 h after the last meal, the application of this equation to the enrichment of 4 h postprandial (t_1) predicts an elimination curve for the $^{13}CO_2$ which is present in the CO_2 pool at that very moment. We subtracted the elimination curve thus calculated from the measured data. The resultant curve reflects the $^{13}CO_2$ actually produced

and entering the CO_2 pool. Since the liver glycogen pool will be the only pool supplying ^{13}C-enriched carbohydrate under sedentary conditions, the resultant curve will reflect the contribution of labelled liver glycogen towards glucose production and oxidation.
During the night, a plateau phase in $^{13}CO_2$ enrichment can be observed. (Tanis et al, 1998) The level of enrichment at plateau level, the duration of the plateau phase and the subsequent decline in $^{13}CO_2$ enrichment were used for analysis. The plateau phase is defined by the average $^{13}CO_2$ enrichment of the data measured between 7 and 12 hours postprandial. The end of plateau phase was determined by the point in time at which a consistent decline of $^{13}CO_2$ enrichment of more than 10% started. The decline was considered consistent if for at least two measuring points the following measurement showed a lower enrichment than the previous one.
The data from this time point and onwards were used for a linear regression analysis. The time at which the regression line crossed the horizontal axis was calculated and considered as the return to baseline time (RTBT).
The maximum rise in $^{13}CO_2$ enrichment, which occurred after the second cycling session, was compared with the $^{13}CO_2$ enrichment data at the start of that session.
Student's t test was used for paired and unpaired data. A $P \leq 0.05$ was considered to be significant.

Table 1. Particulars of the control group and the cycling group.

control group					cycling group				
sex	age	height	weight	BMI	sex	age	height	weight	BMI
	yr	cm	kg	kg/m^2		yr	cm	kg	kg/m^2
M	26	182	70	21.1	F	24	181	66	20.1
M	24	177	64	20.4	F	23	176	56	18.1
M	23	174	74	24.4	M	29	185	78	22.8
M	25	172	64.8	21.9	M	24	170	74	25.6
M	22	198	87	22.2	F	23	173	62	20.7
F	24	169	58.5	20.5	M	22	184	64	18.9
mean	23.9	178.7	69.7	21.8		24.2	178.2	66.7	21.0
sd	1.4	10.5	10.0	1.5		2.48	6.11	8.07	2.76

Results

The cycling group consisted of three women and three men, and the control group of five men and one woman (Table 1).

The mean basal $^{13}CO_2$ enrichment measured during day 1 was 1.0816 Atom% (SD 0.0007) in the cycling group as compared to 1.0821 Atom% (SD 0.0003) in the control group (not significant).

After the first cycling session in the cycling group no rise in $^{13}CO_2$ enrichment was observed: -0.0008 Atom% (SD 0.0005) pre-cycling versus 0.0002 Atom% (SD0.0006) post-cycling, (not significant).

As soon as the ^{13}C-enriched diet was started the breath $^{13}CO_2$ enrichment rose briskly to a maximal mean value of 0.0061 APE (SD 0.0013) 1 h and 15 min (SD 15 min) after the last meal. The mean maximal value in the control group was 0.0059 APE 2 h and 12 min (SD 72 minutes) after the last meal. The difference was not significant.

The mean rise in enrichment at plateau level was 0.0025 APE (SD 0.0012) for the cycling group and 0.0026 APE (SD 0.0005) for the controls (not significant).

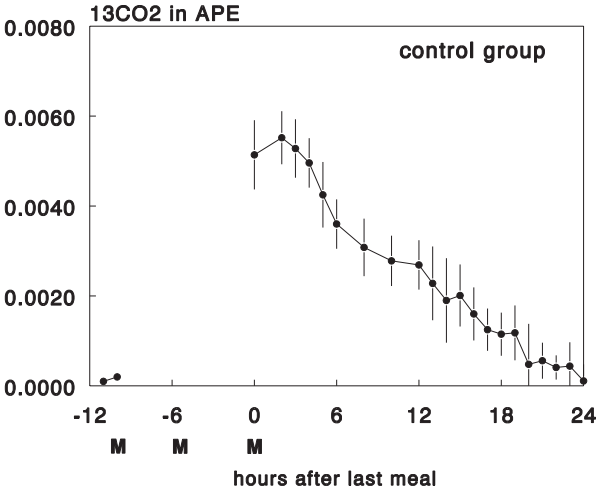
The end of the plateau phase was observed at 13.6 h postprandial (SD 0.86) in the cycling group as compared with 12.8 h (SD 1.17) in the control group (not significant)

In the cycling group the mean return to baseline time following the plateau phase was 19.7 h post-prandial (SD 3.32) and 22.9 h (SD 2.45) in the control group (not significant)

A second cycling session was carried out to analyse the ^{13}C enrichment in muscle glycogen at the end of the fasting period. The mean $^{13}CO_2$ enrichment at the start of this second cycling session was 0.0011 APE (SD 0.0013). After 30 min of cycling, the $^{13}CO_2$ enrichment increased significantly to a mean of 0.0038 APE (SD 0.0019, $P < 0.001$), (Figs 2 and 3).

The top of the enrichment curve was 0.0040 APE (SD 0.0017) 54 min (SD 22 min) after the second cycling session.

In a pilot study 4 subjects followed the same protocol as the control group (i.e. no exercise before the diet started). However they performed a 30 min cycling exercise after 11 h of fasting and repeated this 2 h later. In this group, a small and repeatable temporary decrease in ^{13}C-enrichment in breath CO_2 occurred after each cycling period. (Fig. 4). This suggests a dilution of $^{13}CO_2$ by lower enriched CO_2 from muscle glycogen oxidation.



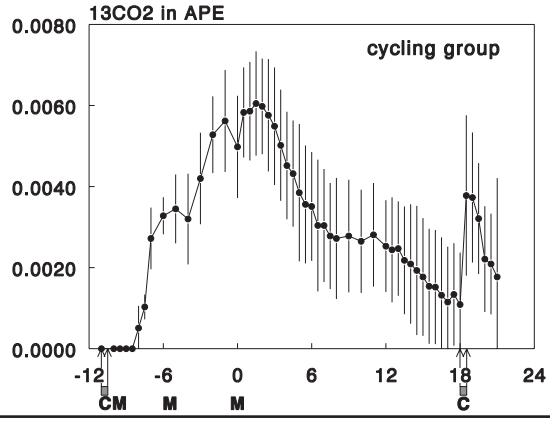


Figure 2. The mean $^{13}CO_2$ enrichment (±SD) in atom percent excess in breath samples of the control group and the study group during day 2 and day 3 of the experiment. During day 2 the special ^{13}C-enriched diet was consumed. The time at which the meals were taken are indicated by a M. Time zero, 18.00h day 2, is the time when the last meal was consumed. The time of the two cycling exercises in the cycling group are presented by a C.

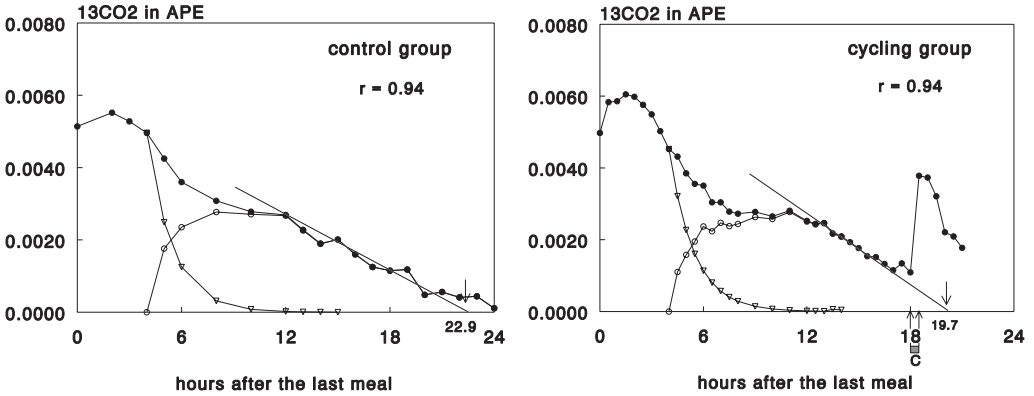


Figure 3. The mean $^{13}CO_2$ enrichment in atom percent excess of the control group and the cycling group from the time the last meal was consumed. The continuous line with the filled dots (●) are the measured data. The dotted line with the open triangles (▽) are the calculated $^{13}CO_2$ elimination curves from the 4 hours postprandial data. The broken lines with the open dots (○) are the resultants of the subtraction of the elimination curves from the measured data, reflecting the oxidation of carbohydrate derived from ^{13}C-enriched glycogen. The straight line is the regression line of the data following the plateau phase. The arrow points at the return to baseline time. The time of the second cycling exercise in the cycling group is indicated by a C.

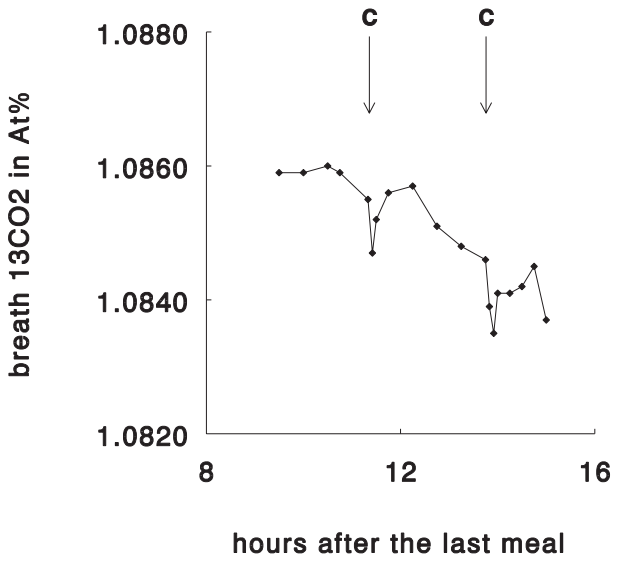


Figure 4. The breath $^{13}CO_2$ enrichment in atom percent in one of the four individuals, who followed the same protocol as the control group with the exception that they performed a cycling exercise 11 and 14 hours after the last meal. A C with arrow indicates the times of the cycling exercises.

Discussion

Recently we described a method to monitor the utilisation of liver glycogen for energy production with a breath test (Tanis et al. 1998).

With this method the glycogen pools are labelled with naturally ^{13}C-enriched carbohydrate. Subsequently during a sedentary fast the rise in ^{13}C enrichment in breath CO_2 reflects the oxidation of ^{13}C-labelled glucose, which is under those circumstances derived from liver glycogen. This method is based on the concept that muscle glycogen is hardly, if at all, utilised as long as muscle exercise is avoided during the test procedure (Andres et al. 1956; Hultman & Bergstrom, 1967; Romijn et al. 1993; Hultman et al. 1994).

In the present study, we elaborated on this assumption by testing the potential contribution of muscle glycogen to the CO_2 production. Through a short period of strenuous exercise the skeletal muscle glycogen depots were grossly depleted. Thereafter muscle glycogen was repleted with naturally ^{13}C-enriched glucose. Starting from this situation we tested whether the higher muscle glycogen ^{13}C enrichment interfered with the measurement of liver glycogen utilization.

The baseline ^{13}C abundance in both the cycling and the control group was at the (low) level expected for persons used to a West-European diet (Lacroix et al. 1973; Schoeller et al. 1980). No rise in $^{13}CO_2$ was observed after the first cycling period before the ^{13}C-enriched diet was started. This confirms that the muscle glycogen pool, which was mobilised as soon as such strenuous exercise was started, had the same low ^{13}C abundance as the other energy sources (liver glycogen, fat and protein) which contributed to the baseline $^{13}CO_2$ production.

A similar low ^{13}C abundance of the different energy sources in the body has been reported earlier in West-Europeans (Wagemakers et al. 1993).

The subjects in the cycling group performed at 80% of their VO_2max. It is well known that during such a degree of exercise muscle glycogen is utilized to a large extent (Bergstrom & Hultman, 1966;

Romijn et al. 1993). Repeated muscle biopsies to measure the degree of ^{13}C enrichment of the glycogen and to verify the degree of skeletal muscle glycogen depletion would have provided more direct information. However, for practical reasons, this was not done.

Shortly after the first cycling session, the intake of the naturally ^{13}C-enriched carbohydrate food started. Due to immediate oxidation of some of the just absorbed ^{13}C-enriched glucose, the breath $^{13}CO_2$ increased as soon as the diet was started. The diet contained enough carbohydrate to ensure an adequate repletion of the glycogen pools. It has been established that the muscle glycogen synthesis is indeed increased after a short high-intensity exercise. Such an increase is much higher than that after a prolonged resistance exercise (Pascoe & Gladden, 1996). It may therefore be assumed that by the end of day 2 the muscle glycogen pool of the subjects in the cycling group had a higher ^{13}C enrichment then in the control group.

A substantial part of the energy that is needed for moderate to intensive exercise comes from an increased utilisation of liver glycogen (Romijn et al. 1993; Hultman et al. 1994). Consequently, one could speculate that the liver glycogen pool would be more depleted after the first exercise session as compared with the liver glycogen pool of the control group. Repletion by ^{13}C-enriched glycogen could result in a higher overall ^{13}C-enrichment of the liver glycogen pool. However, this was not observed, since the plateau enrichment did not differ between the two groups.

Two conclusions can be drawn from this observation. The first conclusion is that a higher ^{13}C enrichment of the skeletal muscle glycogen pool does not interfere with the measurement of the process of liver glycogen depletion. The rise of $^{13}CO_2$ directly after the second cycling session confirmed that the muscle glycogen was still ^{13}C-enriched at that time. These results certainly provide a strong indication that under sedentary fasting conditions the breath test measures selectively the mobilisation of (^{13}C-labelled) liver glycogen and not muscle glycogen. The repeatable decrease in breath $^{13}CO_2$ enrichment after a strenuous cycling exercise in subjects who otherwise followed the same protocol as the control group (Fig. 4) indicates a dilution of $^{13}CO_2$ by lower enriched CO_2 from muscle glycogen oxidation. Thus no significant degree of ^{13}C-labelling of muscle glycogen took place in those individuals, who had not depleted their muscle glycogen stores through muscle exercise before they started the ^{13}C-enriched diet. Otherwise an increase in $^{13}CO_2$ exhalation would have been obtained, as was observed in the present study.

Secondly, we created a situation that led to a higher ^{13}C enrichment of muscle glycogen. This labelled glycogen is presumably conserved in the muscle as long as the study subject is sedentary. We observed a steep increase of ^{13}C enrichment of breath CO_2 as soon as the second cycling session started. Although no quantitative data were obtained, this certainly proves that under fasting conditions a substantial amount of muscle glycogen was preserved and can be used during exercise. It could be argued that the rise of $^{13}CO_2$ after the second cycling session was caused by an increased oxidation of labelled liver glycogen. However, the decline in $^{13}CO_2$ in cycling persons with a low ^{13}C muscle glycogen enrichment argues against this (Fig. 4).

In summary, we studied the effect of a higher degree of ^{13}C enrichment of liver and muscle glycogen on the results of a $^{13}CO_2$ breath test, which was designed to monitor the utilization of liver glycogen. While using test conditions with a different degree of ^{13}C-labelling of muscle glycogen, no difference was observed in plateau enrichment, end of plateau time and the return to baseline time of $^{13}CO_2$ excretion.

This indicates that with this breath test the determination of the time during which liver glycogen mobilisation is demonstrable does not depend on the degree of ^{13}C enrichment of the skeletal muscle glycogen pool.

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4.3

Influence of the ^{13}C-enrichmnet of the habitual diet on a $^{13}CO_2$ breath test used as an index of liver glycogen oxidation: a validation study in Western Europe and Africa

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ABSTRACT

A diet containing naturally ^{13}C-enriched carbohydrate combined with a $^{13}CO_2$ breath test analysis can be used to monitor liver glycogen oxidation in persons used to a diet low in ^{13}C, e.g., the West-European diet. In this study, we further evaluated this test principle further by changing the way we label the glycogen pool. The ^{13}C enrichment of exhaled CO_2 was studied in two groups, one in Europe and one in Africa. The European group (n = 12) was accustomed to a diet low in ^{13}C, and they went on a ^{13}C-enriched study diet to identify liver glycogen. The African group (n = 6) was accustomed to a diet naturally high ^{13}C, and they went on a diet low in ^{13}C. The basal ^{13}C abundance in exhaled CO_2 was higher in the African group (1.0879 At%; atmospheric 1.1 atom percent) than in the European group (1.0821 At%). During the study period, the parameters for liver glycogen oxidation - the $^{13}CO_2$ enrichment plateau, the plateau duration and the return to baseline time - did not differ between groups. The abundance $^{13}CO_2$ in exhaled CO_2 over time in the two groups was similar but inverse. The study confirms the use of a $^{13}CO_2$ breath test to monitor liver glycogen oxidation and demonstrates how to use such a test in persons accustomed to s diet high in ^{13}C.

KEY WORDS

naturally ^{13}C-enriched carbohydrate, liver glycogen, corn, sugar cane, $^{13}CO_2$ breath test

INTRODUCTION

Studies in animals and in man have shown that protracted use of ^{13}C-enriched food increases ^{13}C abundances in the body. As a result, ^{13}C enrichment of the CO_2 pool increases. When rats are fed with (naturally ^{13}C enriched) maize glucose for 35 d, they expire CO_2, which has the same $^{13}C/^{12}C$ ratio as their dietary maize glucose.[1] In humans, the ^{13}C abundance of the CO_2 pool is lower than the atmospheric 1.1 atom percent (At%) and changes in accordance with the dietary ^{13}C abundance and the ^{13}C abundance in the body energy stores. The ^{13}C abundance in the energy stores reflects the average ^{13}C abundance in the diet over a longer period[1-3] because this lower-than-atmospheric abundance is found in different ^{13}C abundance of food and food products.

The difference of the ^{13}C abundance in different foods is determined by the isotopic fractioning of one of the two biochemical pathways that can be used by plants for photosynthesis. The majority of plants use the C3-pathway (the Calvin-Benson cycle), through which isotopic fractioning occurs. Carbohydrates produced through this pathway have a relative low ^{13}C abundance of approximately 1.082 At%. In contrast, a few other plants use the C4-pathway (the Hatch-Slack pathway) for their photosynthesis.[4] Carbohydrates produced by C4 plants have a ^{13}C abundance of about 1.096 At%, which is almost identical to that of the ambient air. The main representatives of the C4 food plants are corn, millet, sugar cane and pine apple.[3] The difference in ^{13}C enrichment of food products in the diet and even in the food-chain is caused by the different contributions of naturally ^{13}C-enriched constituents, e.g., corn and cane sugar. As an example, cheese produced from corn-fed (American) cows has a higher ^{13}C enrichment, 1.0989 At%, than cheese produced from grass-fed (Polish) cows, 1.0807 At%.[3]

The American diet has a higher content of corn and corn-derived food than the usual West-European diet. Consequently, body glycogen, fat and protein stores have a higher ^{13}C abundance. As a result, the exhaled $^{13}CO_2$ abundance is higher in North-Americans, 1.0882 At% than in Europeans, 1.0820 At% .[3,5,6]

Corn and sugar cane are staple food in large areas of the African continent. It can readily be assumed that the inhabitants of those areas will have a higher ^{13}C abundance, though no studies have

been performed to confirm this assumption.
The small but consistent differences in natural ^{13}C enrichment of carbohydrates can be used for metabolic studies because no further isotopic fractioning takes place further along the food-chain or in human metabolism.[1] In subjects accustomed to a diet low in ^{13}C (West-European), the differences in basal ^{13}C abundance between the three energy pools (glycogen, fat and protein) are negligible.[5] This creates a steady basal $^{13}CO_2$ abundance. When such subjects consume a diet with naturally ^{13}C-enriched carbohydrates for 5 d or less, only the glycogen pool shows a significant increase in ^{13}C enrichment.[7] Because, under sedentary fasting conditions, skeletal muscle glycogen is seldom used, the glucose derived from liver glycogen will then be the sole source of ^{13}C enrichment.[6,8,9] Under these circumstances, $^{13}CO_2$ enrichment in breath can be used to monitor the utilization of liver glycogen.
In a previous study on liver glycogen kinetics we used the natural ^{13}C enrichment of corn and sugar cane to label the liver glycogen pool in persons used to a diet low in ^{13}C. The use of this ^{13}C-labelled liver glycogen was subsequently followed during a period of sedentary fasting.[10]
In the present study, we evaluated this test principle further by changing the way we labelled glycogen. In the previous study, the glycogen pool was labelled in persons who had a low basal ^{13}C abundance; in the present study, we reversed the labelling procedure by giving carbohydrates low in ^{13}C to persons accustomed to a diet high in ^{13}C.-enriched carbohydrates. In certain areas of the Africa, corn, millet and sugar cane are staple foods, and, consequently, the basic ^{13}C enrichment of the body energy stores is high. The African part of the study was performed in a rural village in Botswana. The purpose of the study was to investigate whether comparable data could be obtained for the parameters of liver glycogen use. If such data could be obtained, the validity of the concept of the test would be strengthened, and such a method could be applied in populations adapted to a diet high in ^{13}C.

PATIENTS AND METHODS

Subjects

Six healthy female volunteers (age range = 20 - 24 y) participated in the study (African group). All six resided at a boarding school in Mochudi, a rural village in Botswana. Their habitual diet consisted of corn products, sugar cane, meat and vegetables. For baking, sunflower oil or corn oil was used.
The control group (European group) consisted of 12 healthy volunteers living in The Netherlands. This group was used to a diet low in ^{13}C. Some data from this group were published elsewhere.[10] The characteristics of both groups are presented in Table I.
All participants gave informed consent, and the experiment was approved by the local health authorities.

The Diet

Subjects in the African group were allowed to use their habitual diet during the first day of the experiment.
For this study, a diet low in ^{13}C-enriched carbohydrate was prepared for the African group. This diet was consumed during day 2. To avoid hidden sources of ^{13}C enrichment, some food items were imported from Europe. These items were beet sugar, tomato sauce and rice mix (Knorr-Nährmittel AG, Germany) and an artificial sweetener (Natrena, The Netherlands; see Appendix).
In the African group, no caloric restrictions were imposed during day 1 and 2. During the fasting pe-

riod, consumption of water, coffee and tea without milk or sugar was allowed ad libitum.
The diet of the European group has been described elswhere.[10] In brief, during day 1, all subjects consumed their habitual diet. During this day, they had a caloric intake of 80% of their calculated daily need (Harris-Benedict formula). During day 2, a balanced diet with a caloric intake of 120% was used (see Appendix).
We used 80% of the calculated caloric intake in the study design was to obtain a sufficient depletion of the liver glycogen pool at the end of the day and even more after an overnight fast. The intake of 120% during day 2 was used to ensure an ample refill with ^{13}C-enriched glycogen during that day. For practical reasons, this scheme was not applied in the African group.
During day 2, the carbohydrate part had a ^{13}C enrichment of 1.0960 At% in the European group and of 1.0820 At% in the African group.

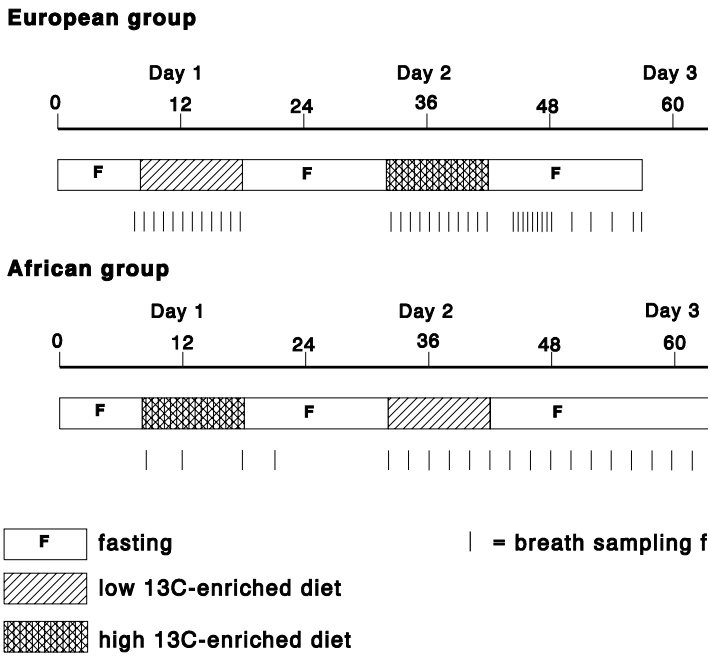

Figure 1. Overview of the 3-day experimental design for the African and for the European group.

TABLE 1.
Characteristics of African and European subjects studied.

	African group n=6	European group n=12
Age (y)	21.7 (1.4)	36.3 (6.1)
Height (cm)	161.7 (5.7)	178.6 (9.9)
Weight (kg)	61.2 (7.2)	75.6 (10.0)
BMI (kg/m2)	23.4 (2.1)	23.7 (2.2)
Sex male : female	0 : 6	6 : 6

Mean values (SD)
BMI, body mass index

Experimental Protocol

For both the African group and European groups, a 3-day study protocol was used (Fig. 1).
Baseline breath samples were collected during study day 1, when subjects ate their habitual diet. After an overnight fast, a fasting breath sample was collected. Then the group-specific diet was consumed during day 2. During that day, breath samples were taken at fixed intervals. The last meal was consumed at 6 p.m. From this time onwards all subjects fasted under sedentary conditions. The European group fasted for 15 h, and the African group for 24 h. Breath samples were collected at the times indicated in Figure 1.
The first data from the European group came in before the African group started. In order to get better information of the last part of the curve, we then decided to extend the breath collecting period in the African group.

Breath Sampling

End-expiratory air samples were collected in 20-mL vacationer tubes. The rubber plug of the tube was removed, and a drinking straw was inserted. After a normal inhalation, the subject quietly exhaled through the straw. The straw was then removed and the tube was quickly closed. All breath samples were analysed in one laboratory.

Analytical Procedures

$^{13}CO_2$ enrichment in breath CO_2 was measured using an Isotope Ratio Mass Spectrometer (SIRA-10, Series II, VG Isogas, Middlewhich, UK) fitted with an autosampler for the vacationer tubes. Breath CO_2 was harvested by condensation in a liquid nitrogen trap. Subsequently the CO_2 was released and led through the mass spectrometer. The measured isotope ratio was reported as percentage $^{13}CO_2$ using the PBD (Belemnitella americana) standard of 1.1235 At% as a reference.

Calculations and statistics

During day 1, the $^{13}CO_2$ enrichment in breath was measured serially to observe any influence of the habitual diet consumed during the day.
For each subject, the baseline $^{13}CO_2$ abundance was calculated as the mean of the two fasting observations at the start of day 2. The individual change in $^{13}CO_2$ enrichment measured during day 2 and 3 was expressed in atom percent excess (APE) above this baseline.
In the analysis, the body CO_2 pool was considered to be one compartment. The ^{13}C-enrichment of the CO_2 pool at any given time is the result of the simultaneous processes of elimination and production. The elimination of $^{13}CO_2$ follows the half-life time of the CO_2 pool. When the half-life time is 60 minutes the first-order rate constant related to the half-life of the process (k) is 0.0115 (time in minutes^{-1}).[11] With this constant, an elimination curve can be constructed by application of the equation

$$APE_{t2} = APE_{t1} \times e^{-0.0115 \times (t2-t1)}$$

Assuming that carbohydrate absorption from the gut is completed 4 h after the last meal, then the application of this equation to the 4-h postprandial data (t1) predicts a theoretical elimination curve for $^{13}CO_2$ present at that time. We subtracted the so calculated elimination curve from the actually measured data. The resultant curve reflects the $^{13}CO_2$ produced after t1 and entering the CO_2 pool.

As stated before, the liver glycogen pool will be the only pool supplying ^{13}C-enriched substrate under sedentary conditions. Hence, the resultant will reflect the contribution of labelled liver glycogen toward oxidation.

During the night, a plateau phase in $^{13}CO_2$ enrichment can be observed. The ^{13}C enrichment at plateau level, the duration of the plateau and the subsequent time interval to return to baseline $^{13}CO_2$ enrichment were measured. The duration of the plateau was calculated as the postprandial time at which, after the plateau was reached, the $^{13}CO_2$ enrichment started to decline for more than 10%. Student's t test was used for paired and unpaired data. $P \leq 0.05$ was considered significant.

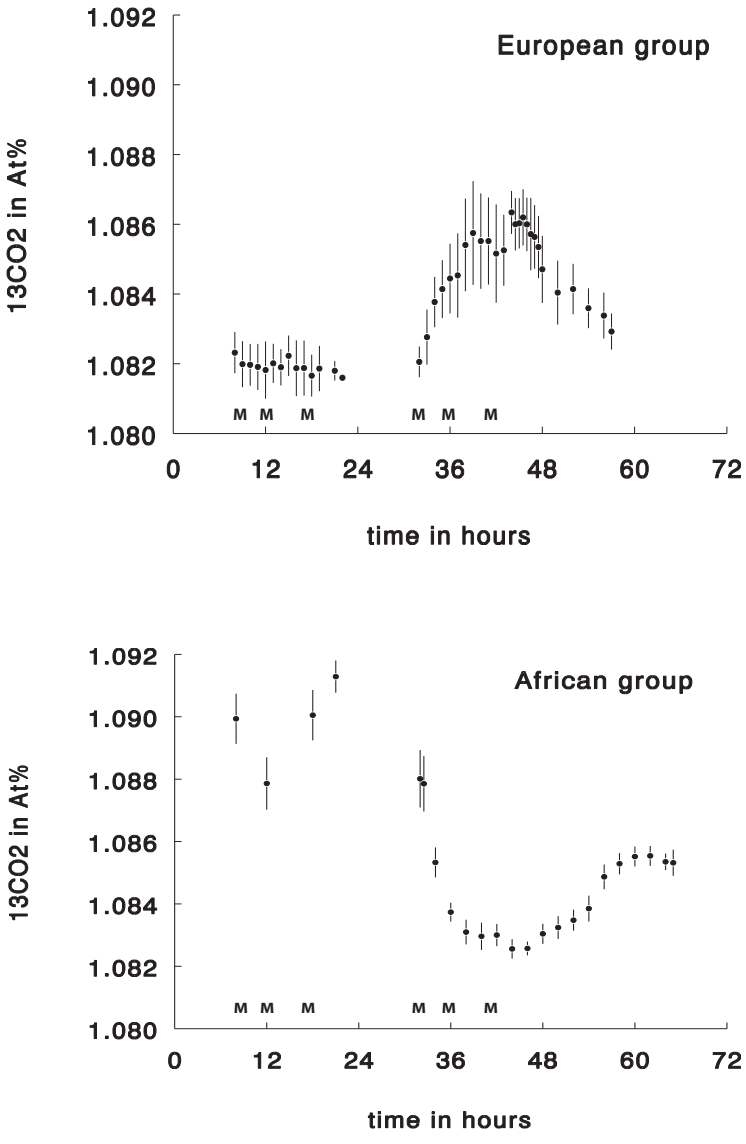

Figure 2. $^{13}CO_2$ enrichment (mean and SD) in exhaled breath in the European group and the African group during the 3 days of the experiment. **M** is the time when a meal was consumed.

RESULTS

The results of the ^{13}C-enrichment analysis of the breath CO_2 are presented in Figure 2.
In the African group, the ^{13}C enrichment showed a considerable variation during day 1. The mean $^{13}CO_2$ enrichment was 1.0899 At% (SD = 0.0008) after breakfast, 1.0879 At% (SD = 0.0008) before lunch, 1.0901 At% (SD = 0.0008) directly after dinner and 1.0911 At% (SD = 0.0005) 3 hours after dinner.
The breath samples from before lunch had a significantly lower $^{13}CO_2$ enrichment than those of the other three measuring points. All values were significantly higher than the day one values of the European group (mean = 1.0820 At%, SD = 0.0006, $P < 0.001$). No significant variation in the $^{13}CO_2$ enrichment was observed in the European group during day 1, while they used their habitual diet.
During day 2, the mean basal (fasting) $^{13}CO_2$ enrichment was 1.0879 At% (SD = 0.0009) in the African group and 1.0821 At% (SD = 0.0004) in the European group ($P < 0.001$).
In the African group, $^{13}CO_2$ enrichment declined briskly when the diet low in ^{13}C was started. The lowest abundance was reached 2 hours after the last meal, at a mean $^{13}CO_2$ enrichment of 1.0824 At% (SD = 0.0002). This value was not significantly different form the baseline $^{13}CO_2$ abundance in the European group, as was expected.
During the sedentary fasting period, a gradual increase in $^{13}CO_2$ enrichment was observed in the African group. The increase lasted until after about 18 hours of fasting, and $^{13}CO_2$ enrichment levelled out at 1.0854 At% (SD = 0.0003), which was different from the basal $^{13}CO_2$ enrichment of 1.0879 At% ($P = 0.003$).
The $^{13}CO_2$ enrichment pattern in the African group followed the inverse pattern of the control group. The inverse relationship became even more evident when the 1.0854 At% enrichment level was taken as baseline for the African group. (Fig 3)
Using the baseline of 1.0854 At% for the African group the end of plateau time was 11.7 h (SD = 0.82) in the African group and 13.5 h (SD = 1.17) in the European group (NS). The return to baseline time was 18.0 h (SD = 1.79) in the African group and 19.0 h (SD = 1.79) in the European group (NS).

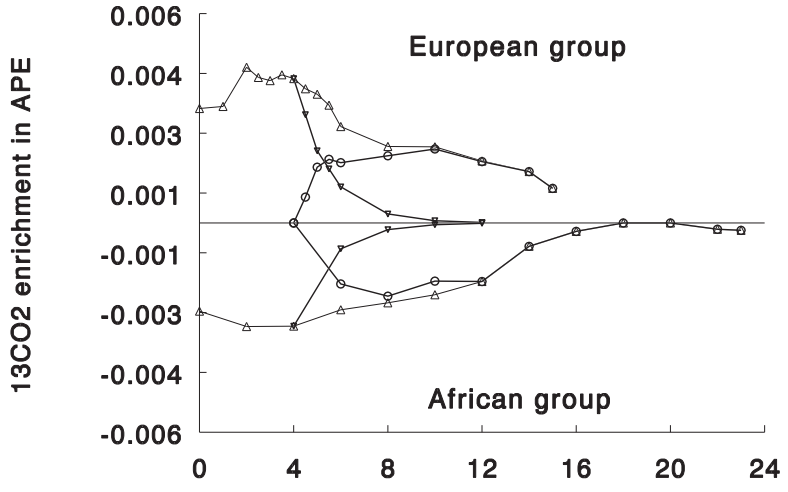

Figure 3. ^{13}C-enrichment in breath CO_2 in atom percent excess (APE) starting at the time of the last meal. The basal enrichment was the 8 am fasting value at day 2 for the European group and the 18 and 20 hours after the last meal value for the African group. (see discussion).
Δ are the actual data, ▼is the CO_2 wash-out curve and o is the subtraction of the wash-out curve and the actual data.

DISCUSSION

The ^{13}C enrichment of breath CO_2 depends on the ^{13}C enrichment of the substrates used for CO_2 production. The purpose of the present study was to validate the $^{13}CO_2$ breath test as an index of liver glycogen oxidation. Validation was done by comparing the effect of giving ^{13}C-enriched carbohydrate to non-^{13}C-enriched persons and non-^{13}C-enriched carbohydrate to ^{13}C-enriched persons.

The non-^{13}C-enriched persons group consisted of a group of Europeans accustomed to a diet low in ^{13}C, and the group of ^{13}C-enriched persons consisted of Africans accustomed to a ^{13}C-enriched diet. An inverse pattern of data was expected between the two groups.

In the literature, no data were available on the CO_2 ^{13}C-abundance in persons living in Africa.

In view of the data from North-America and Europe, $^{13}CO_2$ abundance in African inhabitants used to a staple food of corn and sugar cane was expected to near the North-American level.

Our study confirms this assumption. In the African group, the mean CO_2 ^{13}C enrichment was 1.0879 At% (SD = 0.0009) after an overnight fast. Data of basal $^{13}CO_2$ abundance in different cities in the USA showed a mean of 1.0882 At%, which is in the same range as the African data.[3] In the African group, when compared with the basal ^{13}C-enrichment after an overnight fast (0800-h value at day 2), the ^{13}C enrichment of breath CO_2 was higher after breakfast (1.0899 At%, SD = 0.0008, P = 0.01) and after dinner (1.0913 At%, SD = 0.0005, $P < 0.001$). The CO_2 ^{13}C enrichment measured just before lunch did not differ from baseline value, which suggests a direct influence of the ^{13}C-enriched food on breath $^{13}CO_2$ enrichment. Part of the absorbed food will be directly oxidized. This direct oxidation will cause a rise in ^{13}C enrichment of the CO_2 pool if the ^{13}C enrichment of the oxidized food is higher than the basal ^{13}C enrichment.

In contrast to the African group, the $^{13}CO_2$ enrichment in European group did not show any dietary influence during the first day of the experiment (Fig. 2).

In the African group, $^{13}CO_2$ enrichment decreased to basal European level during day 2, when the diet low in ^{13}C (as in West-European) was consumed.

In the European group, the opposite pattern was observed during consumption of the diet higher in ^{13}C. However the maximum values in this group did not completely reach the African basal value of ^{13}C abundance in breath CO_2.

This result may be explained by the differences in ^{13}C enrichment of the body protein and fat pools between the two study groups. Animal studies showed that the consumption of ^{13}C-enriched carbohydrate over a prolonged period increases the ^{13}C-enrichment of the protein and fat pools[1]. Therefore, ^{13}C enrichment of the body protein and fat pools in the African group should be higher than that in the European group. Thus, the higher ^{13}C values in the Africans can be explained by the oxidation of ^{13}C-enriched protein and fat.

A remarkable observation was that the $^{13}CO_2$ enrichment in the African group did not return to the original enrichment level measured after an overnight fast (1.0879 At%) but levelled out to approximately 1.0854 At% after 18 h of fasting. A likely explanation for this phenomenon is that the actual mean ^{13}C-enrichment of the fat and protein energy stores are 1.0854 At%. After about 18 hours of fasting, the contribution of liver glycogen to oxidation has declined, and muscle glycogen is hardly used during a sedentary fast. Therefore, at this time in the fasting state, the $^{13}CO_2$ enrichment of 1.0854 At% reflects the mean ^{13}C-enrichment of fat and protein as the sources of CO_2 production.[6,8-10]

Taking this value of 1.0854 At% as baseline value, we compared the enrichment profiles over time during the study of the two groups. The curves show a striking similarity, almost a mirror image of one another. The $^{13}CO_2$ wash-out formula was applied to the actual, measured $^{13}CO_2$ enrichment measured at 4 hours postprandial. Thus, the constructed curve was subtracted from the measured one. In the African group, the inverse of the plateau of the European group was observed. The return-to-baseline time was identical in both groups (Fig. 3).

This outcome confirms the idea that the liver glycogen is used for oxidation at a steady rate during

the plateau phase and gradually declines after about 14 hours of sedentary fasting. After about 20 hours of fasting, the contribution of previously labelled liver glycogen is reduced to such a level that it cannot be measured anymore with this technique.[10]
In the control group, no data were collected beyond 15 hours of fasting. However, preliminary results of a later study in which data were collected during a 24 hours sedentary fast (unpublished observations) show a pattern of return-to-baseline beyond 15 hours of fasting, similar to the one observed in the African group.
The caloric intake in the European group was 80% of their habitual intake on day 1 and 120% on day 2 of the study. We assumed that this change will result in a higher ^{13}C-enrichment level of the liver glycogen and we expected a greater change in ^{13}C-labelling in the European group than in the African group. This change should produce a higher peak value in the Europeans, but the duration of the plateau phase and the return to baseline time were not altered.
It could be speculated that the area under the curve represents the amount of labelled glycogen used for oxidation. However, for such an estimation, other parameters such as CO_2 production and the size of the CO_2-pool are needed. Those data were not available in this experiment.
The observed smaller standard deviation of the data at all points in time in the African group can be explained by the fact that the African group lived at a boarding school. As a result all six had used the same diet for a long time. The European volunteers lived individually and had a much more diverse dietary pattern.
This study shows that the ^{13}C abundance of the breath CO_2 in Africans accustomed to a staple diet of corn and sugar cane is significantly higher than in Europeans used to a staple diet low in ^{13}C. Moreover, it confirms our hypothesis that studying liver glycogen kinetics with a naturally ^{13}C-enriched carbohydrate label in a low-^{13}C-enriched individual produces similar results as using a low-^{13}C-enriched carbohydrate label in a high-^{13}C-enriched individual. This corroborates the use of a $^{13}CO_2$ breath test as a tool to monitor liver glycogen oxidation. It also opens possibilities to carry out studies in persons used to a ^{13}C-enriched diet.
This study substantiates again that knowledge about the ^{13}C enrichment of the habitual diet is essential in metabolic studies using naturally ^{13}C-enriched labels.

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APPENDIX

Diet instructions African Group, low ^{13}C-enriched diet for day 2

Breakfast

bread
peanut butter
coffee or tea with “Dutch” beet sugar
margarine
coco-bread

Between Breakfast and Lunch

coffee or tea with beet sugar
and / or cola light
and / or an apple or an orange

Lunch

macaroni or potatoes
beans
“Dutch” tomato sauce
tomatoes
salad made of salad, paprika, apple and oranges
sunflower oil

Between Lunch and Dinner

coffee or tea with beet sugar
and / or cola light
and / or an apple or an orange

Dinner

rice
peas
cabbage with onions baked in sunflower oil
“Dutch” rice mix

desert of apple and orange
coffee or tea with beet sugar
Do not use milk, except for tea and coffee. Do not use any other animal products, no meat, no maize and maize-products, no sugar (except for the sned “Dutch” beet sugar), no cookies, no pineapple.
Salt, pepper, curry and paprika are allowed.
During the fasting period coffee and tea with the Natrena artificial sweetener or cola light are allowed.

Diet instructions for the European Group, ^{13}C-enriched diet during day 2 (Example of 2800 kcal [11700 kJoules] diet)

Breakfast of Porridge Consisting of

150 g	50% skimmed milk
120 g	fresh cream
150 g	water
30 g	custard powder (derived from maize-flour)
38 g	cane sugar

Between Breakfast and Lunch

300 g	50% skimmed milk
30 g	cornflakes
7.5 g	cane sugar

Lunch of Porridge Consisting of

150 g	50% skimmed milk
120 g	fresh cream
150 g	water
30 g	custard powder (derived from maize-flour)
38 g	cane sugar

Between Lunch and Dinner

300 g	50% skimmed milk
30 g	cornflakes
7.5 g	cane sugar

Dinner

150 g	minced meat (beef) fried in 25 g butter
100 g	sweet pepper
70 g	maize for maize polenta
20 g	sauce powder
	Unlimited use of tea or coffee without sugar and milk and non-caloric drinks is al lowed.

4.4

The $^{13}CO_2$ breath test to monitor liver glycogen oxidation in man. A study of its reproducibility.

A. A. Tanis, T. Rietveld, J. L. D. Wattimena, J. W. O. van den Berg
and G. R. Swart.

Submitted

Abstract

Background: Recently we described a $^{13}CO_2$ breath test to monitor liver glycogen oxidation over time. The present study evaluated the reproducibility of this breath test.

Methods: *In six healthy volunteers, liver glycogen was labelled with naturally ^{13}C-enriched carbohydrate. Subsequently the oxidation of this labelled liver glycogen was studied by measuring the changes in ^{13}C-enrichment of breath CO_2 during a period of 24 hours fasting. The procedure was repeated after an interval of six weeks.*

Results: The mean baseline $^{13}CO_2$ abundance did not differ, neither between the two experiments, 1.08215 (sd 0.00014) At% vs. 1.08201 (sd 0.00020) At% (n.s.); mean difference 0,00014 At%, sd_{diff} of 0,00038 At%, nor did it differ from expected values in the Dutch population (1,0820 At%), n.s. The breath $^{13}CO_2$ -enrichment showed a steady state excretion (plateau phase) at 2.62×10^{-3} (sd 0.48×10^{-3}) atom percent excess (APE) in the first series and at 2.53×10^{-3} (sd 0.75×10^{-3}) APE in the second series (n.s.). The end of the plateau phase was 13.2 (sd 1.5) h postprandial in the first and 13.3 (sd 1.5) h in the second experiment (n.s.);

Comparison of both series according to Bland and Altman showed a mean difference of 0.0 h, (sd_{diff} 2.1). Breath $^{13}CO_2$ enrichment returned to baseline values at 23.1 vs. 23.2 h postprandial (n.s.) with a mean difference of –0.2 h, (sd_{diff} 2.8).

Conclusion: The $^{13}CO_2$-breath test to study changes in liver glycogen oxidation in the postprandial period shows reproducible results at the group level, when mean values for selected criteria are taken as endpoints. However, the large standard deviations suggest that measuring at more time points would improve the results. This would also increase the potential for reliable clinical interpretation in testing individual cases.

Key words

^{13}C-carbohydrate
$^{13}CO_2$ breath test
glycogen
natural ^{13}C-enrichment
liver

Introduction and Background

Recently we described a breath test that was designed to monitor the oxidation of liver glycogen over time.[1-3] In this test liver glycogen is labelled with naturally ^{13}C-enriched carbohydrate.[4-6] There is a continuous process of glycogen synthesis and degradation, mainly in fed state conditions, the net result being positive in the fed state and negative while fasting. Part of the glucose moieties released from glycogen degradation will be oxidised, part will be recycled to glycogen or enter other metabolic routes. When liver glycogen is labelled with ^{13}C-enriched glucose, the mobilisation and oxidation of this ^{13}C-labeled glucose results in an increase in ^{13}C-enrichment of the body CO_2 pool. This increase will be reflected in an increase in the ^{13}C-enrichment of breath CO_2. Monitoring this increase in breath $^{13}CO_2$ over time to thereby gauge glycogen oxidation is the concept of the $^{13}CO_2$ breath test. Changes in $^{13}CO_2$ excretion are representative only of changes in that part of liver glycogen that is broken down and oxidised; they do not represent net glycogen breakdown. Glycogen is stored in both liver and skeletal muscle. Thus, muscle glycogen metabolism could interfere with the measurement of liver glycogen mobilisation. However, under sedentary conditions muscle glycogen is hardly utilised, and thus interference should then be negligible [3,6]. Consequently, when the breath test is

performed in the sedentary fasting state, the changes in ^{13}C-enrichment of breath CO_2 can be directly linked to the oxidation of ^{13}C-labelled liver glycogen.
A glycogen oxidation breath test could be used to study net liver glycogen breakdown for energy production in patho-physiological studies in patients with a deranged glycogen metabolism like diabetes mellitus and liver disease. To be applicable, the breath test should provide reproducible results. This was tested in the present study in a group of six healthy volunteers who performed the breath test twice with an interval of six weeks in between.

Methods

Diets

The habitual diet of all participants was a typical West-European diet, which contains a low ^{13}C-abundance [7,8]. All subjects followed a three-day study protocol as shown in figure 1. The details of this protocol have been described previously [1-3]. In short, all participants used their habitual diet (80% of calculated caloric requirements; Harris-Benedict, light activities) during day one. This hypo-caloric intake was chosen to obtain a relative depletion of the liver glycogen stores. The basal ^{13}C-abundance in breath CO_2 was determined during this day. On the second day a standardised labelling diet containing naturally ^{13}C-enriched carbohydrate (120% of the individually calculated caloric requirements; Harris-Benedict, light activities) was consumed. In actual practice, three semi-solid meals (custard pudding, polenta) were used, and some semi-solid “snacks” (corn flakes and milk). This was followed by a period of 24-hours fasting under sedentary circumstances. The composition of the ^{13}C-enriched diet has been described before [1,2].

Measurements

At defined intervals breath samples were collected (figure 1). The CO_2 production was measured by indirect calorimetry (Deltatrac®, Datex, Finland) at 8 AM, at noon and at 4 PM during day three, i.e. after 14, 18 and 24 hours of fasting. The study was repeated after an interval of six weeks. Between the two studies the subjects had no dietary restrictions. The technique of breath sampling has been described before [1,2]. For the ^{13}C-enrichment measurements the ABCA Isotope Ratio Mass Spectrometer (Europa Scientific, Crewe, Cheshire, UK) was used. The calibration was carried out with a certified standard reference gas (GS-17).

Calculations

The calculations used to interpret the data have been described before [1,2]. In short, in these calculations it is assumed that four hours after the last meal, intestinal glucose absorption and direct oxidation will have been completed. This assumption was based on the study of $^{13}CO_2$ gastric emptying breath tests where it was found that the half-time for gastric emptying of semi-solid diets was 23 min indicating that gastric emptying must be practically completed at 2 h. (5 x T/2) postprandial [9] . In practice, four hours is taken as a safe limit point for direct absorption of glucose. A wash-out curve is then calculated, representing the excretion of ^{13}C-labelled CO_2 already present in the body at that point in time (i.e. 4 h postprandial), and supposedly representing oxidation of labelled glucose directly after absorption. Then, any later increase in ^{13}C-labelled CO_2 must arise from oxidation of tissue (i.e. glycogen) derived glucose. This process can be calculated by subtracting the computed

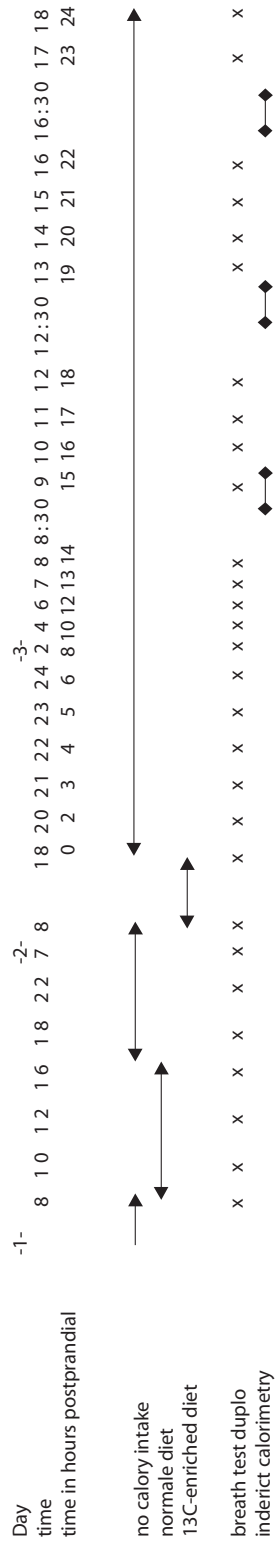


Fig. 1 The study design

washout curve from the actually measured $^{13}CO_2$ excretion. The resulting curve typically shows a plateau in $^{13}CO_2$ excretion, to be followed by a gradual linear decline to baseline levels of excretion. Two characteristics of this curve have been studied, the end of plateau time (EOPT) and the return to baseline time (RTBT). [1-3].
The beginning of the plateau phase was defined by the average $^{13}CO_2$ enrichment measured at 6, 8 and 10 hours postprandial. The end of the plateau phase (EOPT) was defined as the point in time where a decline of more than 10% in $^{13}CO_2$ enrichment was observed. In fact, a moving plateau was calculated using three sequential data points. When the next (fourth) point showed a value more than 10% lower than the preceding plateau, the last point of the three sequential points was designated as the end of plateau. The data following the end of the plateau phase were analysed with linear regression analysis. The time at which the regression line reached the initial baseline value was defined as RTBT.
The study protocol was approved by the hospital medical ethics committee.

Statistical analysis

The mean results, the standard deviations, the mean differences between groups and the standard deviations of the mean differences were computed. The paired T-test and the Bland-Altman analysis for repeated measurements [10] were performed to compare the two experiments. For the T-test a p-value of ≤ 0.05 was considered to be significant. In the Bland-Altman analysis the 95% range of agreement for individuals was calculated by multiplying the standard deviation of the difference by 1.96. This range of agreement was then viewed in relation to the mean value and the mean difference of the mean. Based on this information, a decision on acceptability was made.

Results

Six healthy volunteers, 1 female and 5 male, participated in the study. The mean age was 23.5 (SD 1.5) year, the mean height 1.79 (SD 0.10) meter, the mean weight 69.7 (SD10.0) kg and the mean body mass index was 21.7 (SD1.5) $kg \cdot m^{-2}$.
The results of the repeated breath tests in all 6 individuals are stated in table 1. A detailed example of the measured and calculated data of a single breath test in one individual is shown in fig. 2. An overview of all repeated $^{13}CO_2$ excretion curves in the 6 subjects is depicted in figure 3.
The mean basal $^{13}CO_2$ abundance during day one was not different in the first and the second experiment, 1.8257 and 1.0821 At% (n.s.). This level of enrichment is not different from previously measured values (1.0820 At%; n.s.) in the Dutch population. At day two after the consumption of the ^{13}C-enriched diet the mean maximum rise in ^{13}C-enrichment in breath CO_2 was $5.7x10^{-3}$ APE (SD $0.3x10^{-3}$) for the first and $5.4x10^{-3}$ APE (SD $0.9x10^{-3}$) for the repeated experiment (n.s). The mean ^{13}C-enrichment at plateau phase was 2.62×10^{-3} APE (SD 0.48×10^{-3}) and 2.53×10^{-3} APE (SD 0.75×10^{-3}) respectively. Taking the 4 h postprandial cut-off point, the end of plateau time was observed at 13.2 (SD 1.5) h. and 13.3 (SD 1.5) h. postprandial in the repeated studies (n.s.). The return to baseline time was 23.1 (SD 2.4) h. postprandial and 23.2 (SD 1.7) h respectively.
The Bland-Altman analysis was performed showing mean differences of 0 and –0.2 hours for EOPT and RTBT times respectively (fig 4). The CO_2 production at 14, 18 and 22 h postprandial in the first series was 192 (SD 33.8) ml/min at 14 h, 187 (SD 24.3) ml/min at 18 h and 193 (SD 19.8) ml/min at 22 h. In the second series these values were 187 (SD 20.7) ml/min, 186 (SD 22.0) ml/min and 188 (SD 19.7) ml/min respectively. These differences are not significant; Individual results of CO_2 production measurements can be read from table 3.

	Baseline 13CO2		13CO2 at plateau		End of plateau time		Return to baseline time	
	in At%		in APE		in h postprandial		in h postprandial	
Exp	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Subj.1	1.08214	1.08182	0.00255	0.00287	13	13	25.6	25.2
Subj.2	1.08218	1.08221	0.00290	0.00313	12	12	22.1	24.4
Subj.3	1.08218	1.08173	0.00178	0.00260	12	10	19.2	23.2
Subj.4	1.08221	1.08229	0.00242	0.00120	13	12	22.8	20.5
Subj.5	1.08162	1.08208	0.00295	0.00220	15	14	25.3	22.2
Subj.6	1.08257	1.08201	0.00309	0.00319	10	14	23.6	23.8
Mean	1.08214	1.08182	0.00255	0.00287	12.5	12.5	2.32	3.2
SD	0.00030	0.0002	0.00048	0.00075	1.6	1.5	2.4	1.7
Mean diff	0.00014		0.00008		0		-0.1	
SD diff	0.00038		0.00075		2.1		2.7	

Table 1. The repeated breath tests in six individuals, showing the individual and the mean results on the four main features of the test: the baseline $^{13}CO_2$ abundance in atom percent, the $^{13}CO_2$ enrichment at plateau level in atom percent excess (APE), the end of the plateau time (EOPT) and the return to baseline time (RTBT) in hours postprandial. The mean difference and the standard deviation of the difference are given.

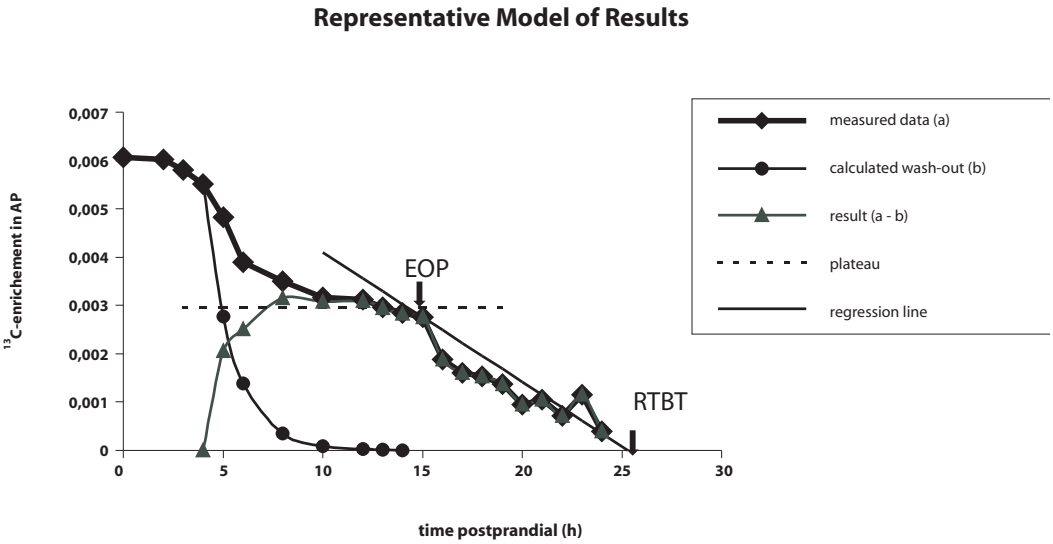


Figure 2.
An example of the results of the breath test in one of the individuals. The bold line with the diamonds indicates the measured values. The dotted line indicates the calculated elimination curve of the $^{13}CO_2$ produced starting at four hours postprandial. The line with the triangles results from subtraction of the $^{13}CO_2$ elimination curve from the measured $^{13}CO_2$ data curve. The horizontal line is plotted at the plateau level. "EOP" indicates the end of plateau time (EOPT). The point where the regression line crosses the X-axis is the return to baseline time (RTBT).

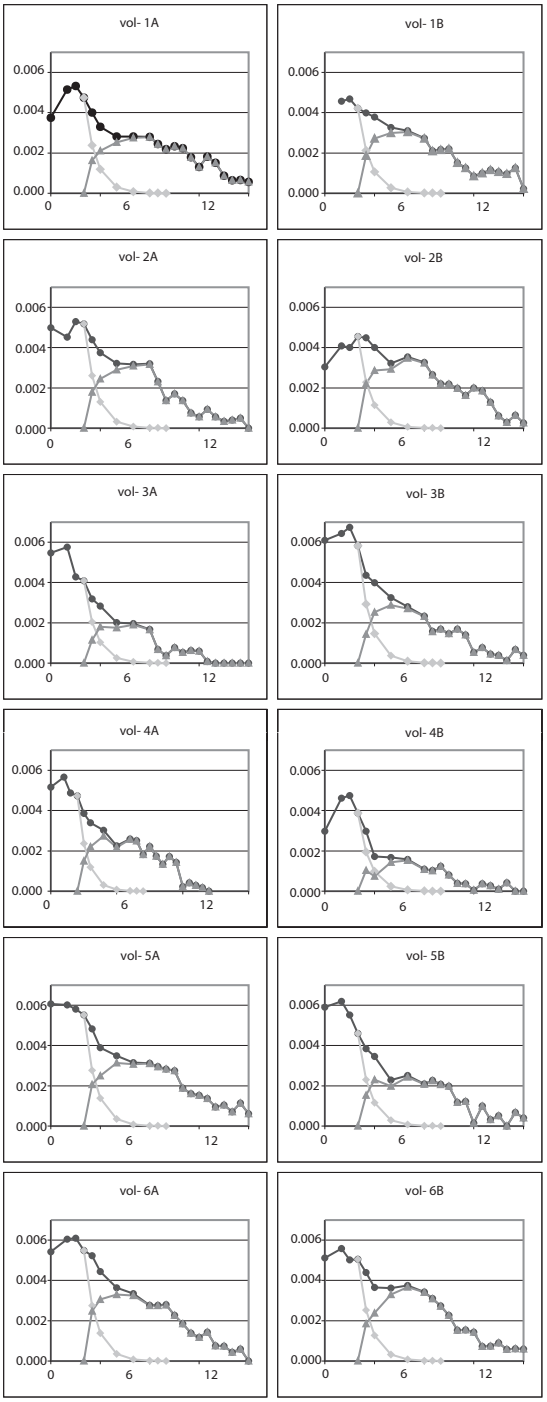


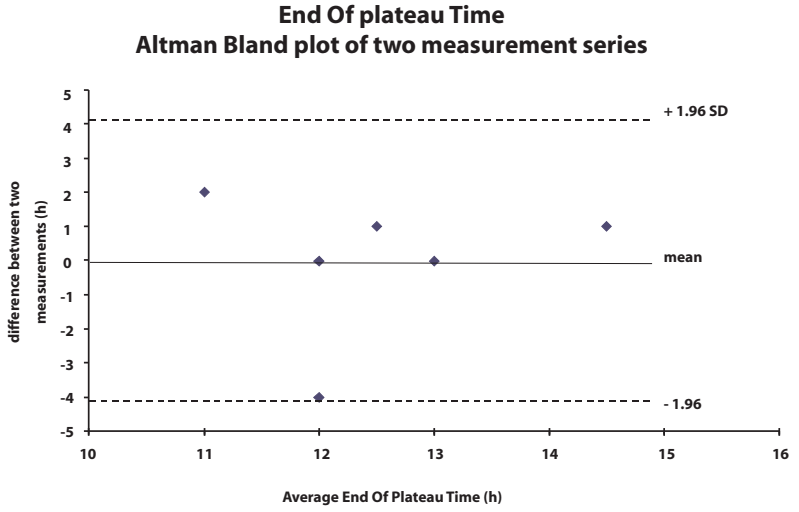
Figure 3.

The ^{13}C-data of the two experiments are combined to show the differences in a graphic way (vol A , B). The dotted lines show the measured data, the diamonds show the calculated elimination and the triangles show the resultant after the calculated elimination data were subtracted from the measured data.

The x-axis represents hrs postprandial. The y-axis represents $^{13}CO_2$ as APE in breath.

Table 2
CO_2 production – comparison of two series

Time postprandial experiment		14 hr 1 st	2nd	18hr 1st	2nd	22hr 1st	2nd
Mean VCO2 ml/min	191.7	199.2	187.0	185.7	193.2	197.7	
SD		33.8	24.3	20.7	22.0	19.8	19.7
Mean diff in ml/min			2.5		1.3		5.5
SD diff			15.0		7.0		14.0



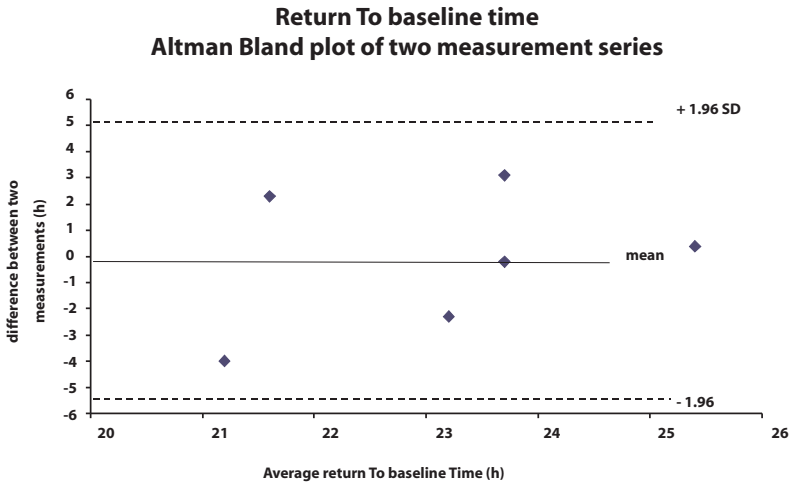


Figure 4.
Two series of glycogen breath tests compared with the Bland-Altman technique. Results of the end of plateau time (EOPT) and Return to baseline time (RTBT) are plotted.

Discussion

Recently we described a $^{13}CO_2$ breath test to monitor the oxidation of liver glycogen in the fasting state [1-3]. This test measures net ^{13}C-labelled glycogen consumption for energy production. It is well known that in the fed state there is extensive recycling of glucose moieties, with up to 50 % of just liberated glucose being used anew for glycogen synthesis. In the fasting state, recycling continues, be it at a low level [11]. It may thus be presumed that this cycling process will not appreciably reduce the value of a $^{13}CO_2$-breath test that is performed in fasting state conditions. Since glucose, liberated from glycogen, can either be oxidised, used in other metabolic processes, or be recycled to glycogen again, a breath test measures only net glycogen use (i.e. oxidation). Because it is unlikely that ^{13}C-glucose originating from ^{13}C-glycogen breakdown will be selectively used for processes other than oxidation, and since cycling is low during fasting, the end of $^{13}CO_2$ excretion in the breath test may be taken as the end of previously deposited ^{13}C-labelled glycogen. In this breath test the liver glycogen is labelled with naturally ^{13}C-enriched carbohydrate. During subsequent fasting the excretion of ^{13}C-enriched CO_2 in breath almost exclusively reflects the oxidation of labelled glycogen derived glucose. It has been demonstrated that the contribution from other potentially labelled sources like protein and fat can be ignored, even after three days of labelling [3]. When this test is performed under sedentary conditions, when there is hardly any mobilisation of muscle glycogen [3], it is actually liver glycogen oxidation that is being monitored. Thus, the performance of a $^{13}CO_2$-breath test is relevant to study when one is interested in liver glycogen stores. The present study was performed to evaluate the reproducibility of this breath test.

Six healthy volunteers followed the same protocol twice with a period of six weeks in between. Since no dietary restrictions were imposed on the subjects either before the first test or in the interval between the two tests, the basal ^{13}C-enrichment of the breath CO_2 measured during the two experiments reflects the basal ^{13}C-enrichment of the habitual diet. The observed ^{13}C-enrichment compared well with the known values of the West-European society [8]. This relative low basal ^{13}C-enrichment is a prerequisite for this breath test in which the increase in ^{13}C-enrichment through labelling with naturally enriched material is rather low [7]. In our repeated test, there was no effect noticeable on basal ^{13}C-enrichment as a result of the labelling during our first study. It is unlikely that during our studies ^{13}C-labelled material, after incorporation in fat or protein and subsequent oxidation of that new product, will have contributed to $^{13}CO_2$ formation and thus contaminated our results. A higher basal ^{13}C-enrichment of the breath CO_2 would then have been observed.

Comparing the results of the two experiments there were no significant differences in end of plateau time or return to baseline time when the paired t-test was applied. This indicates that the test will give reproducible results when groups of individuals are studied. Plateau values, according to the protocol, were calculated on three measured values for $^{13}CO_2$ excretion; standard deviations of the mean value were rather large. Consequently, any errors in these measurements will have a relatively large impact on the resulting plateau level. In future studies, more frequent breath sampling should be considered to improve the data.

For clinical purposes the end of the plateau phase and the return to baseline time are of special interest. The first indicates a decline in the availability of (labelled) liver glycogen for oxidation. Assuming ongoing and equal energy expenditure, this suggests recruitment at that point in time of other metabolic routes for energy production, like an increase in the rates of gluconeogenesis and lipolysis. The latter indicates the time when the contribution of (labelled) glycogen reaches such a low level that it cannot be measured anymore, suggesting the end of the availability of labelled glycogen for oxidation. Frequent sampling especially around those two points in time could delineate those points in time with even more precision.

The Bland - Altman analysis showed quite a wide range in the standard deviation of the difference. However the mean differences of repeated measurements for End of Plateau time and Return to Baseline time were almost zero.

Conclusions

We conclude that the naturally ^{13}C-labelled breath test is a reproducible tool for studying groups of individuals. However, more frequent measurements are indicated to get a better definition of the end of plateau and return to baseline times. This might improve the test variation to a level where is will be applicable in individual cases.
The $^{13}CO_2$ breath test is an interesting and promising tool to study the oxidation of the liver glycogen during fasting as a (patho-)physiological or clinical test in patients suspected of having a deranged liver glycogen storage capacity.

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4.5

The $^{13}CO_2$ breath test for liver glycogen oxidation after a three-day labelling of the liver with a naturally ^{13}C-enriched diet.

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Nutrition 2003;19: - -

Abstract

OBJECTIVE: When naturally ^{13}C-enriched carbohydrate is used to label hepatic glycogen, ^{13}C-liver glycogen oxidation can be monitored subsequently by measuring the ^{13}C-enrichment of breath CO_2 during a sedentary fast. In our previous breath test studies, we used a 1-d labelling protocol to enrich liver glycogen. Others found that after 3-d of labelling the liver glycogen ^{13}C enrichment is identical to the dietary carbohydrate ^{13}C enrichment.

METHODS: We compared a diet protocol in which naturally ^{13}C-enriched carbohydrate was given for 3 d before the breath test with our previously applied 1-d labelling design. The $13CO_2$ breath test was combined with indirect calorimetry. The results were compared with those from our previous studies. In addition, we compared liver glycogen oxidation rates with those from our present technique and different techniques as used in other published studies.

RESULTS: Six healthy volunteers were included in this study. The ^{13}C enrichment of breath CO_2 at the plateau level excretion level did not differ after 1 or 3 d on a labelling diet. However, the end of plateau time tended to be later after the 3-d diet, 14.3 h versus 12.5 to 13.5 h postprandially in the 1-d labelling studies. Also, the return to baseline time was later in the 3-d study, at 25.8 h versus 19.0 to 23.2 h postprandially after 1 d of labelling. The liver glycogen oxidation rate was similar in both techniques until 17h postprandially. After this time the 3-d labelling protocol showed a higher level of liver glycogen oxidation.

CONCLUSION: The results indicated that the labelling of liver glycogen is slightly less complete after 1 d on a ^{13}C-enriched diet as compared with 3-d labelling. Our ^{13}C breath test results compared rather well with studies from the literature using the ^{13}C-NMR technique, the D_2O technique, or the $^{13}CO_2$ breath method to measure liver glycogen oxidation.

KEY WORDS:

$^{13}CO_2$, breath test, naturally ^{13}C-enriched diet, glycogen oxidation, glycogenolysis, liver

INTRODUCTION

We previously described a non-invasive way to monitor liver glycogen oxidation.[1-4]

In this method the body glycogen stores are labelled with a diet that contains ^{13}C-enriched carbohydrate. When the labelled glycogen is oxidized, $^{13}CO_2$ is produced and the ^{13}C-enrichment of the CO_2 pool will increase. This increase can be measured in breath CO_2.

During a sedentary fast muscle glycogen is barely used.[3,5] Thus, under those conditions, the increase in ^{13}C-enrichment of the CO_2 is caused by the oxidation of liver glycogen. Therefore the ^{13}C-enrichment of breath CO_2 can be used as a parameter for liver glycogen oxidation during a sedentary fast.

In our previous experiments, the period during which glycogen is labelled with naturally ^{13}C-enriched carbohydrate was 1 d. On the day before the ^{13}C-labelling, subjects used a hypo-caloric diet (80% of Harris-Benedict calculated energy requirements). We assumed that such a diet, followed by an overnight fast, would adequately deplete the liver glycogen stores. During the day of labelling, a hyper-caloric diet (120% of Harris-Benedict calculated energy requirements) was consumed to ensure an ample supply of carbohydrate to refill the glycogen stores.

Gay et al. demonstrated that on a ^{13}C-enriched carbohydrate diet the ^{13}C-enrichment of liver glycogen is identical to the dietary ^{13}C-enrichment after 3 d.[6]

In contrast to our study design, they did not attempt to deplete the liver glycogen pool before they started the 13C-diet. Their experience implicated that the labelling period of 1 d, which we used in our experiments, was to brief. Consequently, we could have calculated erroneously low rates of liver

glycogen oxidation.
The present study addresses this issue. The breath test was carried out after 3 d of labelling the glycogen pool. During these 3 d, an iso-caloric (100% Harris-Benedict calculated energy requirements), naturally ^{13}C-enriched carbohydrate diet was consumed. The results were compared with those obtained in our previous studies, which were based on a one-day labelling period.

MATERIALS AND METHODS

Subjects

Six healthy volunteers, two male and four female, participated in the study. Their mean (± standard deviation) age was 24.2 (1.7) year, the mean height was 176.7 (10.5) cm, the mean weight 65.2 (12.1) kg, and the mean body mass index was 20.8 (2.5) kg/m^2. All participants were all in good physical condition and they did not take any medication. They habitually consumed a low ^{13}C-abundance (European) diet. Before the study there were no diet restrictions. All gave informed consent. The local ethics committee approved the protocol.

Subjects in control studies

Data from three of our previous studies were compared with the present breath test results. Study 2,[4] (Table I) was performed in 12 healthy subjects, six male and six female, with a mean age of 36.3 (6.1) y, mean height of 178 (9.9) cm, mean weight of 75.6 (10.0) kg, and an mean body weight of 23.7 (2.2) kg/m^2. Twelve healthy individuals, six male and six female, were included in study 3,[2] with a mean age of 36.3 (6.1) y, mean height of 178 (9.9) cm, mean weight of 75.6 (10.0) kg, and an mean body weight of 23.7 (2.2) kg/m^2. In study 4,[3] six healthy subjects, five male and one female, participated, with a mean age of 23.9 (1.4) y, mean height of 178.7 (10.5) cm, mean weight of 69.7 (10.0) kg, and a mean body mass index of 21.8 (1.5) kg/m^2. As a control group for the glycogen oxidation rates, subjects from study 2,[4] were used.

Study protocol

The study protocol is shown in Figure 1. Background and details about the study diet have been described previously.[1,2] The study was carried out over a period of 4 d. The night before the start of the ^{13}C-enriched diet, the subjects fasted from midnight onward. Basal $^{13}CO_2$-enriched breath samples were taken at the start of day 1. The ^{13}C-enriched diet was consumed between 8 a.m. and 6 p.m. on day 1, 2 and 3.

Indirect calorimetry

Energy expenditure was measured by indirect calorimetric measurements (Deltatrac®, Datex, Finland) every morning before breakfast and 3-hourly during the 24 hour sedentary fasting period, which started at day 3 at 6 p.m.

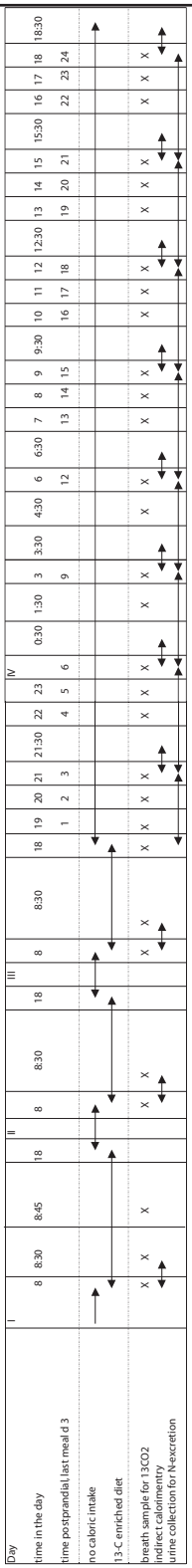

Fig. 1. Outline of the study protocol

The naturally ^{13}C-enriched carbohydrate diet

Caloric intake was calculated (Harris-Benedict, light activities) and all subjects consumed the prescribed amount during all study days. Calorie-free drinks were allowed ad libitum.
The study-diet consisted of 60% (wt/wt) carbohydrate, 27% fat and 13% protein. Eighty percent of the carbohydrate was naturally ^{13}C-enriched and came from corn and sugar cane. The overall calculated ^{13}C-enrichment of the carbohydrate in the diet was 1.093 atom percent (at%; the usual Western European diet has a ^{13}C-enrichment of 1,082 at%).[7,8]

Breath sampling

Alveolar air samples were collected in duplicate in 20-mL Vacutainer tubes (Becton Dickinson Vacutainer Systems Europe, Plymouth, UK). After the rubber plug was removed a drinking straw was inserted. After a normal inhalation, the subject quietly exhaled through the straw. The straw was removed and the tube closed.

Isotope analysis

An Isotope Ratio Mass Spectrometer (ABCA, Europe Scientific, Crewe, Cheshire, UK) fitted with an autosampler was used to measure the ^{13}C-enrichment in breath CO_2 samples. The measured isotope ratio was reported as atom percent ^{13}C in CO_2.
A certified reference gas standard (GS-17) was used for calibration.
The $^{13}CO_2$ enrichment after an overnight fast on days 1, 2, 3, and 4 were expressed in atom percent. All other data were expressed in atom percent excess of ^{13}C above baseline.

Calculations

The baseline ^{13}C abundance in breath CO_2 was defined as the $^{13}CO_2$ abundance on day 1 before breakfast. Combining the half-life time of the CO_2 pool (60 minutes)[9] with the assumption that the absorption of carbohydrate from the gut is completed four hours after the last meal, the contribution of liver ^{13}C-glycogen toward ^{13}C-enrichment of breath CO_2 could be calculated as described previously.[1,2]
The $^{13}CO_2$ enrichment at plateau level, the duration of the plateau phase, the subsequent decline in $^{13}CO_2$ and the moment when enrichment decreased to baseline $^{13}CO_2$ value were used to describe the various phases of liver glycogen catabolism.
Glycogenolysis was calculated from the ^{13}C-enrichment of breath CO_2 and the respiratory gas exchanges. Three considerations were made. The first consideration was that, in 3 d, the diet would not affect the ^{13}C-enrichment of the body lipids and proteins; this was demonstrated by Gay et al.[6] The second one was that muscle glycogen would not significantly contribute to the carbohydrate oxidation during a short sedentary fast. This was found in one of our previous studies in which we created a higher level of ^{13}C enrichment of muscle glycogen; this higher enrichment did not alter the outcome of the test.[3] Third, ^{13}C enrichment of the liver glycogen pool was assumed to be equal to the ^{13}C enrichment of the carbohydrate of the ingested diet, 1.092 at%, once steady basal CO_2 ^{13}C enrichment had been reached (at day 3 in this study).

The rate of liver glycogen oxidation could then be calkculated as:

(measured ^{13}C enrichment of CO_2 – basal ^{13}C abundance of CO_2)/(^{13}C enrichment of liver glycogen –basal ^{13}C abundance of CO_2)

Because the RQ for carbohydrate is 1 and the oxidation of 1mg of glycogen requires 0.829 mL of O_2, the amount of labelled glycogen that was oxidized could be calculated as:

Glycogen (mg/min) = (rate of liver glycogen oxidation x CO_2 consumption per min)/0.829

Comparison of the results with data from our previous studies:

We used historical controls from three of our previous studies. These studies had in common a ^{13}C-labelling period was 1 day and energy intakes of 80% of the required energy intake on prelabelling day 120% during the day of labelling.
The protocol of the study in which the repeatability of the breath test was studied was identical to the present study with the exception of the labelling period (Table I, study 2[4]). In study 3 and 4 (Table I), no CO_2 production was measured. In this comparison only the data from the European group study 3[2] and from the non-cycling group in study 4[3] were used.

Statistical analysis

Mean values and standard deviations were calculated and compared with data from our previous studies. For comparison within the study group, paired t test was used. $P < 0.05$ was considered statistically significant. To compare between two different groups the two-sample t test was applied. $P < 0.05$ was considered to be statistically significant.

RESULTS

^{13}C-enrichment
At the start of the study (before breakfast at 8 a.m.) the ^{13}C-abundance in breath CO_2 was 1.0819 (0.0005) at%. This value gradually increased during the following 3 d, when measured at the same point before breakfast. At day 2it was 1.0838 (0.0007); $P < 0.001$ versus day 1); at day 3 it was 1.0845 (0.0003; $P < 0.01$ versus day 2); and at day 4it was 1.0845 (0.0006; not significantly different from day 3; Fig. 2). The postprandial ^{13}C-enrichment data are shown in Figure 3. The maximum rise in breath $^{13}CO_2$, 0.0065 (0.0011) at% excess, was reached 2.8 (0.4) h postprandially during day 3. The mean plateau level was 0.0030 (0.0001) at% excess. The end of plateau was observed at 14.8 (1.2) h postprandially. The calculated return to baseline time was 25.8 (2.1) hours postprandial with a regression line correlation coefficient of 0.90 (0.06).

Blood glucose levels
During the fasting period the blood glucose levels initially were steady. However, after 14 h postprandially, a significant decline in blood glucose level was observed (Fig. 4).

Glycogen oxidation rates

The glycogen oxidation rate calculated from the $^{13}CO_2$ enrichment and the measured CO_2 production are presented in Figure 5, left. For comparison, the results of our study 2[4] are presented in Figure 5, right. The oxidation rates in both studies correlated well for the first 17 h postprandially. After 17 h the rates were at least 10% higher in the 3-day labelling group (Fig. 6).

Comparison with the other ^{13}C-breath test studies

In general the findings in the present study compare quite well with those of our previous studies. The end of plateau time and the return to baseline time were significantly shorter in study 2.[4] The present results versus those of study 3[2] showed that the peak ^{13}C enrichment and the ^{13}C enrichment at plateau level were higher in the present study and the return to baseline time was significantly longer (Table I).

DATA OF THE PRESENT STUDY COMPARED WITH OUR THREE PREVIOUS STUDIES*

	present study (n = 6)	Study 2[4] (n = 12)	study 3[2] (n = 12)	study 4[3] (n = 6)
Basal abundance (at%)	1.0819 (0.0005)	1.0821 (0.0006)	1.0820 (0.0006)	1.0816 (0.0007)
Maximum rise in enrichement (APE)	0.0065 (0.0011)	0.0056 (0.0007)	0.0048 (0.0010)	0.0059 (0.0013)
Maximum rise in enrichement (h postprandial)	2.83 (0.41)	2.67 (0.65)	2.67 (0.98)	2.20 (1.20)
enrichement at plateau level (APE)	0.0030 (0.0001)	0.0026 (0.0006)	0.0020 (0.0007)	0.0026 (0.0005)
End of plateau (h postprandial)	14.3 (1.20)	12.5 (1.51)	13.5 (1.17)	12.8 (1.17)
Return to baseline time (h postprandial)	25.8 (2.06)	23.2 (1.95)	19.0 (1.79)	22.9 (2.45)

*Data are expressed as mean (standard deviation). APE, atom percent excess; at%, atom percent

Table 1. Data of the present study compared with our three previous studies. Data are expressed as mean (standard deviation). APE, atom percent excess; at%, atom percent

* p value ≤ 0.01, # p value ≤ 0.05 using the two sample t-test.

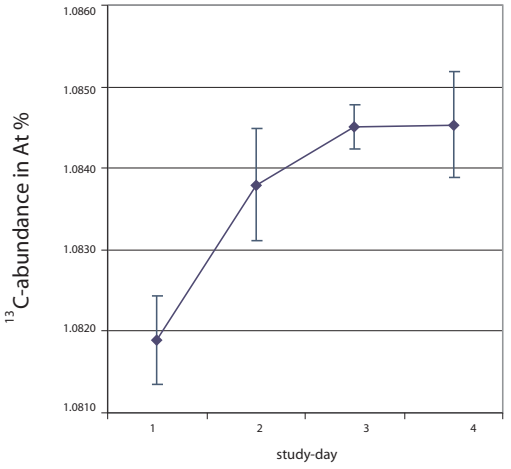

Fig.4-5 1. Outline of the study protocol.

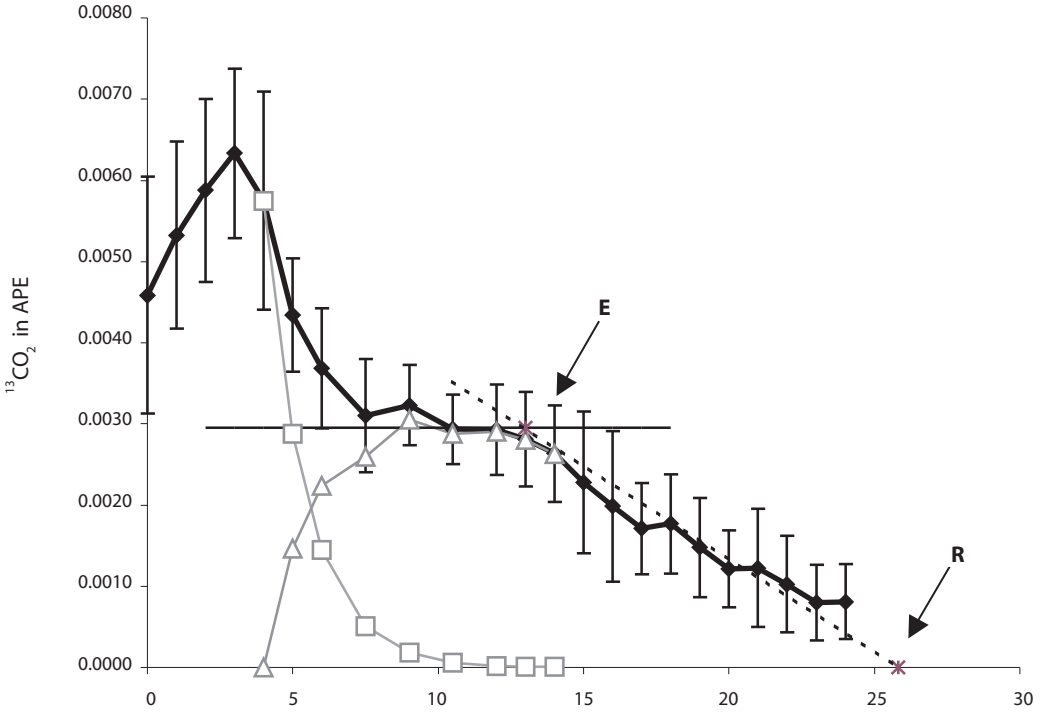

Fig. 2. ^{13}C-enrichment of breath CO_2 after an overnight fast at the start of the study (study-day 1) and after the ^{13}C-enriched diet had been consumed for 1, 2, and 3 d (study-day 2, 3 and 4).

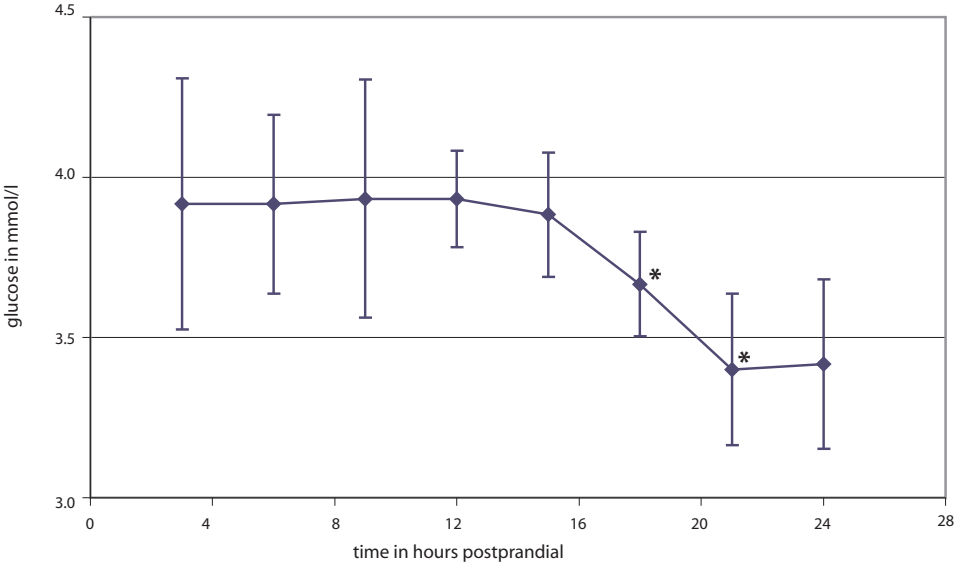

Fig. 3. ^{13}C-enrichment of breath CO_2 in APE above baseline presented as mean and standar deviation. The black dots (●) represent the measured data, the open squares (❑) represent the calculated wash-out curve of the $^{13}CO_2$ present at 4 hours postprandial and the open triangles (Δ) indicate the results of the subtraction of the wash-out curve from the measured data. Open triangles also represent the ^{13}C-enriched liver glycogen oxidation. APE, atom percent excess; **E**, end of plateau time; **R**, return to baseline time.

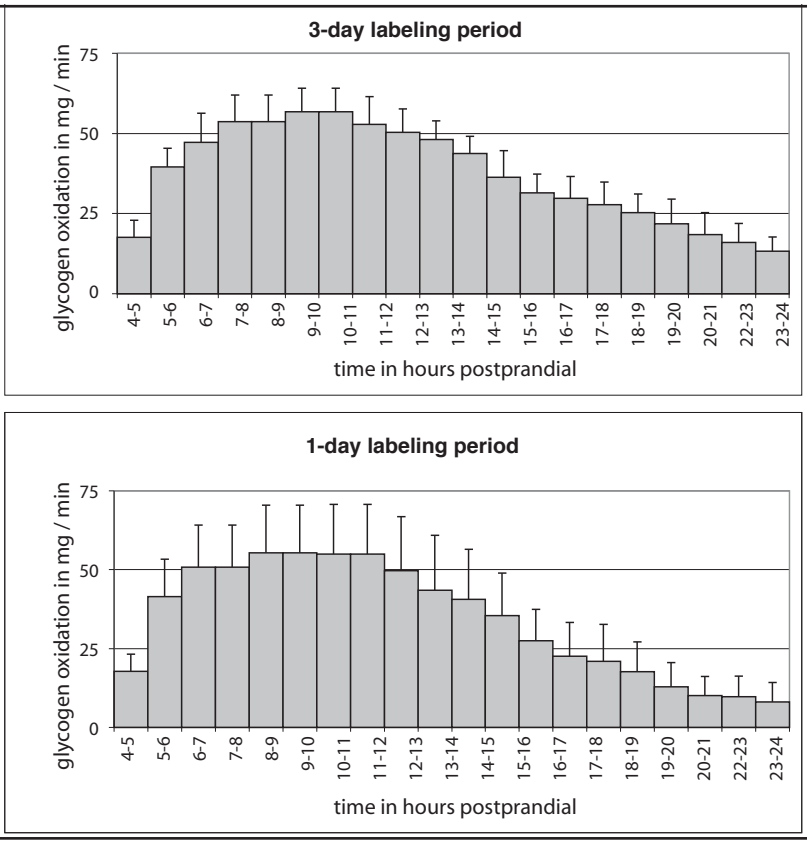

Fig. 4. Postprandial blood glucose levels measured every 3 h for 24 h starting 3 h after the last meal. Values are expressed as mean and standard deviation. *$P \leq 0.05$ by *t* test when data are compared with the previous value in time.

Fig. 5. Liver glycogen oxidation rates calculated from the data from the present, 3-d labelling study (n=6), and from the 1-d labelling study[4] (n=12). The study design for both studies was identical with the exception of the ^{13}C-labelling time.

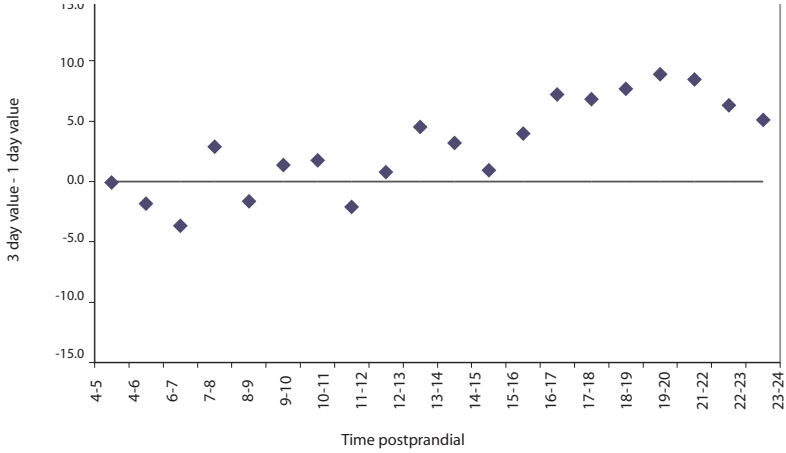

Fig. 6. Difference in liver glycogen oxidation rates (mg/min) between the 3-d labelling (n=6) and the 1-d labelling study[4] (n=12). The 3-d labelling data were used as reference values. From 17 hours postprandially onward, the difference was greater than 10% of the actual oxidation rate.

DISCUSSION

In our previous studies we explored the possibility of monitoring liver glycogen oxidation with a breath test and a diet of naturally ^{13}C-enriched carbohydrate. The present study assessed the effect of prolonged, 3 d, labelling on the outcome of this breath test. The present 3-d and previous 1-d findings were compared.

The results of the present study showed a basal ^{13}C-abundance at the start of the study that was within the range of the Dutch (West-European) population.[2,8,11] While the subjects used the study diet, the fasting (8 a.m.) $^{13}CO_2$ measurements showed an increase at days 2 and 3. A steady state was reached at day 3 (Fig. 2). This was in agreement with the presumption of the study design, which was based on the results of Gay et al.[6] It indicated that, after 1 day of labelling, the ^{13}C enrichment of liver glycogen is not yet maximal. It cannot be excluded that, after 3 or even 5 d of labelling, the core of the glycogen granules would not be fully labelled with ^{13}C.[6] It has been suggested that the core of the glycogen granules has a different pattern of metabolism.[12]

The means and standard deviations of the measured breath $^{13}CO_2$ enrichment on day 4, the day of fasting after 3 d on a labelling diet, are shown in Figure 3, where the curve strongly resembles the curve we found in our earlier studies.[1-4] The plateau level indicates a constant flux of enriched $^{13}CO_2$. We interpret this as a constant liver glycogen oxidation rate during that period. The plateau phase and the decline in ^{13}C-enrichment after 13 to 14 h postprandially are consistent findings in all our studies.

However, the end of plateau time in the present study was later in one of the three comparative studies and the return to baseline time was later than in two of the three comparative studies. Thus the interpretation of the $^{13}CO_2$ curve changed slightly with the longer labelling protocol.

After 17 h of fasting the blood glucose level showed a steady decline from 3.9 mM/L to 3.4 mM/L (Fig. 4). We have no explanation for this phenomenon. However, it is an interesting observation because the decline in $^{13}CO_2$ enrichment started simultaneously. Whether or not this is just a coincidence can only be guessed. The exact mechanisms through which hepatic glucose output (glycogenolysis and gluconeogenesis) are regulated are not completely clear.[13]

The calculated glycogen oxidation rate compared well with the data from the previous study for the first 16 h postprandially (Figs. 5 and 6). The higher glycogen oxidation rates after 16 h fasting might be explained by a deeper penetration of the ^{13}C-label into the glycogen granules after 3 d on a labelling diet. This would mean that after a 1-d labelling procedure the liver glycogen granules are not yet completely labelled. After an overnight fast the liver of a healthy individual still holds 70 to 150 gram of glycogen. Part of this will be consumed during the day, and glycogen formation will occur as soon as feeding starts again. Glycogen formation and glycogenolysis take place at the same time. Thus, after 1 d of consuming a ^{13}C-enriched diet, a rather homogeneous mix of labelled and unlabelled glycogen can be expected.

In our original 1-d labelling protocol the day before the ^{13}C labelling was administered, energy intake was restricted to 80% of the calculated energy need. This was done to achieve greater depletion of the liver glycogen pool during the subsequent night of fasting. The present study showed that such a restriction is not sufficient to reach a level of ^{13}C-enrichment of liver glycogen that equals the ^{13}C enrichment after 3 d on a labelling diet. Recent studies have suggested that a more extensively depleted glycogen pool induces the uptake of glucose for glycogen formation.[14,15]

The extent of mixing of ‘old’ and ‘new’ glycogen is still a matter of debate.[16]

Some studies have suggested a last-in first-out pathway,[17,18] but other studies do not confirm this.[16,19-21]

Do these findings have consequences for the future design of a glycogen-consumption breath test? The present findings indicated that, with the 3-d labelling period, a higher ^{13}C-enrichment of liver glycogen is reached. This is reflected in a slightly higher glycogen oxidation rate. The absolute dif-

ferences are rather small, however, and observed only after 17 h postprandially. Whether this difference will be relevant in the clinical interpretation of the test results will have to be shown in future (patho-) physiological studies. The 3-d labelling protocol certainly makes the test longer and more cumbersome.

We compared the present results with those of some recent studies that focused on liver glycogen depletion. The overall glucose production is usually agreed to be about 2 mg □ kg^{-1} □ min^{-1} in healthy adults. This corresponds to 140 mg □ min^{-1} for a 70 kg person. In our study the estimated glycogen oxidation was 55 mg/min over 6 to 12 h postprandially. This amounts to 40% of those 140 mg/min. The contribution of gluconeogenesis must therefore be about 60%. This last figure fits well with the estimates for gluconeogenesis in the NMR study of Peterson et al. of 55 ± 6% between 6 and 12 h.[22] Hundal et al., estimated a contribution of gluconeogenesis to glucose output of 50% by NMR and about 50% by the D_2O technique over 15 h.[23] Gay et al., who also performed breath test studies, described a contribution of gluconeogenesis of 51 ± 5%.[6] These results from the literature accord well with our findings concerning liver glycogen oxidation rates in this study.

Of the presently available techniques to measure liver glycogen content, NMR seems to be the most direct one. It could be considered the current gold standard. With repeated NMR studies, net changes over time in glycogen content can be measured. In combination with other techniques such as stable isotope studies of the total glucose production and the rate of gluconeogenesis, the kinetics of liver glucose metabolism can be further delineated.[18,22,23] The $^{13}CO_2$ breath test only measures the oxidation of labelled liver glycogen. Simultaneous synthesis of glycogen or gluconeogenesis from non-labelled substrate could occur. If such non-labelled glycogen or non-labelled glucose synthesized through gluconeogenesis is subsequently oxidized during the breath test, this will not be detected. Indeed, the $^{13}CO_2$ breath test only measures the oxidation of ^{13}C-enriched glycogen that is present in the liver at the end of the labelling period. However, because no significant cycling has been observed in fasting healthy subjects,[18] the breath test might be a valid clinical tool to study the contribution of liver glycogen to energy availability and production under fasting conditions.

It would be interesting to make a direct comparison between the $^{13}CO_2$ breath test as a tool to study liver glycogen metabolism with the NMR technique and the isotope measurements of total glucose production and gluconeogenesis.

Obviously, there could be some ^{13}C contamination in other energy sources such as fat or protein that could contribute to ^{13}C-labelled CO_2 production under fasting state conditions. This phenomenon is probably reflected in the basal ^{13}C enrichment if the CO_2 pool that is not solely dependent on labelled carbohydrate oxidation. However, Gay et al. demonstrated that the ^{13}C abundance of plasma proteins and very low-density lipoproteins remains constant even during 5 d of use of a ^{13}C-enriched labelling diet.[6] Thus, we believe that the oxidation of labelled substrates other than liver glycogen is not a problem in our study design.

The breath test has the advantage of being a rather simple, investigatory tool suitable for clinical application. In this context and for practical reasons, a 1-d labelling period might be preferable to a 3-d diet.

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Chapter 5

An orientation in cirrhotic patients

5.0

A $^{13}CO_2$ breath test to assess liver glycogen oxidation.

An orientation in patients with cirrhosis of the liver.

A.A. Tanis, J.W.O. van den Berg, T. Rietveld, J.L.D. Wattimena and G.R. Swart.

Part of this study has been published in “Advances in hepatic encephalopathy & metabolism in liver disease”. Eds. Christopher Record and Hanan Al-Mardini. ISA 1996, Medical Faculty, University of Newcastle upon Tyne, Newcastle upon Tyne. Chapter 7. “Depletion of liver glycogen in cirrhosis using a ^{13}C-enriched diet and $^{13}CO_2$ breath samples”, AA Tanis, JWO van den Berg, T Rietveld, JLD Wattimena and GR Swart.

Abstract

In a pilot study the $^{13}CO_2$ breath test to assess liver glycogen oxidation was applied in 12 healthy volunteers and 12 patients with advanced liver cirrhosis. Patients with liver cirrhosis are thought to have a small liver glycogen pool, resulting in a more rapid change from glycogenolysis to gluconeogenesis during fasting. We used the blood glucose response to intravenous glucagon as an indicator of reduced hepatic glycogen storage. The insulin : glucagon ratio was monitored in the postprandial period in both patients with cirrhosis of the liver and in healthy subjects. Both insulin and glucagon values were increased in cirrhosis. However the insulin : glucagon ratio was lower in cirrhosis. After 10 hours fasting this ratio could be used to discriminate between the two groups.
The $^{13}CO_2$ enrichment was higher in the patients with cirrhosis at the time of 6 hours postprandial.
The decline in $^{13}CO_2$ between six and fourteen hours postprandial was more in the patients with cirrhosis. Glucose response to glucagon was lower in cirrhotics than in healthy controls.
The combination of insulin : glucagon ratio and decline in ^{13}C-enrichment of breath CO_2 could be a tool to select patients for further metabolic studies or clinical intervention.

Introduction

In chapters 3 and 4 a new method to examine hepatic glycogen oxidation and thus indirectly hepatic glycogen reserves is described. Patients with liver cirrhosis are believed to have smaller hepatic glycogen stores than healthy individuals, and this results in depletion of the glycogen pool at an earlier stage during fasting and in an earlier increase of gluconeogenesis to maintain an adequate blood glucose level. [1]
We therefore decided to compare the results of the $^{13}CO_2$ breath test in patients, who could be predicted to have a small liver glycogen pool, with healthy controls. Patients with advanced cirrhosis of the liver (Child-Pugh classification B and C) were chosen. As an indirect confirmation of the small hepatic glycogen pool in this category of patients, we measured the blood glucose response to a standard dose of glucagon after a 15 hour fast. If after fifteen hours of fasting the glycogen pool in patients with cirrhosis is depleted to a greater extent than in controls, then the increase of the blood glucose level after i.v. administration of glucagon is predicted to be less marked. [2]
The study was perfomed as a pilot study to compare the changes occurring in carbohydrate metabolism during fasting in patients with liver cirrhosis with those in healthy controls. We analysed the blood levels of different molecules involved in glucose and glycogen metabolism in twelve healthy volunteers and in twelve patients with cirrhosis during a two to fifteen hour postprandial period.
The hepatic metabolism of glucose and glycogen is mainly regulated by hormones. Although there is a demonstrable neuronal influence, this probably plays a minor role as after liver transplantation glucose and glycogen metabolism is not greatly disturbed. [3] The two key hormones involved in the regulation of glucose and glycogen metabolism are glucagon and insulin. The ratio of these two hormones is of importance in determining if there is a net glycogenesis or a net glycogenolysis. [1,2,4]
The insulin: glucagon ratio was determined repeatedly during the fasting period. We postulated that the insulin:glucagon ratio would be lower or decrease at an earlier stage during fasting in patients with cirrhosis . Blood glucose levels were expected to be higher in cirrhosis since many patients with cirrhosis have a relative insulin resistance.
We postulated that patients with cirrhosis of the liver have a smaller liver glycogen pool. We assumed that, before administering the ^{13}C-enriched diet, this pool is at least as empty as in healthy controls. Should the liver glycogen reserves in cirrhotics be depleted to a greater extent by the dietary restriction before loading with ^{13}C-enriched carbohydrates, then the relative ^{13}C-enrichment of the glycogen pool will be higher in patients with liver cirrhosis, and this will be reflected by a higher ^{13}C-enrich-

ment at plateau level. In this study the $^{13}CO_2$ enrichment at 6 hours postprandially was considered to represent the plateau in both groups.
Several studies have emphasised that postprandial protein depletion starts early in cirrhosis, and the most likely explanation is that this is to compensate for an early depletion of the liver glycogen pool. [1,4-6]. We therefore postulated that a smaller liver glycogen pool which is depleted at an earlier stage during fasting will result in a steeper decline in the $^{13}CO_2$ enrichment of breath CO_2 in patients with cirrhosis of the liver.

Study subjects and methods

Subjects

Twelve healthy volunteers and twelve patients with cirrhosis of the liver and a Child Pugh classification B (8-9 points) and C (10 points) participated in the study. The cirrhosis was biopsy-proven in all patients.

Study protocol

The healthy volunteers did not use any medication and consumed less than two units of alcohol per day. Patients known to have diabetes mellitus were excluded. One patient, who was initially included, received insulin treatment and her data were not used in the analysis. All participants gave informed consent.
All participants received a standard diet during the day. The details of the diet have been described elsewhere. [7] Three meals were consumed during the day. The last meal was taken at 6 pm.
Blood samples for the analysis of glucose, glucagon, insulin, alpha-amino-acids, beta-hydroxybutyrate, free fatty acids and alanine were drawn at 8pm, midnight, 4am, 8am. (two, six, ten and fourteen hours postprandially). At 9am (fifteen hours postprandially) blood was taken to measure glucose and 1mg glucagon (Novo, The Netherlands) was administered via an antecubital vein. Blood samples were taken 10, 15 and 20 minutes after the injection. Glucose was analysed in these 4 blood samples.

Methods

Standard hospital laboratory techniques were used for the analysis of the hormones and metabolites.
The molar insuline:glucagon was calculated as:

(insuline in mMol/l : glucagon nanogramm per liter)*23.3 [8]

For the $^{13}CO_2$ breath test all participants followed the same protocol. This protocol has been described in detail elsewhere [7] The day before the ^{13}C-enriched diet was consumed all used a standard diet, which contained 80% of the calculated daily energy need. The ^{13}C-enrcihed diet supplied 120% of the calculated energy need. The diet was consumed during three meals. The last meal was taken at 6pm. Breath samples were taken at regular intervals till 9am the next morning (15 hours postprandial).

Statistical analysis

The mean and standard deviation of each group was calculated for each parameter. Since the two groups were not matched, the Mann-Whitney test (Wilcoxon two sample test) was used to compare the two groups. A p-value of < 0.05 was considered to be significant.
For paired data the student T-test for paired data and two-sided variance was used. A p-value of < 0.05 was considered to be significant.

Table 1: Data from patients (n=11) and volunteers (n=12) expressed in mean (SD), in hours postprandial with the exception of the rise in glucose concentration.

Item	h postprandial	Patients	Volunteers	p value
Glucose (mmol/l)	2	7.1 (3.6)	4.5 (0.8)	< 0.001
	6	6.1 (3.4)	4.2 (0.3)	< 0.05
	10	5.0 (2.2)	4.1 (0.4)	NS
	14	4.4 (1.3)	4.2 (0.5)	NS
	15	4.8 (1.4)	3.9 (0.5)	NS
Rise glucose (mmol/l)	10 min	- 0.1 (0.8)	0.5 (0.7)	NS
In minutes after glucagon	15 min	0.1 (0.8)	1.2 (1.0)	< 0.02
	20 min	0.4 (0.9)	1.6 (1.1)	< 0.02
Insulin mU/l	2	56.5 (15.9)	32.9 (20.6)	< 0.01
	6	31.9 (14.7)	13.4 (12.0)	< 0.001
	10	18.7 (6.7)	13.1 (10.4)	< 0.01
	14	17.2 (6.7)	13.2 (4.3)	NS
Glucagon ng/l	2	125.9 (114.9)	32.9 (14.7)	< 0.01
	6	92.1 (83.8)	15.8 (5.7)	< 0.01
	10	105.0 (76.2)	14.8 (10.0)	< 0.001
	14	103.5 (91.5)	14.8 (12.6)	< 0.001
Molar insulin / glucagon	2	23.1 (31.9)	33.7 (35.5)	NS
	6	22.6 (35.5)	21.6 (20.0)	NS
	10	5.2 (1.9)	28.5 (24.9)	< 0.001
	14	4.5 (2.2)	32.2 (22.8)	< 0.001
a-amino-N	2	4.5 (0.5)	5.0 (0.7)	NS
	6	4.0 (0.5)	4.6 (0.5)	< 0.01
	10	3.9 (0.5)	4.3 (0.4)	< 0.05
	14	3.7 (0.4)	4.3 (0.5)	< 0.02
beta-OH-butyric acid	2	19.5 (6.4)	47.1 (35.9)	< 0.01
	6	49.7 (39.4)	167.5 (89.7)	< 0.001
	10	66.0 (39.6)	158.9 (92.2)	< 0.01
	14	109.1 (49.3)	162.6 (116.4)	NS
free fatty acids	2	303.6 (121.7)	689.2 (590.2)	< 0.02
	6	496.4 (351.5)	725.8 (302.2)	NS
	10	711.8 (342.7)	575.0 (203.1)	NS
	14	959.1 (170.9)	690.0 (273.3)	NS
alanine	2	409.1 (61.7)	358.3 (81.6)	NS
	6	320.9 (69.2)	300.0 (41.3)	NS
	10	281.8 (60.0)	269.2 (33.7)	NS
	14	276.4 (63.6)	310.0 (66.1)	NS

Results

One patient, who was initially included, had an insulin dependent diabetes mellitus. The data from this patient were excluded from the analyses. Thus data from twelve volunteers and eleven patients were analysed.
The mean (± standard deviation) age of the patients group was 58.6 ± 8.9 years and of the healthy volunteers 36.2 ± 5.8 years ($p<0.01$). The mean height of the patients was 173.6 ± 7.4 cm and of the volunteers 178.6 ± 9.9 cm (n.s.). The mean weights were respectively 72.3 ± 12.6 kg and 75.5 ± 10.0 kg (n.s.) and their body mass indices were 23.9 ± 3.7 and 23.6 ± 2.2 $kg*m^{-2}$ (n.s.).
Though the age was significantly different between the two groups, body height, weight and body mass index were not. The patients had no ascites detectable by physical examination during the observation period.
The other data are presented in table 1.

Blood glucose levels were different in the early postprandial phase, two and six hours after the last meal. One patient had blood glucose values exceeding 10 mMol/l two, six and ten hours postprandially.
Exclusion of the data of this patient changed the significance of the difference from $p < 0.05$ to $p > 0.05$ at six hours postprandially. No change was observed at the other points in time.
The increase in blood glucose concentration in response to the glucagon injection was higher in the volunteers fifteen and twenty minutes after the glucagon injection.
Insulin levels were higher in the patient group the first ten hours postprandially, while the glucagon levels stayed higher throughout the observation period.
The alpha-amino acids levels were lower in the patient group, except at two hours postprandially. In contrast, beta-hydoxybutyrate levels differed during the early postprandial hours, while free fatty acid concentrations showed little difference. The alanine levels did not differ significantly between the two groups throughout the period of observation.

10 hours postprandial									
insulin:glucagon ratio	patients	volunteers	total		insulim:glucagon ratio	patients	volunteers	total
=< 10	11	2	13		=<15	11	8	19
>10	0	10	10		>15	0	4	4
totaal	11	12	23			11	12	23
sensitivity	100				sensitivity	100		
specificity		83.3			specificity		33.3	
14 hours postprandial								
insulin:glucagon ratio	patients	volunteers	total		insulim:glucagon ratio	patients	volunteers	total
=< 10	10	1	11		=<15	10	3	13
>10	1	11	12		>15	1	9	10
totaal	11	12	23			11	12	23
sensitivity	90.9				sensitivity	90.9		
specificity		91.7			specificity		75.0	

Table 2: The molar insulin : glucagon ratio of 11 patients with cirrhosis (P) and 12 healthy volunteers (V). In the left column the data were divided above 10 and below 10, while in the right column the data were divided above 15 and below 15. Data were collected 2, 6, 10 and 14 hours postprandially (pp).

The individual molar insulin : glucagon ratios are depicted in figure 1.
After 10 hours a ratio of 10 was found to be a cut off point between the two groups. The only person with a ratio above 10 in the cirrhosis group had a ratio of 228, which is extremely high. This outlying value has been excluded in the data presented in figure 1 but was included in the analysis presented in table 2.
The relation between the rise in blood sugar after glucagon injection and the insulin : glucagon ratio is shown in figure 2 .

The ^{13}C-enrichment of breath CO_2 at plateau level (6 hours postprandial) was 0.00431 ± 0.00152 APE in the patient group and in the healthy group 0.00276 ± 0.00094 APE, $p < 0.05$. Figure 3.
The decline in $^{13}CO_2$ enrichment between 6 and 12 hours was for patients –0.00174 ± 0.00096 APE and –0.00105 ± 0.00061 APE in controls (n.s.).
The decline in $^{13}CO_2$ enrichment between 6 and 14 hours was for patients –0.00264 ± 0.00089 APE and in controls –0.00136 ± 0.00058 APE (<0.01).
The decline in $^{13}CO_2$ enrichment between 6 and 15 hours postprandial was for patients –0.00293 ± 0.00105 APE and in controls –0.00184 ± 0.00064 APE (<0.05.)
See figure 4.

The combined data of the insulin : glucagon ratio and the decline in $^{13}CO_2$ in both groups are shown in figure 5. The two groups are well separated by the combination of these two parameters.

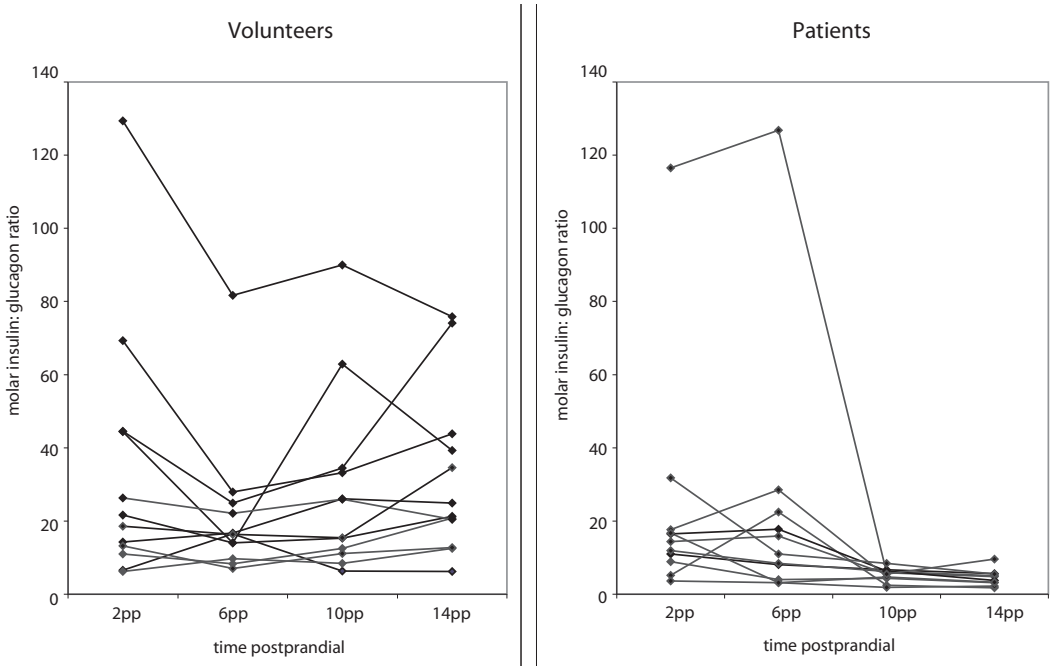

Figure 1: The molar insulin : glucagon ratio in 11 patients and 12 volunteers at 2, 6, 10 and 14 hours postprandial.

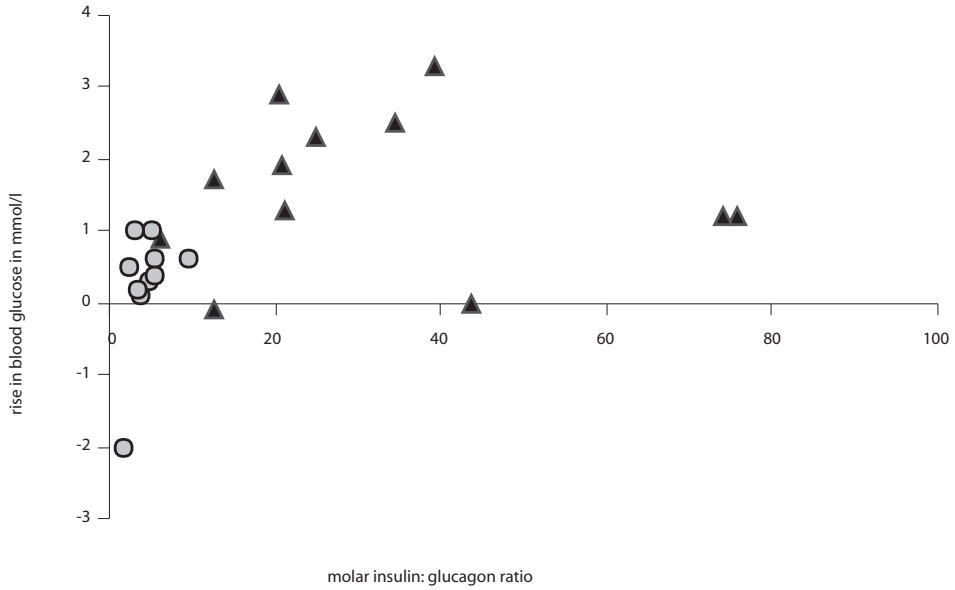

Figure 2:
The relationship between the molar insulin : glucagon ratio fourteen hours postprandial and the increase in blood glucose level after administration of 1 mg glucagon iv in healthy subjects (triangles) and cirrhotics (open dots). Data of one patient who had a extreme high ratio of 288 has been omitted.

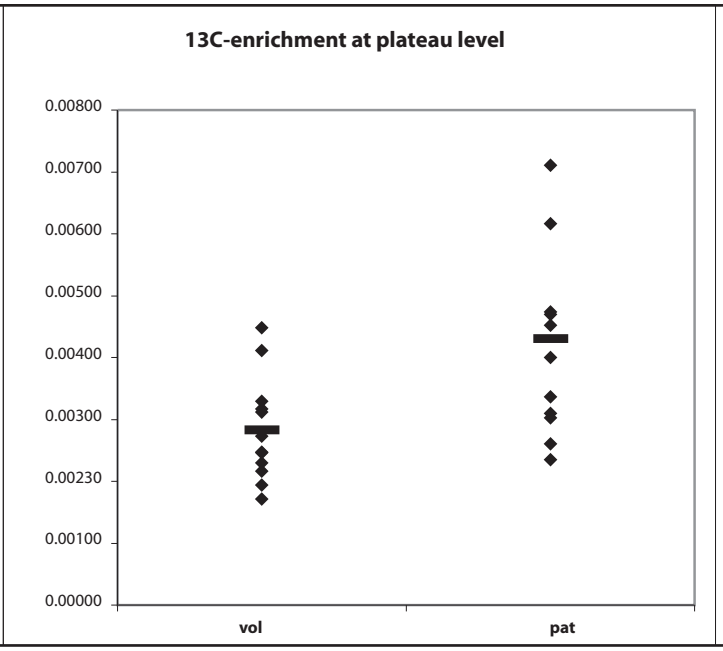

Figure 3:
The ^{13}C-enrichment of breath CO_2 in atom percent excess six hours postprandial in twelve healthy volunteers (vol) and eleven patients with cirrhosis (pat). The horizontal bar represents the mean value in each group.

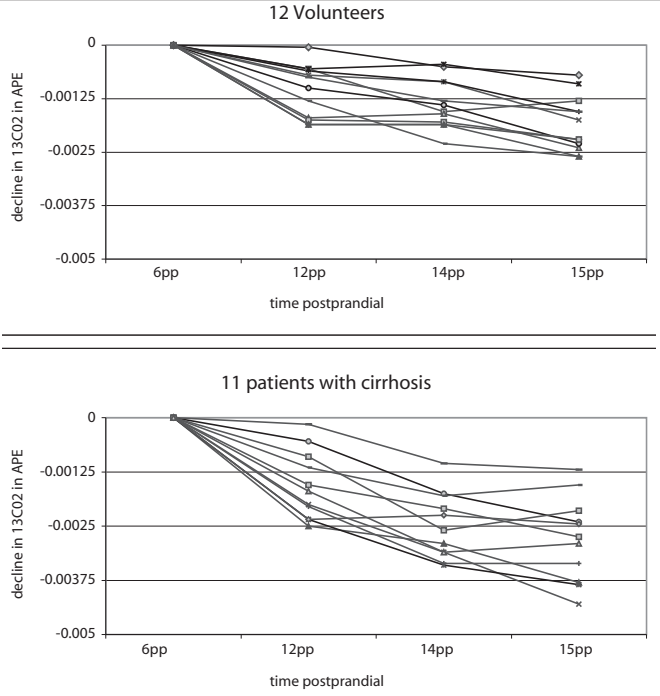

Figure 4:
Decline in ^{13}C-enrichment of breath CO_2 in atom percent excess (APE) during the period from six to fifteen hours postprandial (pp) in cirrhotic patients and healthy controls.

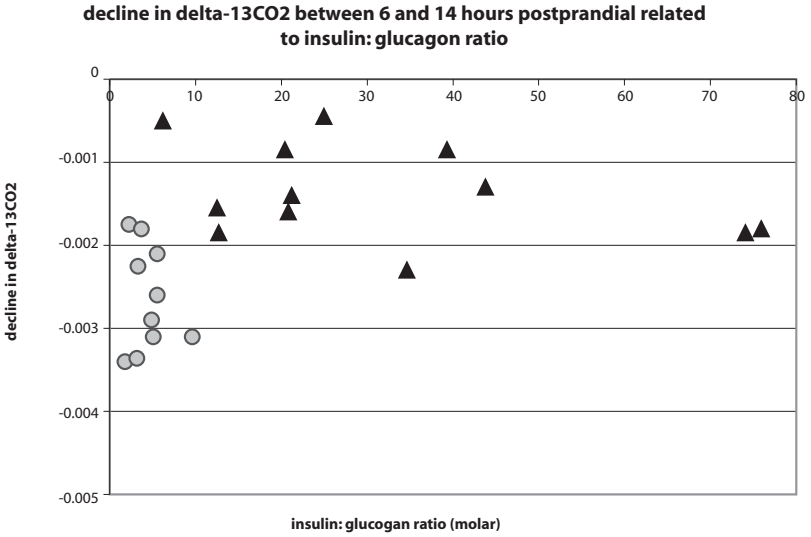

Figure 5:
Relationship between the decline in ^{13}C-enrichment of breath CO_2 in atom percent excess over the period six to fourteen hours postprandial and the molar insulin : glucagon ratio at fourteen hours postprandial in 10 patients and 12 healthy controls. Data from one patient with a ratio of 288 has been omitted.

Discussion

Elevated blood glucose levels are often observed in patients with cirrhosis. [1,9] An decreased insulin sensitivity of the peripheral tissue could contribute to this phenomenon. In our study we only found significantly raised blood glucose levels in one patient, as we excluded patients treated for hyperglycaemia from the study.
The reduced glycemic response to glucagon injection in patients with cirrhosis has been ascribed to depletion of hepatic glycogen stores and is similar to that found in healthy volunteers after 3 days of fasting. [1,10] Kabadi found a increase in blood glucose after an intravenous bolus of 1 mg glucagon of 3.58 ± 0.22 mmol/l in healthy volunteers and 1.45 ± 0.21 mmol/l in cirrhotics after an overnight fast. They measured the increase of glucose from fasting level till the highest level measured in a period between 15 and 180 minutes after 1 mg glucagon intravenously, 9 measuring points [2] Zillekens reported an increase of 2.35 ± 0.19 mmol/l in healthy volunteers and 0.79 ± 0.08 mmol/l in cirrhotics. [1]
Hyperinsulinemia in cirrhosis has been documented in many studies and is attributed by some authors to an increased production of insulin, while others have reported a decreased clearance of insulin. The hyperglucagonemia is thought to be caused by hypersecretion. [1,6,9]
An interesting observation was the decrease in insulin levels over time while the glucagon levels hardly changed. This supports the suggestion that the higher levels are a result of an increased production rather than of an impaired clearance.
Marchesini suggested that the decreased insulin/glucagon ratio would enhance muscle protein catabolism, which they found to be increased in cirrhotics. [4] In contrast Fabri did not find an increase in hepatic nitrogen metabolism after glucagon infusion in patients with cirrhosis. [9] Swart found a negative correlation between the nocturnal protein loss and the increase of the serum glucose level after a standard dosage of glucagon given intravenously after an overnight fast. A higher protein loss correlated with a lower increase in serum glucose concentration, $r^2 = 0.79$, p 0.05 (personal communication)

Plasma free fatty acid levels increased in patients after 14 hours of fasting. Zillekens and co-workers also found a higher level of plasma free fatty acids in cirrhosis, and suggested that the higher free fatty acid level could be due to a decreased hepatic removal in cirrhosis. [1] However an early depletion of the liver glycogen pool could initiate a stimulation of lipolysis and an increased contribution of oxidation of free fatty acids to energy production. The levels of ß-hydroxybutyrate were lower in patients with cirrhosis up to and including the ten hours postprandial measuring point. Zillekens observed no difference after an overnight fast. This emphasizes again the importance of multi-point measurements. The interpretation of the differences can only be speculative since turnover studies were not performed. It is possible that the liver , which is the only organ to add significant quantities of ketone bodies to the blood, has a higher fatty acid oxidation rate during these hours, or that the utilization of ß-hydroxybutyrate by extrahepatic tissues is relatively reduced. [11]
The blood levels of alpha-amino acids were higher in the control group but the alanine levels were not different. Zillekens found the opposite in the fasting state. Alanine is a main source for gluconeogenesis. Again only turnover studies will provide answers on metabolic questions concerning substrate utilization. No rise in alanine levels does not necessarily mean that alanine utilization is not increased.
The insulin : glucagon ratio observed in this study suggests that the ratio may help to detect patients with the metabolic abnormalities at risk for early protein depletion in cirrhosis. If this observation is confirmed by future studies this could be an easy parameter to correlate with other metabolic changes. Previous studies have shown a wide variation in both glucagon and insulin values. In the study of Zillekens, glucagon was 23 ng/l in healthy volunteers and 266 ng/l in patients with cirrhosis. The insulin values were 5.7 and 17.5 mU/l respectively. The molar insulin : glucagon ratio was found to be significantly lower in cirrhosis. [1]

Almdal measured a glucagon value of 85 ng/l and an insulin value of 6.8 mU/l in healthy volunteers, [12] while Vilstrup reported a glucagon level of 75 ng/l and an insulin level of 10 mU/l. [5]
Kabadi found in healthy controls a glucagon of 173 ng/l and in cirrhotics 487 ng/l together with an insulin level of 7 mU/l in controls and 18 mU/l in cirrhosis. [2]

All reported that the blood-samples were taken after an overnight fast. The great variation will be a drawback in using insulin : glucagon ratios for the individual patient unless the blood samples are taken under strictly standardized dietary and time conditions.

The relation between the increase of blood glucose and the molar insulin ratio is shown in Figure 2. Kabadi showed an inverse relation between the glucagon level and the response to glucagon injection during fasting. As expected this relation is even more clear when related to the insulin : glucagon ratio. [2]
The study is in agreement with the hypothesis that the ^{13}C-enrichment of breath CO_2 would be higher in patients with cirrhosis at the time of the plateau level.

It can be argued that in liver cirrhosis gastric emptying could be delayed and that therefore the ^{13}C-enrichment of the breath CO_2 would not only be derived from liver glycogen but also from delayed absorption of carbohydrates from the intestine. We did not measure stomach emptying. However if delayed absorption were to play a role then the $^{13}CO_2$ curve should increase more slowly and reach a top at a later stage. This was not observed.

The insulin : glucagon ratio discriminates quite well between the two groups. If combined with the $^{13}CO_2$ decline the discrimination becomes even better.
If further studies confirm these findings the combination of the breath test and the insulin : glucagon ration could be used in clinical practice, for instance to decide whether specific nutritional interventions such as late night carbohydrate supplementation are necessary [1]

We thank JHP Wilson for helpful discussions and a critical revision of an earlier draft of the paper.

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Chapter 6

Summary and concluding remarks

In the body carbohydrate is stored as glycogen in liver and skeletal muscle. The glycogen is used on demand as a source of glucose and it is replenished after a meal.
In the interpretation of previous research it was postulated that patients with cirrhosis of the liver have a smaller liver glycogen pool. As a result of an earlier depletion of the glycogen pool, gluconeogenesis would be enhanced in cirrhosis during relatively short term fasting – like overnight. This enhanced gluconeogenesis could be the cause of a more negative nitrogen balance, as had been observed in cirrhosis.
While the studies described in this thesis were being performed, Peterson et al confirmed this hypothesis by an elegant study in which they combined NMR and stable isotope techniques. [1]

We wished to develop an easy bedside method to study liver glycogen kinetics, especially liver glycogen depletion. We designed a theoretical model on which to base our test, taking into account the availability of a mass spectrometer, experience with ^{13}C-isotopes and our limited budget. This model was tested in healthy volunteers.

We developed the following method.
The liver glycogen pool of persons, who are used to a low ^{13}C-enriched West-European diet, is depleted during an overnight fast. They then are given a naturally ^{13}C-enriched carbohydrate diet to replete the liver glycogen pool with naturally ^{13}C-enriched carbohydrate.
During subsequent fasting the enriched glycogen is used as a source of glucose and is oxidized. This oxidation enriches the body CO_2 pool with ^{13}C and thus the $^{13}CO_2$ proportion of breath CO_2 increases. This breath CO_2 can easily be collected and the ^{13}C-enrichment can be measured.
Since muscle glycogen is hardly utilised under sedentary conditions the sole source of ^{13}C-enrichment will be the enriched liver glycogen.

Different aspects of the proposed method were tested.
The stability of the baseline $^{13}CO_2$ values, essential for this kind of studies, was shown in chapter 3. The mean ^{13}C-abundance of breath CO_2 was 1.0828 SD 0.00082. The distribution of the data followed a normal distribution pattern. Repeated measurements showed a small and acceptable variation in day to day measurements and after 3 months.
We concluded that the baseline ^{13}C-abundance is stable and can be used as a baseline value in this study-design.
A matter of concern could be the increasing trend in Europe to use imported foodstuffs from all over the world. This makes it necessary to measure baseline ^{13}C-abundance in every single study subject.

The studies reported in chapter 4 show that it is possible to measure in a reliable fashion the slight differences in ^{13}C-enrichment that we induce in our model. This made an interpretation of the $^{13}CO_2$ enrichment data possible. The observation that a plateau in ^{13}C-enrichment could be observed for some hours is very interesting. Knowing that the only ^{13}C-enrichment excess above baseline comes from liver glycogen it strongly suggests a constant oxidation of enriched liver glycogen. This can only be concluded if the CO_2 excretion rate remains constant during the observational period.
The later studies confirmed that the CO_2 excretion rate is indeed quite constant during the plateau period.
The end of plateau time is another interesting point. At this time something changes. Again assuming

a constant CO_2 excretion rate the change in ^{13}C-enrichment indicates a change in substrate that is used for oxidation. In more specific terms, it means that instead of ^{13}C-enriched carbohydrate non-^{13}C-enriched substrate is now being oxidized. This could mean an enhanced gluconeogenesis.
Based on the literature we assumed that the muscle glycogen does not interfere with the breath test as long as study subjects are sedentary. This was confirmed by a study in which this was specifically tested. Healthy volunteers depleted a substantial part of their muscle glycogen pool by intense exercise prior to consuming the ^{13}C-enriched food. It was shown that muscle glycogen was enriched through the use of the diet, but that it did not interfere with the test results as long as the subjects stayed sedentary.

We investigated the hypothesis that it must be possible to carry out the designed test in the inverse version in persons used to a ^{13}C-enriched diet. Africans used to a diet with the naturally ^{13}C-enriched corn and sorghum as staple food, received a West-European diet for one day and breath CO_2 samples were collected for ^{13}C-enrichment analysis. The hypothesis was confirmed. Inversed curves were observed. However the ^{13}C-enrichment of the CO_2 pool did not completely return to the starting values, probably because of a slightly lower ^{13}C-enrichment of the fat and protein pool.

In order to be useful, a test should be reproducible. Although this seems obvious Altman complained: “Replicate studies are rarely made in method comparison studies, so that an important aspect of comparability is often overlooked. A method with poor repeatability will never agree well with another method.” [2]
Our reproducibility study showed that the designed method showed an acceptable reproducibility. Compared with the indirect calorimetry method to analyse carbohydrate oxidation, the oxidation rate that was calculated from the $^{13}CO_2$ breath test data was lower. A possible explanation could be the relatively large and slow reacting nitrogen pool in the calorimetry studies. Another possible explanation could be an incomplete or lower ^{13}C-enrichment in the liver glycogen pool in our studies.

Incomplete labelling of the liver glycogen pool after an only one-day labelling diet could influence the results. Our fifth study showed that after a three-day labelling diet there seemed to be a slightly more extensive labelling of the liver glycogen pool. The end of plateau phase and the return to baseline time were significantly longer in the three-day labelling group. However the difference that was observed between the two groups was rather small. The question is whether this difference is clinically relevant and warrants the advice to always use a three-day labelling in future studies. Preparatory to answering this question our method should be compared with another method, such as the NMR-method.
A requirement for a comparison of the method described in this thesis with the NMR-technique is that no cycling occurs during the observation period, since the described $^{13}CO_2$ method does not take cycling into account, while the NMR method measures the net disappearance of liver glycogen.

Some orientation data were collected from patients with a biopsy proven cirrhosis of the liver and reported in chapter 5.
Although the model appears to be a valid representation of liver glycogen oxidation during fasting and might be useful for measuring glycogen depletion in the liver of healthy individuals, other factors can complicate the interpretation in patients with liver disease.
A smaller glycogen pool at the start of the enrichment period in a patient with cirrhosis will produce a ^{13}C-enrichment of the liver glycogen pool that is higher or at least equal to the healthy controls. Oxidation of this glycogen would results in a higher or at least an equal ^{13}C-enrichment of breath CO_2. However a relatively increased contribution of gluconeogenesis towards oxidation of glucose will cause the ^{13}C-enrichment to be lower. From this point of view an even and maximal enrichment of the liver glycogen should be the starting-point for all subjects.

If such an maximal 13C-enrichment of the liver glycogen is achieved the variation in breath $^{13}CO_2$ will be due to the relative contribution of oxidised ^{13}C-enriched liver glycogen.
The orientation showed a higher ^{13}C-enrichment at plateau level, a steeper decline in ^{13}C-enrichment over the period 6 to 14 hours postprandial. The combination of this decline in ^{13}C-enrichment and the molar insulin : glucagon ratio gave a clear discrimination between the healthy controls and the patients with cirrhosis. This set of data could provide a useful tool to select patients for nutritional intervention or further studies.

In conclusion we developed a model to monitor the oxidation of liver glycogen. Our studies showed that it was possible to label the liver glycogen with naturally ^{13}C-enriched carbohydrate and to monitor its oxidation. ^{13}C-enriched muscle glycogen did not interfere with the test within the test conditions. The test design and the tests results were confirmed by using the inverse test set-up in persons who were used to a habitual diet of ^{13}C-enriched carbohydrates. The test was reasonably reproducible within the same persons, though the study group was small. Extending the dietary ^{13}C-enrichment from one to three days did alter the test results. These alterations were small and possibly of no clinical importance.
A pilot study shows that the test indeed is capable of detecting differences in glycogen oxidation between cirrhotics and healthy individuals.
The clinical application of the test needs further validation by comparing it with other techniques such as the NMR.
The studies described in this thesis demonstrate the feasibility of using naturally isotope enriched products for metabolic studies. This breath test is relatively simple to perform as a bedside test and makes use of inexpensive substrates. For a metabolic test to evaluate a complex process, it is reasonably reproducible.

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Chapter 7

Samenvatting (summary in Dutch)

Vrijwel alle koolhydraten, die het lichaam opneemt, worden omgezet in glucose.
Van dit glucose wordt 98% gebruikt voor energie productie (verbranding).
Dit kan op twee manieren. Of glucose wordt direct opgenomen in de lichaamscel en daar gebruikt of het wordt eerst opgeslagen als glycogeen, om later weer te worden gemobiliseerd en verbrand. Deze opslag gebeurt in lever- en spiercellen.
Als er een tekort aan glucose dreigt, dan wordt glycogeen weer omgezet in glucose.
Bij een tekort aan glycogeen vormt het lichaam glucose uit glycerol uit vet en uit aminozuren afkomstig van eiwitten (gluconeogenese). Als de glycogeen voorraad klein is, dan zal het proces van gluconeogenesis eerder een rol van betekenis gaan spelen. Deze eerder optredende gluconeogenese kan zo een verhoogde eiwitafbraak in het lichaam veroorzaken.
Om in de klinische praktijk inzicht in het proces van glycogeenverbruik tijdens vasten te verkrijgen is een relatief eenvoudige test nodig om de verbranding van glucose uit het leverglycogeen zichtbaar te maken.
De in dit proefschrift beschreven studies betreffen het ontwerp en het toetsen van een methode om de verbranding van leverglycogeen te kunnen volgen.
Hierbij werd gebruik gemaakt van een op natuurlijke wijze met ^{13}C-verrijkte koolhydraten. Dit wordt gevormd via een in de natuur uniek fenomeen van isotopische discriminatie bij de opbouw van plantaardige koolhydraten.

In de natuur komen twee stabiele isotoopvormen van het koolstof atoom (C) voor, namelijk ^{12}C en ^{13}C. In de atmosfeer komen de twee isotopen van het koolstofatoom voor in de verhouding ^{12}C (98,9%) en ^{13}C (1,1%).
Gedurende de koolhydraatsynthese treedt een isotopische discriminatie op ten gunste van het ^{12}C atoom. Het percentage ^{13}C is dan niet meer 1,1% maar slechts 1,08%.
Een uitzondering hierop zijn plantensoorten die hun koolhydraten via een ander chemisch pad synthetiseren. Het percentage ^{13}C in de koolhydraten van deze planten is vrijwel gelijk aan de atmosfeer. De meeste plantensoorten, die dit pad gebruiken, komen in de tropen voor. Voorbeelden van deze planten zijn maïs, rietsuiker, sorghum, millet en ananas.
Het verschil in ^{13}C percentage is klein, doch zeer stabiel.
In alle overige metabole processen, zoals bij opslag en verbranding in het menselijk lichaam, treedt geen isotopische discriminatie meer op. Derhalve is het mogelijk om dit natuurlijk verschil te gebruiken in metabole studies bij mens en dier.

De meeste planten in de voedselketen in West-Europa, hebben een lage ^{13}C-verrijking. Dit werkt door in de totale ^{13}C-verrijking van de voeding, bijvoorbeeld ook het vlees en de melk.
Dit heeft als gevolg, dat de stoffen die het lichaam verbrand ook een lagere ^{13}C-verrijking hebben en dus heeft ook het bij deze verbranding gevormde koolzuurgas (CO_2) een lagere verrijking. Het door verbranding gevormde koolzuurgas verlaat het lichaam voornamelijk via de uitademinglucht. Het ^{13}C-gehalte van dit koolzuurgas kan dus in de uitademinglucht worden gemeten.

Slechts als bijvoorbeeld suiker of snoep uit suikerriet, cornflakes of vlees van maïsgevoede kippen wordt geconsumeerd, krijgt de West-Europeaan koolhydraten, eiwitten en / of vetten van hogere ^{13}C verrijkingsgraad binnen. Als nu voedsel met een hogere ^{13}C-verrijking wordt verbrand, dat is dit te meten door de stijging in de ^{13}C-verrijking van de uitgeademde CO_2.

Dit concept werd door ons toegepast in een studieontwerp om de verbranding van leverglycogeen te bestuderen.
Het studieontwerp werd gebaseerd op de volgende overweging.
Tijdens vasten in rust wordt de glucose concentratie in het bloed op peil gehouden door afbraak van leverglycogeen en de nieuwvorming van glucose uit vet of eiwit.
Als nu het leverglycogeen als enige een hogere ^{13}C verrijking heeft, zal een verhoging van het ^{13}C gehalte in de uitademinglucht plaatsvinden als glycogeen uit de lever wordt verbrand.
Wordt er dus een extra verrijking in adem CO_2 gemeten, dan is dit afkomstig van glucose gevormd uit leverglycogeen. De bijdrage van ^{13}C-verrijkt leverglycogeen kan op deze manier redelijk eenvoudig worden gevolgd door tijdens rustend vasten op verschillende tijdstippen ademlucht te verzamelen en dit te meten op $^{13}CO_2$ verrijking.

Er werden een aantal oriënterende studies uitgevoerd. [hoofdstuk 3] In de eerste plaats werd aannemelijk gemaakt dat het basale ^{13}C-gehalte in de uitademing CO_2 in Nederland laag is en een normale verdeling heeft. Tevens werd aangetoond dat de dagelijkse fluctuatie in basale $^{13}CO_2$ verrijking gering is. Dit bleek ook te gelden voor de fluctuatie op langere termijn.

Het volgende model werd geconstrueerd. Bij personen, die een lage ^{13}C-verrijking hebben, wordt gedurende een korte periode de laag ^{13}C-verrijkte koolhydraten vervangen door de natuurlijk verrijkte koolhydraten. Deze koolhydraten zullen in de lever worden opgeslagen. Als deze weer worden verbrand, dan zal dit een stijging geven in de ^{13}C-verrijking van de CO_2 in de uitademinglucht.
Het model werd bij een12 gezonde proefpersonen uitgetest. [hoofdstuk 4.1]
Alle 12 proefpersonen waren gewend aan een West-Europees, laag ^{13}C-verrijkt, dieet. Gedurende een dag waarop men het gebruikelijke laag ^{13}C-verrijkte dieet volgde trad er geen fluctuatie op in $^{13}CO_2$ verrijking.
Allen kregen een dag een dieet waarvan de koolhydraten afkomstig waren van natuurlijk ^{13}C-verrijkte planten. Het volgende werd waargenomen. Gedurende de dag van ^{13}C-verrijkte koolhydraten inname werd een toename van de $^{13}CO_2$ verrijking waargenomen, piekend na iedere met ^{13}C-verrijkte maaltijd. De voornaamste stijging werd bereikt ongeveer 2 uur na de laatste maaltijd.
Na een initiële daling in $^{13}CO_2$ verrijking trad gedurende rustend vasten een plateau fase op in de $^{13}CO_2$ verrijking. Een plateau fase in $^{13}CO_2$ verrijking betekent bij gelijkblijvende CO_2 productie een stabiele aanvoer van ^{13}C verrijking. Dit impliceert, dat in een dergelijke situatie een constante hoeveelheid verrijkt leverglycogeen per tijdseenheid wordt verbrand.
De plateau fase werd gevolgd door een vrijwel lineaire daling in $^{13}CO_2$ verrijking De observatie periode bleek tekort om de $^{13}CO_2$ verrijking tot de basaal waarde terug te laten keren. Dit laatste punt werd berekend door extrapolatie van de lineaire daling.

In het model werd ervan uitgegaan dat spierglycogeen nauwelijks verbrand wordt gedurende vasten in rust. En dat dus de ^{13}C-verrijking van het spierglycogeen door de ^{13}C-verrijkte maaltijd de interpretatie van onze test als test van verbranding van leverglycogeen niet zou storen.
In een tweede studie werd dit (indirect) getoetst. [hoofdstuk 4.2] Dit werd bereikt door de hoeveelheid spierglycogeen fors te reduceren door middel van een flinke fiets exercitie, voordat de proefpersonen de ^{13}C-verrijkte maaltijd tot zich namen. Vervolgens werd het spierglycogeen weer aangevuld door ^{13}C-verrijkte koolhydraten. Als spierglycogeen substantieel zou bijdragen aan de CO_2 productie, dan zou een hogere ^{13}C-verrijking van spierglycogeen een verstorende invloed hebben op de gemeten curve, die bedoeld is om de verbranding van ^{13}C-verrijkt leverglycogeen te kunnen beoordelen. Dit bleek niet het geval.

Er werd gezocht naar een andere benadering om de methode te valideren.
We redeneerden als volgt. Als het model de verbranding van ^{13}C-verrijkt leverglycogeen weer-

spiegelt, dan moet het mogelijk zijn om dit ook in een omgekeerd model aan te tonen. Namelijk door personen, die in hun dieet gebruikelijk veel ^{13}C-verrijkte koolhydraten gebruiken, te belasten met juist niet verrijkte koolhydraten. Het was namelijk bekend, dat, als proefpersonen langdurig met ^{13}C-verrijkte koolhydraten (zoals maïs en rietsuiker) worden gevoed, de basale verrijking van $^{13}CO_2$ op een substantieel hoger niveau komt te liggen.
Gebaseerd op deze gegevens hebben we in een derde studie [hoofdstuk 4.3] de volgende hypothese getoetst. Bij personen, die een dieet hebben van hoge ^{13}C-verrijking, wordt de leverglycogeen voorraad na een nacht vasten aangevuld koolhydraten met een laag ^{13}C-verrijkingsgehalte (bietsuiker en tarweproducten). Een omgekeerde curve vergeleken met de voorgaande studies werd verwacht. Deze studie werd uitgevoerd in Botswana (Zuidelijk Afrika) en toonde inderdaad de verwachte omgekeerde curve. Daarnaast bleek dat de vastende basale ^{13}C-verrijkingswaarde na 24-uur vasten niet helemaal terugkwam op het hogere basale niveau van voor het laag ^{13}C-verrijkte dieet. Dit suggereert, dat de ^{13}C-verrijking in vet en eiwitten bij deze personen niet dezelfde ^{13}C-verrijking heeft als de koolhydraten, maar wel hoger is dan bij personen die gebruikelijk geen maïs en rietsuiker eten.
In een vierde studie [hoofdstuk 4.4] werd de reproduceerbaarheid van het ontworpen model getoetst. Een groep van 6 proefpersonen voerde het experiment twee maal uit met een tussenliggende periode van 6 weken. Er bleek geen duidelijk verschil tussen de beide groepen te bestaan. Wel was er in 2 van de 6 personen een behoorlijke variatie. Dit limiteert mogelijk het gebruik van deze methode voor een individuele beoordeling.

In het ontwikkelde model werd uitgegaan van het toedienen van natuurlijk ^{13}C-verrijkte koolhydraten voor de periode van 1 dag. Teneinde ervoor te zorgen dat de leverglycogeen voorraad, welke 's nachts aangesproken wordt om energie via glucose te leveren, voldoende leeg zou zijn om zo veel mogelijk nieuw (^{13}C-verrijkt) glucose weer op te nemen, werd de dag voor de toediening van het verrijkte dieet maar 80% van de berekende energie behoefte door de proefpersonen geconsumeerd. Of dit voldoende ^{13}C-verrijking van de leverglycogeen voorraad veroorzaakte was onduidelijk.
Uit de studie van Guy et al bleek dat gedurende een vijf dagenlang gebruik van een ^{13}C-verrijkt dieet na 3 dagen een constante hoge verrijking van het leverglycogeen werd bereikt.
Om te toetsen of de door ons gebruikte methode van 1 dag een ^{13}C-verrijkt dieet voldoende was, werd in een vijfde studie [hoofdstuk 4.5] het 1-dags protocol vergeleken met een 3-dagen 13C-verrijkt dieet protocol. Deze studie toonde aan, dat een dieet gedurende 3 dagen de leverglycogeen voorraad meer verrijkte. De tijd nodig voor het uitputten van de leverglycogeen voorraad was enkele uren langer. Dit verschil was echter dusdanig klein, dat de vraag rijst of het voor het gebruik in de kliniek echt noodzakelijk is om een 3-dagen dieet protocol te volgen.
Er werden ook observaties verricht bij een 12-tal patiënten met een levercirrose. De resultaten worden in hoofdstuk 5 weergegeven. Er werd een hogere verrijking gedurende de plateaufase gevonden, wat verenigbaar is met de gedachte dat de glycogeen voorraad kleiner is. Ook de relatief snelle daling van het $^{13}CO_2$ gehalte in uitademinglucht aan het einde van de plateaufase past binnen dit concept.

Samengevat blijkt het dus mogelijk met een dieet, dat natuurlijk ^{13}C-verrijkte koolhydraten bevat, de glycogeenvoorraad van de lever te verrijken en de verbranding van dit verrijkte lever glycogeen te volgen in de ^{13}C-verrijking van uitademing CO_2. Als de CO_2 productie tegelijkertijd wordt gemeten, dan is het mogelijk met deze methode een redelijke kwantitatieve schatting te maken van de hoeveelheid leverglycogeen, die werd verbrand gedurende de meetperiode. Validatie van deze methode met bestaande methoden, zoals de NMR methode of isotopen studies, is wenselijk om tot een verdere validering te komen. Vervolgens zal in patiënten groepen, met name bij levercirrose patiënten de klinische toepasbaarheid getoetst moeten worden.
Gezien de eenvoud van handeling en bepaling is deze ademtest, mits voldoende gevalideerd, een veelbelovende methode voor klinische vraagstellingen en voor research doeleinden.

Nawoord

Om met suikerriet, maïsmeel, ademlucht en een minimum aan geld de gerezen onderzoeksvraag te lijf te gaan was een hele uitdaging.
De genoemde ingrediënten herinnerden me sterk aan mijn verblijf in Afrika. Dit samen met het feit dat niet eerder dit idee was uitgewerkt brachten mij er toe de handschoen op te nemen.

In de loop der jaren, die het onderzoek en de bewerking in beslag nam, hebben velen mij geholpen en een steentje bijgedragen.
Enkelen wil ik hier persoonlijk bedanken.

Paul Wilson, internist.
Paul, je gaf ons de volledige vrijheid en het vertrouwen om het onderzoek naar eigen inzicht op te zetten en uit te werken. Hartelijk dank voor je kritiek en voor je support in de laatste fase.
Jij was bereid om mij na 10 jaar tropen geneeskunde (toch nog) op te leiden tot internist. Daar ben ik je nog steeds dankbaar voor. Je scherpe en snelle klinische analyse is voor mij nog steeds een voorbeeld.

Roel Swart, internist.
Het was een lange weg vanaf onze eerste contacten tot de voltooiing van dit boekje. Je onderzoek naar het eiwitmetabolisme in patiënten met cirrhose was de basis voor mijn eigen onderzoek. Je stimulerende kritische geest, het steeds relativeren en het streven naar perfectie hebben mijn geest vaak verrijkt. De samenwerking en de uitgebreide discussies met jou en Wim zijn voor mij van grote waarde geweest. Jullie geduld en begrip als het weer langer duurde dan voorzien heb ik erg gewaardeerd.

Wim van den Berg, biochemicus, hoofd lab interne geneeskunde II.
De eerste vragen rondom het bestudeerde model zijn aan jou brein ontsproten. Je ideeën, je technisch inzicht, je zorgvuldigheid en je humor laten iets blijvends na. De Rhoonse avonden, waarbij ik bij jou thuis met jou en Roel kon brainstormen, ideeën toetsen en scherpslijpen zijn onvergetelijk.

Trinet Rietveld, analiste op het Lab Interne II.
Trinet, ik heb groot respect voor de manier waarop jij het analisten vak uitoefent. Je enthousiaste inzet en je kritische nauwkeurigheid stralen van de resultaten af. Zonder dit was het niet mogelijk geweest om met relatief zulke kleine marges resultaat te boeken.
We delen een levendige interesse voor improvisatie en nieuwigheden. Het onderzoek vroeg om beide in ruime mate. Dit heeft veel plezier gebracht. Jouw ideeën maakten het mogelijk om thuis bij vrijwilligers bloed af te nemen en 's nachts in mijn keuken te verwerken. (Is er geen geld voor een opname op de balans afdeling? Dan doen we het toch thuis of op het lab).

Darcos Wattimena, analist.
Darcos, je kritische benadering van probleemstellingen en de mogelijke antwoorden daarop hebben met name in de beginfase van het onderzoek veel duidelijkheid gebracht. Je nauwkeurigheid van werken zijn samen met die van Trinet spreekwoordelijk. Hartelijk dank voor je werk en voor je geduld om mij in te wijden in de wereld der isotopen.

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The Tanis' family.
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De vraag naar de zin van dit alles kan alleen maar worden gesteld in het grotere verband van zingevingvragen. "There is more between earth and heaven than is dreamed of in your philosophy" (Shakespeare). God als schepper en verlosser biedt de mogelijkheden en perspectieven om ons te ontplooien, zelfs in een gebroken wereld. Dit impliceert een grote verantwoordelijkheid, ook voor hen die wetenschap bedrijven. Het omgaan met onze gebrekkige kennis, de diversiteit in mensen en culturen vereist dan ook zorgvuldigheid en bescheidenheid. De bijbelse Prediker vatte het zo samen: "Aan het maken van boeken is geen einde, en veel studie vermoeit het lichaam. Alles gehoord hebbend, is de conclusie: Dien God en volg zijn geboden, want dat betaamt alle mensen."

Sonja, je trouw, je relativeringsvermogen en je houding van
“een mens is wat hij is en niet wat hij heeft”
zijn van onschatbare waarde.

Curriculum vitae

De auteur van dit proefschrift werd in 1956 te Kampen geboren.
In 1974 behaalde hij het VWO diploma aan de christelijke scholengemeenschap “Johannes Calvijn” te Rotterdam. Datzelfde jaar startte hij zijn studie geneeskunde aan de Erasmus Universiteit. Deze werd in 1981 afgerond. Vervolgens bereidde hij zich voor op het werk als arts in een ontwikkelingsland in de ziekenhuizen Bergzicht en St. Anna te Goes en het Ikazia Ziekenhuis te Rotterdam.
Vanaf 1983 tot 1991 was hij werkzaam als arts in het Deborah Retief Memorial Hospital te Mochudi, Botswana. Van dit ziekenhuis was hij van 1984 tot 1991 tevens medisch directeur. In 1991 startte hij zijn opleiding tot internist in het Ikazia ziekenhuis te Rotterdam, opleider Dr. R. Ouwendijk. Vanaf 1993 vervolgde hij deze opleiding op de afdeling inwendige geneeskunde II van het Academisch Ziekenhuis Rotterdam – Dijkzigt, opleider Prof. J.P.H Wilson. In 1997 volgde inschrijving als internist. In 1998 werd de opleiding tot maag-, darm- en leverarts afgerond, opleiders M. van Blankensteijn en Prof. S.W. Schalm.
Sinds 1999 is hij werkzaam als gastroenteroloog in het Ziekenhuis Walcheren.
Hij is gehuwd en heeft 4 kinderen.