Fermentation as a Means of Optimizing the Glycaemic Index - Food Mechanisms and Metabolic Merits with Emphasis on Lactic Acid in Cereal Products

Östman, Elin

2003

Link to publication

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Fermentation as a Means of Optimizing the Glycaemic Index

Food Mechanisms and Metabolic Merits with Emphasis on Lactic Acid in Cereal Products

Elin Östman

Department of Applied Nutrition and Food Chemistry
Lund University
2003


Academic thesis which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Friday 28th March, 2003, at 10.30 in lecture hall B, Center for Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering. Faculty opponent: Dr. Martine Champ, Human Nutrition Research Centre, INRA, Nantes, France.
Contents

LIST OF PAPERS V
CONTRIBUTION TO THE PAPERS VI

1 BACKGROUND 1

1.1 Carbohydrates in human nutrition 1
  1.1.1 Metabolic diseases related to carbohydrate intake 2
  1.1.2 Bioavailability of carbohydrates 3
    1.1.2.1 Starch 3
    1.1.2.2 Starch digestion and absorption 4
    1.1.2.3 Small-intestinal absorption of monosaccharides 4
  1.2 Energy metabolism 5
    1.2.1 Metabolism of glucose 5
    1.2.2 Metabolism of fat and protein 6
    1.2.3 Glucose supply during the postabsorptive state 7
  1.3 The glycaemic index 8
    1.3.1 Role of dietary GI in relation to the insulin resistance syndrome 10
      1.3.1.1 Intervention studies of low-GI diets 11
      1.3.1.2 Satiety and body weight regulation 15
      1.3.1.3 Epidemiological and prospective data 16
    1.3.2 Mechanisms for metabolic effects of low-GI foods 18
    1.3.3 GI in relation to other physiological effects 19
      1.3.3.1 Physical and cognitive performance 19
      1.3.3.2 Growth-stimulating properties of a high-GI diet 20
      1.3.3.3 Caries 21
  1.4 Food factors affecting GI 22
    1.4.1 Type of carbohydrate 22
    1.4.2 Starch interactions as affected by structure and processing 22
    1.4.3 Botanical structure and dietary fibre 23
    1.4.4 Antinutritional factors 25
    1.4.5 Organic acids 25
  1.5 The GI concept; current status and objections 26
  1.6 Conclusions 27

2 OBJECTIVES 28

3 MATERIALS AND METHODS 29
## Contents

3.1 Description of the test products 29  
3.1.1 Bread products 29  
3.1.2 Fermented barley gruel 29  
3.1.3 Dairy products 29  
3.1.4 Pickled cucumber 30  
3.1.5 Model system – simulated baking of starch/gluten mixtures 30  
3.2 Chemical analyses 30  
3.2.1 Starch 30  
3.2.2 Monosaccharides 30  
3.2.3 Fat and protein 30  
3.2.4 Lactic acid 31  
3.3 Amylase kinetics 31  
3.4 Rate of *in vitro* starch hydrolysis 31  
3.5 Microscopic studies 32  
3.6 Meal studies in healthy volunteers 32  
3.6.1 Subject characteristics 32  
3.6.2 Postprandial effects 33  
3.6.3 Second meal effects 33  
3.6.4 Evaluation of glucose and insulin responses 33  
3.7 Animal study 34  
3.7.1 Evaluation of metabolic parameters 34  
4 RESULTS AND DISCUSSION 35  
4.1 Effects of lactic acid on the rate of starch hydrolysis and microstructure in bread and bread-like systems 35  
4.1.1 Barley bread baked with addition of lactic acid 35  
4.1.2 Rate of *in vitro* amylolysis 35  
4.1.3 Microstructure of lactic-acid-containing bread 38  
4.2 Impact of lactic acid on glycaemia and insulinaemia to cereal- and dairy products in healthy subjects 40  
4.2.1 Fermented barley gruel 40  
4.2.2 Fermented *vs.* non-fermented dairy products 41  
4.3 Second meal effects 43  
4.4 Effects of lactic acid on metabolic parameters in rats 46  
5 CONCLUSIONS 48  
ACKNOWLEDGEMENTS 49  
REFERENCES 50
List of papers

This thesis is based on the following publications:

Paper 1  Inconsistency between glycemic and insulinemic responses to regular and fermented milk products
Elin M Östman, Helena GM Liljeberg Elmståhl and Inger ME Björck
*American Journal of Clinical Nutrition* 2001; 74: 96-100
The article is reproduced with permission by the *American Journal of Clinical Nutrition.*
© 2001 American Society for Clinical Nutrition

Paper 2  On the effect of lactic acid on blood glucose and insulin responses to cereal products: mechanistic studies in healthy subjects and *in vitro*
Elin M Östman, Mikael Nilsson, Helena GM Liljeberg Elmståhl, Göran Molin and Inger ME Björck
*Journal of Cereal Science* 2002; 36: 339-346
The article is reproduced with permission by Elsevier Science.
© 2002 Elsevier Science Ltd.

Paper 3  Barley bread containing lactic acid improves glucose tolerance at a subsequent meal in healthy men and women
Elin M Östman, Helena GM Liljeberg Elmståhl and Inger ME Björck
*Journal of Nutrition* 2002; 132: 1173-1175
The article is reproduced with permission by the *Journal of Nutrition.*
© 2002 American Society for Nutritional Sciences.

Paper 4  A diet based on wheat bread baked with lactic acid improves glucose tolerance in hyperinsulinaemic Zucker (*fa/fa*) rats
Elin M Östman, Helena GM Liljeberg Elmståhl, Göran Molin, Ingmar Lundquist and Inger ME Björck
*Journal of the Science of Food and Agriculture* (submitted)
Contribution to the papers

**Paper 1** The author, EÖ, took part in the design of the study, administered the human study, evaluated the results and wrote the manuscript.

**Paper 2** The author, EÖ, took part in the design of the different experiments, administered the human study, performed some of the laboratory work, was responsible for the evaluation and wrote the manuscript.

**Paper 3** The author, EÖ, took part in the design of the study, administered the human study, evaluated the results and wrote the manuscript.

**Paper 4** The author, EÖ, took part in the design of the study, administered the animal study, performed parts of the analytical work, was responsible for the evaluation and wrote the manuscript.
1 Background

1.1 Carbohydrates in human nutrition

Carbohydrates constitute an important source of energy in most mixed diets. In a global perspective, the total intake of carbohydrates varies from 40% to 80%, based on energy content (FAO/WHO 1998). According to recent recommendations, 55% of the total energy intake should preferably come from carbohydrates obtained from a variety of food sources. A classification of dietary carbohydrates is presented in Table 1. The main sources of carbohydrates are cereals, representing over 50% of the carbohydrates consumed in both low-income and high-income countries. Other important sources of carbohydrates are fruits, vegetables and roots, as well as milk products and free sugars. Intake of non-starch polysaccharides (i.e. dietary fibre) ranges from about 19 g/day in European countries and North America, to nearly 30 g/day in rural Africa (FAO/WHO, 1998).

Table 1. Dietary carbohydrates classified according to their degree of polymerisation.

<table>
<thead>
<tr>
<th>Carbohydrate class</th>
<th>Sub-group</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars</td>
<td>Monosaccharides</td>
<td>Glucose, fructose, galactose</td>
</tr>
<tr>
<td></td>
<td>Disaccharides</td>
<td>Sucrose, lactose, maltose</td>
</tr>
<tr>
<td></td>
<td>Sugar alcohols</td>
<td>Sorbitol, xylitol, mannitol</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Malto-oligosaccharides</td>
<td>Maltodextrins</td>
</tr>
<tr>
<td></td>
<td>Other oligosaccharides</td>
<td>Fructo-oligosaccharides</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Starch</td>
<td>Amylose, amylpectin</td>
</tr>
<tr>
<td></td>
<td>Non-starch polysaccharides</td>
<td>Cellulose, hemicellulose, pectin</td>
</tr>
</tbody>
</table>
1.1.1 Metabolic diseases related to carbohydrate intake

In the industrialized parts of the world, there is food in abundance and the unlimited access to palatable and readily prepared foods has a great impact on health. Today, there is such an increase in obesity and type 2 diabetes that it is being referred to as an epidemic. The relations between diet-related metabolic disorders are complex, and they are usually grouped together under the term insulin resistance syndrome (IRS). Components of IRS, including obesity, glucose intolerance, hypertension, dyslipidaemia and impaired fibrinolytic capacity are major risk factors for type 2 diabetes and cardiovascular disease (CVD). Diet and exercise constitute two very important tools in treating and preventing disorders linked to IRS.

The association between a high fat intake and increased risk of CVD is well established. Consequently, the advice to the public has long been focused on lowering the intake of fat, in particular saturated fat, to prevent CVD. Overall, fat intake has decreased during the past 30 years, and with it the prevalence of CVD. In recent years, however, this decrease in CVD appears to have levelled out, and in some populations even turned into a slight increase, despite a reasonably low intake of total and saturated fat (Spieth et al., 2000). The increase in cases of CVD can be related to the rapidly increasing prevalence of obesity and type 2 diabetes. Consequently, in order to combat health disorders related to IRS, other dietary factors besides the quantity and quality of fat should be considered. One such additional dietary factor is the quality of the carbohydrates.

The concept of glycaemic index (GI) was introduced about 20 years ago to classify carbohydrate-rich foods according to their effects on postprandial blood glucose levels (Jenkins et al., 1981). Several recent studies have reported that diets with rapidly absorbed carbohydrates, i.e. those that evoke high postprandial blood glucose levels and, consequently are characterized by high GIs, increase the risk of coronary heart disease (CHD) (Liu et al., 2000) and type 2 diabetes (Salmerón et al., 1997a and b). These findings have also been substantiated in intervention studies (Frost et al., 1996 and 1998), and highlight the importance of the quality of dietary carbohydrates. Although the role of dietary carbohydrates in metabolic disease has yet achieved little recognition, there is an increasing interest in the potentials of lowering the dietary GI. A practical problem with studies of low-GI diets is, however, the limited number of low-GI foods on the market, particularly among starchy staple foods.
**1.1.2 Bioavailability of carbohydrates**

Dietary carbohydrates can be divided into *glycaemic* or *non-glycaemic*, based on whether or not they affect the blood glucose level after a meal. Glycaemic carbohydrates mainly include starch, sucrose, lactose, glucose, maltose, fructose and galactose. These carbohydrates have in common that they can either be absorbed directly (glucose, fructose, galactose) or digested and absorbed (starch, sucrose, lactose, maltose) in the human small intestine. However, the bioavailability of glycaemic carbohydrates is affected by a variety of food factors.

Disaccharides must be hydrolysed into their component monosaccharides before they can be absorbed in the small intestine. Some people suffer from one form or another of disaccharidase deficiency, which causes malabsorption and intolerance of the corresponding disaccharide, *e.g.* lactose. Only glucose can be used as energy without further transformation. Fructose and galactose must be metabolized into glucose by the liver and therefore cause a lower postprandial rise in blood sugar.

Non-glycaemic carbohydrates include non-starch polysaccharides (dietary fibre), certain types of oligosaccharides, sugars, sugar alcohols and *resistant starch* (RS). The main forms of RS are physically enclosed starch, *i.e.* within intact cell structures (RS₁), some raw starches (RS₂) and retrograded amylose (RS₃) (Englyst *et al.*, 1990 and 1992). Non-glycaemic carbohydrates pass through the small intestine undigested and reach the large bowel where they are fermented by the microflora, yielding *short-chain fatty acids* (SCFAs) (Topping *et al.*, 2001).

**1.1.2.1 Starch**

Starch constitutes a major source of carbohydrate in the diet. The rate, as well as the extent, of starch digestion in the small intestine is dependent on the characteristics of the raw material, as well as the type and extent of processing (Section 1.4). Each starch molecule is made up of the two polysaccharides amylose and amylpectin. Amylose is, in principle, a linear polysaccharide made up of *D*-glucose units connected by *alpha*-1,4-glucosidic linkages. Amylopectin is a highly branched polysaccharide consisting of linear segments of glucose units connected by *alpha*-1,4-glucosidic linkages. The linear segments are connected by *alpha*-1,6-glucosidic linkages to form a three-dimensional molecule. Most of the common cereal starches contain 20-30% amylose but high-amylose starches (50-70% amylose) are also available. Waxy starches contain essentially 100% amylpectin.
Background

In plants, starch is used as a means of energy storage and it is packed in the form of granules. The size and morphology of these starch granules are dependent upon the botanical origin of the plant. Starch granules are not water-soluble but they hydrate easily in aqueous solutions. When an aqueous suspension of starch granules is heated, additional swelling occurs until a temperature is reached at which a transition takes place from an organized to a disorganized structure of the molecules. This transition is known as gelatinization, and it normally occurs over a range of about 10°C. Gelatinization dramatically increases the availability of starch for digestion by amylolytic enzymes. Upon further heating, swelling continues and the amylase and some of the amylopectin are leached from the granule producing a viscous suspension. Cooling of this suspension leads to the formation of a gel. With time, realignment of the linear chains of amylase and the short chains of amylopectin can occur in a process known as retrogradation.

Starch takes part in macromolecular interactions with, for example, proteins. It has been shown that the swelling of starch during cooking of pasta is restricted due to the presence of a structured and continuous protein network (Cunin et al., 1995). These starch-protein interactions form a physical barrier to alpha-amylase action, slowing down the starch digestion in the gastrointestinal tract.

1.1.2.2 Starch digestion and absorption

The digestion of starch is initiated in the mouth, where salivary alpha-amylase cleaves alpha-1,4 links. The starch fragments formed include maltose, dextrins containing the alpha-1,6-glucosidic branching points of amylopectin, and minor amounts of glucose. Upon reaching the stomach, the salivary amylase is more or less inactivated, due to the acidic conditions. Interactions between starch and proteins can be expected to be partly degraded in the stomach by the protease pepsin. In the small intestine, the pancreatic alpha-amylase degrades starch into maltose, maltotriose and alpha-limit dextrins. Disaccharidases with maltase, isomaltase and alpha-limit dextrinase activity are attached to the intestinal brush-border membrane and they accomplish the final hydrolysis into glucose.

1.1.2.3 Small-intestinal absorption of monosaccharides

The brush-border membrane of the small intestine is lined with enterocytes. Glucose and galactose are absorbed from the intestinal lumen by a sodium-dependent glucose transporter (SGLT1) (Longo et al., 1998). SGLT1 is responsible for the active transport of glucose against a concentration gradient into the cytoplasm of the enterocyte. Together with every glucose molecule, two molecules of sodium enter the enterocyte.
Glucose is pumped out through the basolateral membrane and enters the circulation through the action of a *facilitative glucose transporter* (GLUT2), which is the same glucose transporter as the one responsible for hepatic glucose uptake. The absorption of fructose into the enterocytes is mediated by another facilitative glucose transporter (GLUT5) (Longo *et al.*, 1998).

Depending on the characteristics of the carbohydrate-containing food ingested, the rate of glucose delivery to the blood will vary. The rate of gastric emptying, the susceptibility of the carbohydrate moiety to digestive enzymes and the rate of absorption into the enterocytes are important determinants. Rapid absorption of glucose will yield a high blood glucose level followed by a high insulin response. This high insulin secretion causes rapid absorption of the glucose into the peripheral cells with a concomitant drop in the blood glucose level; sometimes even below the fasting level. In contrast, slow glucose absorption causes a moderate insulin response and prevents undershooting of the fasting blood glucose level.

## 1.2 Energy metabolism

### 1.2.1 Metabolism of glucose

Glucose is the major energy source for the body during the absorptive phase and, together with fructose and galactose, it is transported from the enterocytes directly to the liver via the portal vein. Fructose and galactose are converted to glucose in the hepatocytes (Vander *et al.*, 1994). Within the liver, glucose can be stored as glycogen or transformed into *triglycerides* (TGs). However, the largest fraction of glucose is released into the bloodstream and transported into cells by an insulin-dependent mechanism. Insulin is synthesized in the pancreatic beta-cells. A more complex protein called proinsulin is first formed. After the proinsulin chain has folded into a specific pattern, a proteolytic enzyme cleaves the protein, yielding one molecule of insulin and a molecule called C-peptide.

Most important for the control of insulin secretion is the glucose concentration of the blood flowing through the pancreas (Vander *et al.*, 1994). However, insulin secretion depends not only on the degree of glycaemia but also on the plasma concentration of certain *amino acids* (AA) and the secretion of the gut hormones known as *incretins*. The incretins are insulinoipotropic hormones that are released from the intestinal mucosa in response to orally ingested nutrients. There is general consensus that the two most important incretin hormones are *glucose-dependent insulinoipotropic peptide* (GIP) and *glucagon-like peptide 1* (GLP-1) (Juul *et al.*, 2001). Activation of the parasympathetic neurons also stimulates insulin secretion (Vander *et al.*, 1994).
Background

In the membranes of fat and muscle cells, specific glucose transporters (GLUT4) are present and extra membrane-bound glucose transport proteins are stored in intracellular vesicles. When insulin molecules bind to the insulin receptors on the surface of the target cells, a signal tells the cell to add the glucose transporters to the plasma membrane. Once fused with the plasma membrane they increase the rate of glucose uptake (Alberts et al., 1989). This causes the blood glucose level to decrease and, in response, less insulin is released from the pancreas. When insulin levels decrease, the extra glucose carriers are rapidly removed from the cell surface and returned to the intracellular pool. The complex of insulin and its receptor is removed from the cell surface by endocytosis. The insulin molecule is degraded by endosomes and the receptor is recycled back to the plasma membrane (Alberts et al., 1989). Upon reaching the inside of the cell, glucose is rapidly oxidized to form energy via CO₂ and H₂O, or converted to glycogen, which is then stored within the cell.

1.2.2 Metabolism of fat and protein

TGs are formed from \textit{alpha}-glycerol phosphate and \textit{fatty acids} (FAs) that are linked together. Glucose is crucial for the formation of \textit{alpha}-glycerol phosphate, since it cannot be formed from any fat metabolites. Some of the TGs formed in the liver are stored there but most are packaged into molecular aggregates called \textit{very low-density lipoproteins} (VLDLs). Because of their size, VLDLs can not easily penetrate the capillary walls. Lipoprotein lipase is therefore located on the surface of the capillaries and it hydrolyses TGs into monoglycerides and FAs. The FAs can diffuse across the capillary wall and into the adipocytes, where they recombine with \textit{alpha}-glycerol phosphate (supplied by glucose metabolites) to form TG once again (Vander et al., 1994).

There is a net synthesis of protein during the absorptive period, but it is just enough to replace the proteins that are catabolized during the postabsorptive period. Excess AAs are not stored as protein but are converted to carbohydrates or fat.
1.2.3 Glucose supply during the postabsorptive state

During the postabsorptive state, no glucose is absorbed from the intestinal tract. It is, however, necessary for the brain function that blood glucose be maintained at a physiological and constant level (glucose homeostasis). A low glucose level can result in serious effects on neural activity. There are three possible ways to maintain glucose homeostasis, namely: glycogenolysis, proteolysis and fat utilization (Vander et al., 1994).

Although of short duration, hepatic glycogenolysis occurs rapidly and is the first means of maintaining glucose homeostasis. The skeletal muscle cells, however, lack the enzyme to convert glucose-6-phosphate into glucose and can therefore only form lactate and pyruvate. The lactate and pyruvate from the muscle cells are then circulated to the liver, where they are converted into glucose.

A few hours into the postabsorptive state, protein becomes the major source of blood glucose. Body protein can supply large quantities of AAs, which are metabolized by the liver and transformed into glucose. In order to salvage liver-derived glucose for the needs of the nervous system, most organs increase their fat utilization during the postabsorptive phase. Catabolism of adipose tissue yields glycerol and FAs. Glycerol is converted to glucose by the liver and the FA is bound to plasma-albumin and circulated in the blood stream as free fatty acid (FFA). The FFA can enter the Krebs cycle and be catabolized into CO₂ and H₂O, which in turn yield energy (Vander et al., 1994).

Almost all tissues, excluding the nervous system, can metabolize FFAs. The liver differs from other tissues in that most of the acetyl-CoA formed there, do not enter the Krebs cycle, but are processed into ketones. So-called ketone bodies are released into the blood and provide an important energy source during prolonged fasting for the many tissues capable of oxidizing them via the Krebs cycle (Vander et al., 1994). Many areas of the brain are also capable of utilizing ketones for energy. However, an intake of about 50 g/day of carbohydrate is enough to avoid ketosis (FAO/WHO, 1998).
1.3 The glycaemic index

The concept of GI was introduced in 1981 (Jenkins et al., 1981). According to this concept, carbohydrate-rich foods are assigned values on the basis of their influence on the blood glucose level after a meal. In 1997, the FAO/WHO Board of experts stated the following definition of GI:

“The glycaemic index is defined as the incremental area under the blood glucose response curve of a 50 g carbohydrate portion of a test food expressed as a percent of the response to the same amount of carbohydrate from a standard food taken by the same subject”.

Generally, the incremental area under the blood glucose curve is calculated geometrically by applying the trapezoid rule, ignoring the area beneath the fasting concentration (FAO/WHO, 1998). The standard food used in a GI test can be either white bread or a glucose solution; the GI values obtained with bread being 1.37 times those obtained with glucose (Wolever et al., 1991).

GI values for more than 750 different types of food were published recently (Foster-Powell et al., 2002). Using the same mathematical model as for calculating the GI, an insulinaemic index (II) can be determined. There is usually good correlation between the values of GI and II for carbohydrate-rich foods (Björck et al., 2000), implying that high-GI foods are more insulin demanding.

In Table 2, the GIs of common food groups are listed. There is a wide range of GIs within each food group. The most important carbohydrate foods in the Western diet are cereal foods, such as bread and breakfast cereals, together with potatoes and pasta. Apart from pasta products, many of these starchy staple foods belong to the high-GI foods. The number of low-GI products is limited, but with access to GI tables it is possible to chose products, within a food group, that have lower values.

In order to characterize the glycaemic effect of mixed meals and diets the term glycaemic load (GL) has been introduced (Salmerón et al., 1997b). Multiplying the percentage of the total meal carbohydrate by its GI and summing these values for all foods gives the GL for a mixed meal (Wolever et al., 1991). Studies have shown that although dietary fat and protein affect the absolute glycaemic response, they do not affect the relative differences between carbohydrate-containing meals (Bornet et al., 1987; Wolever et al., 1996).
Table 2. GI values (based on white bread) and intervals for monosaccharides and groups of food products (Foster-Powell et al., 2002).

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>140</td>
</tr>
<tr>
<td>Sucrose</td>
<td>85</td>
</tr>
<tr>
<td>Lactose</td>
<td>65</td>
</tr>
<tr>
<td>Fructose</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food group</th>
<th>Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole meal bread</td>
<td>60-110</td>
</tr>
<tr>
<td>Bread with intact kernels</td>
<td>40-90</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>60-120</td>
</tr>
<tr>
<td>Pasta</td>
<td>60-80</td>
</tr>
<tr>
<td>Potatoes</td>
<td>80-130</td>
</tr>
<tr>
<td>Rice</td>
<td>50-120</td>
</tr>
</tbody>
</table>
1.3.1 Role of dietary GI in relation to the insulin resistance syndrome

The IRS is sometimes called the metabolic syndrome or syndrome X, and includes: obesity, type 2 diabetes (also called non-insulin-dependent diabetes mellitus – NIDDM), hypertension, CVD, dyslipidaemia and hyperinsulinaemia. Insulin resistance (IR) is a state where the tissue sensitivity to insulin has declined, which is counteracted by a compensatory increase of insulin secretion from the pancreatic beta-cell (DeFronzo et al., 1991). IR is a characteristic feature of obesity, hypertension and type 2 diabetes and has a high prevalence in the general population.

In an obese non-diabetic person, the compensatory insulin response to IR is nearly perfect, and no change in glucose tolerance occurs. However, in a diabetic individual, the beta-cell response is not perfect, resulting in glucose intolerance. Studies have shown that type 2 diabetes leads to impaired secretion of GLP-1 and impaired insulinotropic activity of GIP (Juul et al., 2001). Although it is not known how important the incretin defect is for the metabolic dysfunctions of type 2 diabetes, it is thought that the defect contributes to the impaired insulin secretion that characterizes the disease.

In both obese non-diabetic and diabetic subjects, persistently elevated plasma insulin levels (hyperinsulinaemia) are present. Hyperinsulinaemia is known to contribute to the development of hypertension, plasma lipid abnormalities and atherosclerosis (DeFronzo et al., 1991). High insulin levels per se are also a major risk factor for the development of CVD, by stimulating formation of atherosclerotic plaque and inhibiting the reabsorption of these plaques once they are formed.

High circulating levels of blood glucose are associated with both immediate and long-term adverse effects. Studies in vitro have shown that glucose can cause oxidation of membrane lipids, proteins, lipoproteins and DNA, as well as activating inflammation (Ludwig, 2002). Excessive postprandial glycaemia decreases blood high-density lipoprotein (HDL) cholesterol concentrations and increases triglyceridaemia (Gavin, 2001) and evidence has also been found that acute hyperglycaemia can increase blood pressure in both diabetic and non-diabetic subjects (Ceriello et al., 1997; Giugliano et al., 1997). Overall, postprandial hyperglycaemia increases the risk of CVD in non-diabetic and diabetic subjects (Ceriello, 2000).

One mechanism associated with the increased risk of CVD is that chronically elevated blood glucose levels lead to the glycosylation of a variety of proteins which may induce the scavenger-receptor-mediated uptake of low-density lipoproteins (LDLs) by the macrophages on and within the endothelium (James et al., 1993). Furthermore, these macrophages are related to stimulation of the atherosclerotic process.
Other adverse physiological effects of elevated blood glucose levels include glycosylation of proteins and a series of reactions producing so-called *advanced glycation end products* (AGEs). Certain proteins undergo significant non-enzymatic glycosylation leading to the production of AGEs, either due to chronic exposure to normoglycaemia caused by ageing or, in the case of diabetics, shorter exposure to hyperglycaemia. The deposition of AGEs into proteins is believed to contribute to the development of several abnormalities associated with ageing and diabetes mellitus, such as atherosclerosis and capillary basement membrane thickening. AGEs are also involved in ocular diseases and contribute to cataract formation (Handa *et al.*, 1998).

Although high postprandial glucose levels are negative for several body functions, subjects are normally not exposed to hyperglycaemia for longer periods. In contrast, persons with IRS suffer from chronic daylong hyperinsulinaemia. Because of its pathogenic role in the development of CVD, the treatment of hyperinsulinaemia is of outmost importance. Consequently, low-GI foods have been suggested to decrease IR and hyperinsulinaemia by lowering the postprandial glucose and reduce the insulin demand (Willett *et al.*, 2002).

### 1.3.1.1 Intervention studies of low-GI diets

Metabolic effects of low-GI diets have been evaluated in healthy, hyperlipidaemic, diabetic, obese or overweight subjects. The studies cover intervention periods lasting from 2 weeks up to a year.

In a study of 6 healthy men, macronutrient equivalent low-GI (63) and high-GI (104) diets were served for 2 weeks in a crossover design (Jenkins *et al.*, 1987b). The study included *intravenous glucose tolerance tests* (IVGTTs) and measurements of several parameters related to glucose metabolism. Significantly lowered fasting total cholesterol (-15%) and serum fructosamine (-7.0%) levels were found after the low-GI period. In addition, the low-GI diet significantly reduced 12-h glucose profile (-37%) and 24-h urinary C-peptide levels (-32%). However, there were no differences in blood glucose levels or *areas under the curves* (AUCs) after the IVGTTs. The same authors have also shown that it was possible to lower the total cholesterol (-8.8%), LDL cholesterol (-9.1%) and serum TG (-19.3%) in a group of 24 hyperlipidaemic subjects with raised TG levels, by reducing the GI of the diet by 11 units for 1 month (Jenkins *et al.*, 1987a).

In a recent study of moderately overweight non-diabetic men (Bouché *et al.*, 2002), low-GI (41) and high-GI (71) diets were served for 5 weeks each in a crossover design. The macronutrient intake did not differ between the test periods. However, the intake of dietary fibre was significantly increased (+38%) during the low-GI period.
The postprandial glucose and insulin AUCs were 30-50% lower after the low-GI than after the high-GI diet. Analysis of blood and tissue samples also showed an improvement in plasma lipid parameters (leptin, lipoprotein lipase and hormone-sensitive lipase mRNA) and a significant decrease in total fat mass (~700 g) after the low-GI period. Body weight did not differ between the two test periods and the reduced total fat mass can probably be explained by an increase in lean body mass.

Low-GI foods are frequently richer sources of dietary fibre than high-GI foods. However, a lowering of dietary GI appears to have metabolic benefits per se in diabetics (Järvi et al., 1999). Twenty type 2 diabetic patients were given a high-GI (83) or a low-GI (57) diet for 24 days in a crossover study, and glucose, insulin, cholesterol, as well as the levels of plasminogen activator inhibitor-1 (PAI-1), were measured. The results showed that a low-GI diet could not only lower the AUC for glucose and insulin by 30% during a 9-h profile day and lower the LDL cholesterol level, but could also normalize the PAI-1 activity levels. This was the first time that a substantial effect on the fibrinolytic activity was reported following a lowering of GI in the absence of a difference in type and amount of dietary fibre.

Metabolic benefits have also been observed in type 1 diabetics. In a study by Collier et al. (1988), 7 diabetic children were given either a test or a control diet with a micronutrient composition that resembled their usual diets. In the test diet (GI=69), approximately 50% of the high-GI carbohydrates in the control diet (GI=82) were replaced by low-GI alternatives and the intervention lasted for 6 weeks. The glucose response to a standard carbohydrate challenge (white bread) was tested at the beginning and end of each test period and blood samples were collected. The results showed that when increasing the proportion of low-GI starchy foods for 6 weeks, glucose control was improved and the level of total cholesterol significantly lowered (~14%).

Moreover, a high-fibre/low-GI (70) diet given to adult type 1 diabetic patients for 24 weeks, was found to significantly reduce mean daily blood glucose concentrations by 18%, HbA1c (glycated hemoglobin) levels by 5% and the number of hypoglycaemic events by 50% compared with a low-fibre/high-GI (90) diet (Giacco et al., 2000). In a long-term prospective study in diabetic children, Gilbertson et al. (2001) found that flexible low-GI dietary advice led to significantly lower HbA1c levels at 12 months (8.05±0.95%), than in those receiving traditional dietary advice (8.61±1.37%). The low-GI dietary advice period was also associated with a better quality of life for both the children and their parents.
Considering that diabetes predisposes sufferers to vascular disease, it is important to consider the potential preventive role of low-GI foods in patients with CHD. Consequently, several studies have been designed to directly evaluate the insulin-stimulated glucose uptake in isolated adipocytes and one of them was conducted in a group of patients with advanced CHD (Frost et al., 1996). Thirty-two patients waiting for coronary artery bypass surgery were enrolled in the study and divided into two dietary treatment groups. The low-GI group was encouraged to change one major carbohydrate source to a low-GI alternative at each meal, while the high-GI group was encouraged to avoid low-GI foods and to eat rapidly absorbed carbohydrates. At the start and end of the 4-week test period an oral glucose tolerance test (OGTT) was performed on each subject, and blood glucose, insulin and FFAs were analysed at several points in time. During heart surgery a fat biopsy was taken for analysis of the insulin-stimulated glucose uptake in isolated adipocytes. The low-GI group showed a significant decrease (30%) in AUC for insulin at 4 weeks, whereas no decrease was observed in the high-GI group. There was no significant change in the glucose AUC in either group, nor were there any significant changes in fasting insulin or glucose levels. From the in vitro measurements in isolated adipocytes the authors concluded that there was a significant increase in insulin-stimulated glucose uptake in the low-GI group, compared with the high-GI group.

Similarly, in a later study by Frost et al. (1998), insulin sensitivity (IS) of the adipocytes in women with a parental history of CHD was improved when the GI of their diet had been reduced by 26% for 3 weeks. The low-GI diet was also associated with improved whole body IS in premenopausal women with and without parental history of CHD.

Taken together, the results presented above support the hypothesis that a lowering of dietary GI improves blood glucose regulation (Giacco et al., 2000), glucose tolerance (Collier et al., 1988) and/or IS (Frost et al., 1998), as well as blood lipids (Jenkins et al., 1987a) and fibrinolysis (Järvi et al., 1999). A summary of evidence from the literature concerning protective effects of low-GI diets on IRS is presented in Figure 1.
Figure 1. Protective effects of low-GI diets on the insulin resistance syndrome.

- Indicates evidence for reduction of risk factors
- Indicates epidemiological evidence for reduced risk of disease
1.3.1.2 Satiety and body weight regulation

Obesity is associated with reduced IS and an increased risk of type 2 diabetes and CVD. The potential difference between foods in facilitating weight regulation is therefore an issue of great concern. Although there is a lack of data concerning the relation between the acute satiating merits of foods and long-term effects on body weight, it could be hypothesized that a diet based on foods with a higher post-meal satiety would reduce the risk of overconsumption and thus prevent obesity.

In a recently published review (Ludwig, 2000), several studies demonstrated either increased satiety, delayed return of hunger or decreased ad libitum food intake after low-GI compared with high-GI foods. For example, in one study performed on obese teenage boys, the voluntary energy intake after high-GI meals was 53% higher than after low-GI meals (Ludwig et al., 1999). The less satiating effect of high-GI foods could be explained by the dramatic postprandial increase in the insulin level, resulting in a rapid reduction in both glucose and FFAs, often below fasting levels 3-5 hours, postprandially. When the two major metabolic fuels (glucose and FFAs) are low at the same time, the central nervous system might interpret the situation as a lack of fuel, which could trigger the signalling of hunger and favour the storage of fat (Brand-Miller et al., 2002).

One mechanism possibly responsible for the increased satiation of low-GI foods is the slower rate of starch digestion and absorption, which results in prolonged feedback signalling of satiety to the brain (Havel, 2001). Moreover, low-GI diets may improve access to stored metabolic fuels, decrease hunger and promote weight loss (Ludwig, 2000). However, when slightly overweight men ingested a low-GI diet for 5 weeks, compared with an energy and macronutrient equivalent high-GI diet, their total fat mass was significantly reduced, despite no difference being observed in body weight (Bouché et al., 2002). The fact that no weight loss occurred suggests that lean tissue increased as the fat mass decreased, probably due to a shift from the oxidation of lean to adipose tissue.

An interesting study of obese, hyperinsulinaemic women (Slabber et al., 1994), showed a significantly larger weight reduction after 12 weeks on a moderately energy-restricted diet that was tailored to evoke a low insulin response (low-GI diet), than following a corresponding diet that elicited a normal insulin response. Fasting insulin levels also dropped significantly more after the less insulin demanding diet, which further strengthens the hypothesis of the metabolic merits of low-GI foods in the treatment of obesity and its associated disorders.
Furthermore, in a study of obese children, a low-GI diet proved to be a promising alternative to treatments with energy-restricted, low-fat diets (Spieth et al., 2000). Consequently, the overall mean change in body mass index (BMI) for the low-GI group was -1.53 kg/m$^2$, compared with -0.06 kg/m$^2$ for the reduced-fat group ($P < 0.001$). Also the decrease in body weight was significantly larger in the low-GI group.

New data are available where healthy overweight women followed a high-carbohydrate/low-fat diet with either a high or a low GI for 10 weeks (Raben et al., 2003). Several parameters were measured, for example, ad libitum energy intake, body weight and risk factors for type 2 diabetes and CVD. No beneficial effects of the low-GI diet were seen on body weight regulation or appetite. However, the low-GI diet resulted in decreased LDL levels with 10% ($P < 0.05$), again suggesting a beneficial effect of low-GI diets on risk factors for CVD.

Although it is difficult to interpret the currently available evidence, low-GI foods appear to have some potential in the prevention and treatment of obesity. More intervention and epidemiological studies are needed to evaluate the potential of a low-GI diet in long-term weight maintenance. A prerequisite for the successful use of low-GI foods in weight regulation is, however, that there is a sufficient amount of low-GI products on the market, providing comparable alternatives to the basic products of the diet of today.

1.3.1.3 Epidemiological and prospective data

In two prospective studies, 6-year follow-ups were carried out in 42,000 men and 65,000 women (Salmerón et al., 1997a and b). Their usual intake of fibre and their dietary GI/GL was calculated from the responses to detailed dietary questionnaires. The results of both studies supported the hypothesis that long-term consumption of a diet with a high GL was a significant, independent predictor for risk of developing type 2 diabetes (RR=2.17 and 2.50 for men and women, respectively).

Similarly, in a prospective (10-year follow-up) study of more than 75,000 female nurses, dietary GL was found to be directly associated with the risk of CHD (RR=1.98 for the highest quintile) after adjustment for age, smoking status, total energy intake, and other coronary disease factors (Liu et al., 2000). In the latter study the association between GL and CHD risk was most evident among women with body weights above average (BMI≥23). Furthermore, in subjects with normal fasting glucose levels, a significant association has been found between the 2-h post-load blood glucose concentration and risk of cardiovascular mortality (RR=3.0) after adjustment for sex, age and known cardiovascular risk factors (deVegt et al., 1999).
In a cross-sectional study of 1400 middle-aged men and women (Frost et al., 1999), a significant negative relation ($r = -0.00724$, $P = 0.02$) was found between serum HDL cholesterol level and the GI of the diet. In fact, the GI was a stronger predictor than dietary fat intake for serum HDL cholesterol levels. Similar negative relations between HDL cholesterol and GI has been found in data from 37,900 US adults (Ford et al., 2001), as well as in type 1 diabetic subjects at European diabetic centres (Buyken et al., 2001). For the 2,000 type 1 diabetic subjects included in the latter study, the GI was also independently related to the HbA1c levels. Furthermore, a strong positive association between GL and fasting TG levels has been found in healthy postmenopausal women (Liu et al., 2001).

Taken together, there is a substantial amount of data suggesting a potential of low-GI diets in both preventing and managing metabolic disturbances. More epidemiological studies are necessary to assess the preventive potential. One problem with epidemiological studies is, however, the possibility that data can be confounded by other factors. It is important to consider whether low-GI diets could, in fact, be markers for a healthy lifestyle in general, with more physical exercise, less smoking, higher consumption of fruit and vegetables etc. Moreover, a short coming with the currently available epidemiological data concerning the preventive value of low-GI diets is that critical product information may not have been included in dietary questionnaires which limits the accuracy of the calculation of dietary GI/GL. Examples of such product data include e.g. the type of rice, the kernel to flour ratio in bread etc. The lack of certain product specifications in the international GI tables is also a problem in this respect. Future epidemiological studies need to allow for the dietary GI to be predicted with higher accuracy.
1.3.2 Mechanisms for metabolic effects of low-GI foods

Low-GI foods are associated with a prolonged digestive phase, minimizing hyperglycaemia, which would otherwise be corrected by a counterregulatory hormonal response. In response to hyperglycaemia, the plasma levels of FFA rise, causing a relative IR in muscle and adipose tissue. It has thus been suggested that low-GI foods improve the IS by suppressing post-meal FFA levels (Wolever, 1990). Studies have shown that low-GI foods ingested at breakfast can improve glycaemia and insulinaemia after a subsequent lunch (4 h later) in healthy subjects (Jenkins et al., 1982; Liljeberg et al., 1999 and 2000). This second meal effect suggests that low-GI foods are associated with mechanisms extending beyond the immediate postprandial phase and thus contribute to the long-term metabolic merits of low-GI diets.

In order to find evidence for the metabolic relevance of a lower rate of glucose delivery to the blood, the impact of increased meal frequency has been studied (Jenkins et al., 1989). When serving a diet of 17 snacks over the day for 2 weeks, the fasting total cholesterol, LDL and apolipoprotein B levels were significantly reduced in normal subjects. In addition, the mean serum insulin level and urinary C-peptide output decreased after the “nibbling” diet. These findings suggest that following a diet with a prolonged absorption time, such as a low-GI diet, plays a role in the prevention of heart disease and promotes glucose homeostasis.

The presence of carbohydrates in the upper gastrointestinal tract stimulates the release of several peptides that are linked to a reduced gastric emptying rate (GER) and an increased insulin secretion and satiety (Hellström et al., 2001). GIP secretion is stimulated by absorbable carbohydrates and by lipids, whereas GLP-1 is secreted as a response to the presence of nutrients in the lumen of the gut (Juul et al., 2001). It is thought that GIP is secreted in the upper small intestine, whereas the secretion of GLP-1 is more potent in the lower part of the small intestine. This suggests that meals containing slowly absorbable nutrients have a more prominent impact on the GLP-1 response than those with rapidly absorbed nutrients. GLP-1 has a lowering effect on the GER, and is also able to inhibit appetite and food intake. Hence, an increased release of GLP-1 by low-GI foods could be one mechanism responsible for improved glycaemia.

The digestibility of carbohydrates in low-GI foods is generally lower than that in high-GI foods. Consequently, low-GI starchy foods frequently contain higher amounts of RS, or low-molecular-weight carbohydrates that for various reasons escape digestion and/or absorption in the small intestine. Low-GI foods thus increase the amount of carbohydrate entering the colon, which increases the colonic fermentation and SCFA production.
Some of the SCFAs produced in the colon enter the portal circulation and appear to activate the *ileocolonic brake* in a dose-dependent manner (Topping *et al*., 2001). The latter causes a slower upper gastrointestinal passage of food, which may lower the rate of starch delivery to the blood. In addition, a potential beneficial effect of SCFAs on the hepatic glucose metabolism has been reported (Thorburn *et al*., 1993) but the mechanisms are not clear.

Other possible mechanisms responsible for metabolic benefits of low-GI diets include the replacement of adipose tissue by lean tissue. A high-GI diet could cause a more pronounced negative nitrogen balance than a low-GI diet, suggesting that fat tissue is oxidized to a lower degree and muscle to a higher degree with the high-GI diet (Agus *et al*., 2000). In contrast, low-GI foods promote fat oxidation at the expense of carbohydrate oxidation (Ludwig, 2000). Consequently, replacing high-GI carbohydrates by low-GI alternatives could result in the replacement of fat mass by lean tissue, which has been reported by Bouché *et al.* (2002).

In most intervention studies regarding metabolic effects of low-GI diets, efforts have been made to keep body weight constant. Thus, although many intervention studies have been performed, they usually do not allow conclusions regarding effects on weight maintenance or reduction. It has been suggested that low-GI foods promote satiety and consequently reduce appetite and decrease the overall food intake (Ludwig *et al*., 1999). This suggests a weight reducing potential of low-GI diets, possibly followed by maintenance of the ideal weight when continuing on a balanced diet. Reduction of obesity is the key factor to overall improvement in glucose and lipid metabolism.

### 1.3.3 GI in relation to other physiological effects

#### 1.3.3.1 Physical and cognitive performance

Available literature regarding the impact of GI on physical performance is yet more difficult to interpret due to differences in the experimental design regarding, for instance, meal timing before exercise, the quantity of carbohydrate ingested, and the method used for measuring performance. Furthermore, the metabolic response to pre-exercise ingestion of carbohydrates with different GIs is complex and responses are thought to be largely individual. Results from two studies (Febbraio *et al*., 2000; Kirwan *et al*., 2001) suggest that ingestion of a low-GI meal prior to exercise increases fat oxidation and maintains blood glucose at a higher level throughout exercise, compared with a high-GI pre-exercise meal. Few studies have, however, reported increased endurance after a low-GI, compared with a high-GI meal (Thomas *et al*., 1991; Kirwan *et al*., 2001). It is common that carbohydrates are ingested during prolonged exercise, which minimizes the importance of the GI of the pre-exercise meal (Burke *et al*., 1998).
Background

After prolonged intensive exercise, it has been suggested that high-GI foods accelerate muscle glycogen resynthesis (Burke et al., 1993; Jozsi et al., 1996). Although this may be of limited importance to the general public, the beneficial effect of high-GI foods after intensive exercise offers a market for products intended for athletes.

As glucose is the main metabolic fuel for the brain, it has been hypothesized that the rate of glucose delivery to the blood also determines the availability of glucose to the brain. In line with that hypothesis there has been some evaluations of the glycaemic features of various carbohydrates in relation to cognitive performance. In some of those studies it has been indicated that having breakfast is associated with improved memory, compared with having no breakfast (Dye et al., 2002). Furthermore, it appears that in some cases, glucose ingestion can enhance the memory and attention span (Dye et al., 2000), although the mechanism behind this effect is not clear. The effects of carbohydrates, other than glucose, are less distinct and they seem to change with the time of day. Little is known about the effects of protein and fat on cognitive performance and, because of differences in macronutrient profile between studies, it is difficult to relate the findings to a single meal component. Several studies also have “no food” as the control, which does not constitute a suitable reference.

Regarding a potential relation between GI and cognitive performance, only limited data are available in the literature. One recent study showed that the consumption of a low-GI breakfast was associated with improved cognitive performance in both humans and rats (Benton et al., 2002). However, the blood glucose levels per se could not be correlated with the improved cognitive performance, suggesting that the underlying mechanism is related to other features of the postprandial metabolic state. The role of insulin is increasingly discussed in relation to cognitive performance (Park, 2001).

1.3.3.2 Growth-stimulating properties of a high-GI diet

The hyperinsulinaemic properties associated with a high-GI diet stimulate the growth of both normal and cancer cells through a mechanism mediated by insulin-like growth factor (IGF-1) and its binding proteins (IGFBP-1 and IGFBP-3). Consequently, in addition to the positive metabolic effects of low-GI/GL diets described above, two recent papers suggest that there are associations between the dietary GI/GL and the risk of breast cancer in women (Augustin et al., 2001) and colorectal cancer in men and women (Franceschi et al., 2001). In addition, recent data suggest that the development of myopia is partly caused by hyperinsulinaemia stimulating an abnormal vitreal chamber growth (Cordain et al., 2002).
1.3.3.3 Caries

Starch degradation in the mouth can be described as a two-step process. Firstly, the starch molecules are cleaved by salivary \textit{alpha}-amylase into maltose, maltotriose and dextrins. Secondly, these starch degradation products, in particular maltose and maltotriose, are metabolized further by plaque microorganisms into lactate and other organic acids. The pH drop in the plaque varies depending on the characteristics of the ingested food. A high correlation has been found between the GI and the fall in plaque pH for starchy foods such as pasta, potatoes, rice and different kinds of bread (Lingstrom \textit{et al.}, 2000). Consequently, high-GI starchy foods not only lead to more accentuated postprandial blood glucose increments; but also to a more prominent pH drop in dental plaque. Since prolonged exposure of the enamel to low pH is one factor in the aetiology of dental caries, high-GI foods can be expected to be more cariogenic.
1.4 Food factors affecting GI

A number of food factors appear to affect the rate of glucose delivery to the blood. Some are related to the characteristics of the raw food, while others are related to the processing conditions. The various food factors responsible for low-GI features are discussed below. According to the literature, there are three main ways at the gastrointestinal level whereby these food factors affect the blood glucose response. These gastrointestinal events include lowering of the GER, lowering of the rate of absorption and/or exposure of the luminal content to the brush-border enzymes, and in the case of starchy foods, lowering of the rate of starch digestion in the upper small intestine. Some food factors may affect more than one of these events.

1.4.1 Type of carbohydrate

The GI of pure, low-molecular-weight carbohydrates decreases in the following order: glucose > sucrose > lactose > fructose (Table 2). Consequently, the GI of carbohydrate foods is dependent on the carbohydrate composition. In addition to the carbohydrate monomer per se, the combination of carbohydrates may affect the rate of absorption. It has, for example, been suggested that the rate of fructose absorption increases when fed in combination with glucose or starch (Ribi et al., 1993). A study in Zucker (fa/fa) rats has shown that small amounts of orally administered fructose or sucrose can be useful in lowering the postprandial glucose response to a carbohydrate challenge (Wolf et al., 2002). The suggested explanation of this phenomenon is that fructose increases the postprandial uptake of glucose in the liver. In contrast, dietary fructose has been associated with increased fasting and postprandial plasma TG concentrations in men (Bantle et al., 2000). However, according to Wolf et al. (2002), the negative attributes of fructose feeding have only been documented at high dietary intakes. Consequently, differences in the type of carbohydrate present may be partially responsible for the important differences seen in GI features among fruits (Foster-Powell et al., 2002).

1.4.2 Starch interactions as affected by structure and processing

In the case of starchy foods, the rate of small-intestinal digestion is an important determinant of the glycaemic response, and hence of GI (Granfeldt et al., 1992). The starch crystallinity greatly affects the availability to amylases. Consequently, the highly ordered structure present in native starch granules constitutes a barrier to enzymatic attack, thereby lowering the rate of digestion (Bornet et al., 1989).
The starch crystallinity induced upon heat treatment and cooling (retrogradation), may create regions of highly ordered structures which are less readily hydrolysed by amylases, or in some cases even rendered totally resistant (Englyst et al., 1985 and 1987).

Processing can also create macromolecular interactions between starch and other food constituents rendering the starch less readily available for digestion. For example, in the case of retrograded starch, the lower digestibility is caused by interactions in between starch molecules. The main macromolecular interactions responsible for the pasta texture are those between starch and protein (Colonna et al., 1990). These interactions are also responsible for the low-GI properties of this food group (Granfeldt et al., 1991). The low-GI features of pasta products have been explained by a limited swelling of starch granules during cooking caused by encapsulation of starch by the protein network (Colonna et al., 1990). According to Holm et al. (1992), canning of pasta products increases the glycaemic response and the suggested mechanism for the increased starch availability was an excessive swelling of starch that promoted weakening and disruption of the protein network.

In bread, it is possible to promote retrogradation of amylose by the use of high-amylose-containing flour (Granfeldt et al., 1995) and/or baking at pumpernickel conditions (120ºC for 20 h), thus reducing the rate of starch hydrolysis and the GI (Åkerberg et al., 1998). In addition, it has been suggested that amylose forms complexes with lipids, which display a lower availability to amylases in the rat small intestine (Holm et al., 1983).

### 1.4.3 Botanical structure and dietary fibre

Both the particle size and the surface-area-to-starch ratio play important roles for the rate and extent of amylolysis (Snow et al., 1981). Dietary fibre as part of an intact botanical structure may be effective in reducing glycaemia (Liljeberg et al., 1994). In contrast, the levels of naturally occurring dietary fibre in common wholemeal flours appear to have no effect on postprandial glucose responses (Snow et al., 1981; Liljeberg et al., 1992 and 1994). However, high levels of viscous, soluble dietary fibre have the capacity to lower the rate of glucose delivery to the blood (Braaten et al., 1991). The physiological mechanism for an effect of the viscous fibre component is a lowering of the GER (Torsdottir et al., 1991), and/or an increase in the thickness of the unstirred layer of the small intestinal mucosa, resulting in the creation of an absorption barrier (Flourie et al., 1984).
Consequently, the inclusion of a barley genotype, *Prowashonupana* (PW), with high levels of viscous dietary fibre was found to lower the GI of bread and porridge products in healthy humans (Liljeberg et al., 1996b).

Recent data on bread baked with flour from PW kernels indicate that the handling of the raw material is crucial in achieving the desired effect (Rossi, Larsson, Elmståhl and Björck, unpublished data). In that study, PW flour was included at levels of 0, 35, 50 and 75% in flat bread, in mixture with wheat flour. Flour from two different batches of scalded and heat-treated PW kernels were used at the 50% level. The viscosity of solutions with isolated fibre fractions (1-4% dry matter) was measured, and a fluidity index (FI) was determined for bread products that had been subjected to simulated enzymatic digestion. All bread products were also tested in healthy subjects in order to characterize the postprandial glucose and insulin responses. With increasing amounts of PW the GI and II were lowered by up to 40%. Furthermore, the GI could be predicted from the determination of FI in a simple enzymatic in vitro system with measurements of fluidity (Bostwick consistometer). A linear correlation was found between GI and FI ($r = 0.9917$, $P = 0.000$), suggesting that the higher the viscosity, the lower the GI. However, one of the PW batches had completely different viscosity properties from the other (intrinsic viscosity=1.90 and 0.88, respectively), and therefore, did not affect postprandial glycaemia. Neither did a barley flour of the commercial type have any effect on the blood glucose response. It was concluded that it was the viscosity and not the level of beta-glucans that was important for the improved glycaemia. Thus, it is important to be careful in the processing of grains in order not to destroy valuable characteristics of the raw material.

Commercial canning has been shown to increase the digestibility of beans both in vitro and in vivo, compared with domestically cooked beans (Traianedes et al., 1986). Both the high temperature and moisture during pressure canning were thought to affect the in vitro rate of starch hydrolysis in canned beans. It was also concluded that the pressure-cooked beans were physically different from the domestically boiled beans, suggesting that the canning process could change the nature of the starch interactions with fibre and/or protein. Another possibility is that certain antinutrients that affect starch digestion are inactivated during the canning process. It is also possible that a loss in fibre viscosity occurred during the canning process of the beans, similar to the loss of viscosity of the PW barley batch mentioned above.
1.4.4 Antinutritional factors

High intake of phytic acid has been found to be negatively correlated with GI in healthy individuals (Yoon et al., 1983; Thompson et al., 1987). The mechanism suggested for lowered glycaemia was that phytic acid may affect starch digestibility through interaction with the amylase protein and/or binding with salivary minerals such as calcium, which is known to catalyse amylase activity (Thompson et al., 1987).

Lectin and polyphenol concentrations have also been shown to be negatively related with glycaemic response in normal and diabetic individuals (Thompson et al., 1984; Rea et al., 1985). The delayed appearance of glucose in the blood after ingestion of lectin-containing foods may be caused by either interference with the mucosal phase of digestion or by direct binding of lectin to the starch and/or the digestive enzymes (Rea et al., 1985).

Similarly, the suggested mechanism for the blood glucose lowering effect of polyphenols is that they either interact directly with starch molecules and/or undergo protein-polyphenol interactions that reduce the protein digestion and, hence obstruct the digestion of starch molecules within the protein network (Thompson et al., 1984).

1.4.5 Organic acids

Organic acids are naturally present in certain foods, can be produced upon fermentation or added, as in the case of pickled foods. Examples of common fermented foods are dairy products such as yoghurt, crème fraîche and cheese, as well as sourdough bread and certain meat products, e.g. salami.

A relatively small number of studies have been carried out where the effects of organic acids on postprandial glycaemia have been evaluated. Inclusion of either lactic acid (added directly or through fermentation) or the sodium salt of propionic acid in bread products (Liljeberg et al., 1995 and 1996a), as well as the presence of lactic acid in mixed meals with vegetables (Torsdottir et al., 1992; Gustafsson et al., 1994), has been reported to lower postprandial glycaemia and insulinaemia. In addition, two studies have reported blood-glucose-lowering effects of acetic acid in bread meals (Brighenti et al., 1995; Liljeberg et al., 1998). In the case of acetic and propionic acids, the suggested mechanism for the glucose lowering effect was a delayed gastric emptying (Liljeberg et al., 1996a and 1998; Darwiche et al., 2001). In addition, for bread to which the sodium salt of propionic acid was added, an increased postprandial satiety was observed (Liljeberg et al., 1996a), suggesting a potential effect on weight regulation. Regarding lactic acid, the literature on mechanisms is limited and no studies appear to have been published on the long-term effects of fermented foods.
1.5 The GI concept; current status and objections

One objection to the GI concept has previously been the lack of long-term clinical investigations. Today, however, a substantial amount of data is available supporting the use of low-GI foods for long-term beneficial effects (Section 1.3.1). On no occasion have low-GI diets shown any adverse effects in intervention studies (Ludwig, 2002). The present lack of an adequate number of low-GI foods, particularly in the group of basic carbohydrate-rich foods, limits the application of the GI concept. In addition, concern has been raised about the complexity of communicating the GI concept to consumers (Pi-Sunyer, 2002). Since many GI studies address factors such as the degree of gelatinization of starch and physical form of the foods, it would be necessary to make the consumer aware of the importance of handling the food in such a way as to maintain its beneficial glycaemic characteristics. In order to be successful in the communication to the consumers, low-GI foods must provide attractive and comparable alternatives to the wide range of convenience foods of today.

A problem with existing international GI tables is that they often lack relevant information regarding food composition and processing conditions (Foster-Powell et al., 2002). It is therefore recommended that GI values be accompanied by such information in the future. Another problem with GI tables is that in some cases there is a relatively wide variation of GI values for similar food products when tested in different laboratories (Foster-Powell et al., 2002). This variation reflects methodological differences and/or differences in the physical and chemical properties of the food products. Examples of differences between laboratories are: 1) how the carbohydrate content of the test food is determined (chemical analysis/calculation by difference), 2) food preparation procedure, and 3) the method of blood sampling (venous/capillary). Work is in progress at GI laboratories around the world to standardize the methodology in order to minimize the variation.

A common objection to the GI concept is also that the fat and protein contents of the diet can counteract the beneficial properties of low-GI foods (Pi-Sunyer, 2002). Simultaneous ingestion of fat and protein lowers the GI of the individual carbohydrate foods somewhat, but does not change their hierarchical relationship with regard to GI (Wolever et al., 1996; Ludwig, 2002). Moreover, in several long-term studies, where only the carbohydrate source was changed from high to low-GI alternatives, beneficial effects on the glucose and lipid metabolism were observed in both healthy and diabetic subjects (Jarvi et al., 1999; Bouche et al., 2002), suggesting that differences in GI characteristics of individual carbohydrate foods affect glycaemia and insulinaemia also as components in mixed diets. In addition, by merely changing the GI of bread in the diets of women with impaired glucose tolerance, their insulin economy was improved (Liljeberg Elmstahl, Frid, Groop and Björck, unpublished data).
1.6 Conclusions

In conclusion, there is a substantial amount of evidence from intervention studies that low-GI diets are useful in the treatment of metabolic disorders, and reduces risk factors associated with type 2 diabetes and CVD. Furthermore, epidemiological and prospective studies indicate a protective role of low-GI diets against the development of type 2 diabetes and CVD. However, there is a need for more carefully controlled studies where, in particular, the original focus and the design of dietary questionnaires relate to the GI concept.

Although there are evidence available that low-GI foods promote a higher post meal satiety and may reduce cumulative food intake at proceeding meals, there is still a lack of information from long-term studies regarding the possible relation between dietary GI/GL, satiety and body weight regulation.

The FAO/WHO expert consultation on “Carbohydrates in human nutrition” supports the choice of low-GI foods (FAO/WHO, 1998). Also in the current European dietary recommendations for type 2 diabetics, the importance of carbohydrate foods rich in dietary fibre or with a low GI, is specifically stated (DNSG, 2000). Furthermore, this is in line with the recommendations from the Swedish diabetic association and the healthy eating guidelines for diabetics in Australia. However, in contrast to this consensus, in recommendations from the American Diabetes Association it is stressed that the total amount of carbohydrates in meals or snacks is more important than the source and type of the carbohydrate (ADA, 2002).

The methodology for GI determination needs to be standardized, and published GI values in international tables should be accompanied by relevant characteristics regarding e.g. carbohydrate composition and processing conditions. There is also a need for an extended list of low-GI foods, particularly among the starchy staple foods.

In Australia a programme was recently launched for labelling of food products with their GI value. In order for such a programme to be successful there is a need for education of the consumers, as well as more low-GI alternatives on the food market. Experiences from the Australian labelling programme may add important knowledge on the consumer acceptance of the GI concept, but more research is needed in the field of consumer communication.
Objectives

2 Objectives

The overall purpose of the work presented in this thesis was to examine the use of fermentation as a means of lowering the GI characteristics of carbohydrate foods. Previous studies have shown that addition of lactic acid to bread, or the use of homofermentative lactic-acid-yielding starter cultures reduce the rate of starch hydrolysis as measured \textit{in vitro}, and the glycaemic and insulinaemic properties in healthy subjects; indicating that lactic acid may interfere with the digestive process. The mechanism at a food level has, however, not been revealed.

The purpose of the present thesis was firstly, to evaluate the food mechanism for a reduced availability of starch in lactic-acid-containing bread. Secondly, the purpose was to study the potential effect of lactic acid in other food systems such as gruel or milk products. Finally, the metabolic effects of lactic-acid-containing bread on glucose tolerance and related parameters were evaluated in a second meal study in healthy subjects, and in a dietary intervention on obese, hyperinsulinaemic rats.
3 Materials and Methods

3.1 Description of the test products

3.1.1 Bread products

The white wheat bread used as a reference (Papers 1 and 2) and for microscopy was based on commercially available wheat flour (Kungsörnen AB, Järna, Sweden) and baked according to Liljeberg and Björck (1994). The lactic-acid-containing wheat bread used for microscopy was baked according to the same recipe, substituting 6 g water by 6 g lactic acid (no. 27714, 88-92 % total acid, Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany).

The flour used for the barley bread (Paper 3) was obtained by milling intact barley kernels without hulls (no. 8775, Svalöf Weibull AB, Svalöv, Sweden). In the barley bread 80% of the flour was barley flour and the remaining 20% wheat flour. The barley bread was baked with or without the presence of lactic acid (see details above). The recipe and baking procedure are described in Paper 3.

The bread used in the diets of the animal study was prepared in a pilot-plant bakery (Nord Mills AB, Malmö, Sweden). Two types of bread were prepared from wheat flour (Bagerivete, Nord Mills AB, Sweden), one with and one without lactic acid (see details above). The recipe and baking procedure are described in Paper 4. Furthermore, lactic-acid-containing bread products based on Extra Bagerivete (Nord Mills AB, Sweden) were baked in the same pilot-plant bakery, with the same baking procedure. One of the latter bread products was enriched with wheat gluten (1.2% of total flour weight) and the detailed recipes are presented in Paper 2.

3.1.2 Fermented barley gruel

Three different kinds of gruel were studied (Paper 2), all based on high-amylose barley flour (hull-less Glacier, 42 % amylose, Svalöf Weibull, Svalöv, Sweden). The recipe is described in Paper 2. One type of gruel was fermented with Lactobacillus plantarum 299 and Lactobacillus plantarum 16M2, yielding both L- and D-lactic acid and the other was fermented with Lactobacillus rhamnosus 271, and thus contained only the L-form of lactic acid. The third kind of gruel was not fermented.

3.1.3 Dairy products

The dairy products studied (Paper 1) were regular milk and three fermented products; yoghurt, “filmjölk” and “ropy milk”. All products had a fat content of 3%. The milk, filmjölk and yoghurt were produced by Skånemejerier (Malmö, Sweden) and the ropy milk was produced by Arla (Gävle, Sweden).
3.1.4 Pickled cucumber

The pickled cucumber studied (Paper 1) was made in the laboratory using 0.5 kg sliced cucumber, 200 ml acetic acid (12%) and 300 ml water.

3.1.5 Model system – simulated baking of starch/gluten mixtures

A simplified model system containing starch and gluten was studied (Paper 2). Wheat starch (Cerestar, Germany) was mixed with wheat gluten (Sigma Chemical Co., MO, USA) and a phosphate buffer. The pH was adjusted with either lactic or hydrochloric acid, to 4.0, which corresponded to the pH in a previously studied lactic-acid-containing bread (Liljeberg et al., 1995).

The starch/gluten mixture was incubated to imitate the baking procedure, i.e. 22ºC for 50 min, then 38ºC for 20 min followed by 100ºC for 15 min. The starch/gluten mixtures were subjected to different kinds of treatment regarding gelatinization and homogenization conditions and after each treatment the rate of in vitro starch hydrolysis was evaluated (analysis explained in Section 3.4).

3.2 Chemical analyses

3.2.1 Starch

The starch content of all bread products was determined by an enzymatic method (Holm et al., 1986). Each sample was suspended in phosphate buffer and incubated with thermostable alpha-amylase (Termamyl, Novo Nordisk A/S, Denmark) in boiling water, followed by incubation with amyloglucosidase (Roche Diagnostics GmbH, Germany) at 60ºC. At the end of this procedure, the released glucose was measured with a glucose oxidase peroxidase reagent and the starch content could be calculated from the amount of glucose multiplied by 0.9.

3.2.2 Monosaccharides

The contents of glucose, galactose and lactose in the dairy products (Paper 1) were analysed by using a commercially available enzymatic kit (Boehringer Mannheim GmbH, Mannheim, Germany).

3.2.3 Fat and protein

Crude protein content was measured according to the Kjeldahl method, using 6.25 as a conversion factor from nitrogen to protein. The fat content was analysed according to the Schmid-Bondzynski-Ratslaff (SBR) method.
Fat analysis using the SBR method includes acidic hydrolysis followed by extraction with ether/petroleum ether (Lange, 1972). Thereafter, the solvent is evaporated and the fat residue weighed.

### 3.2.4 Lactic acid

The amount of lactic acid in the bread products (Papers 2–4) was analysed with a commercially available enzymatic kit (Boehringer Mannheim GmbH, Mannheim, Germany). The samples were prepared for lactic acid analysis according to Lönner et al. (1988).

### 3.3 Amylase kinetics

Soluble starch (acc. to Zulkowsky GR (Zulkowsky, 1880); MERCK 101257, Darmstadt, Germany) was used as a substrate for the pancreatic alpha-amylase (Sigma A-6255, Sigma Aldrich, Steinheim, Germany) and the reaction rate $v$ (mg/ml·s) was measured at different substrate concentrations $[S]$ (Paper 2). Tannic acid was used as a known inhibitor of alpha-amylase. A Lineweaver-Burke plot was drawn, the slopes and intercepts calculated and finally comparisons between the inhibiting effects of alpha-amylase and tannic acid could be made.

### 3.4 Rate of in vitro starch hydrolysis

To measure the rate of starch hydrolysis, the in vitro method described by Granfeldt et al. (1992) was used. The aim of this method is to imitate the starch-degrading process in the oral cavity and small intestine. Six persons were asked to chew a portion of the sample and then spit it out into a mixture of phosphate buffer, proteolytic enzyme and HCl. After incubation at 37ºC at a very low pH (1.5-2.0), the sample was neutralized and alpha-amylase was added. The sample was transferred to dialysis tubing (molecular weight cut-off 12,000-14,000), which was then put in a beaker containing phosphate buffer. The products of starch degradation could pass through the dialysis tubing and samples were taken from the surrounding buffer solution at 30-min intervals for three hours. The amount of reducing sugars was determined spectrophotometrically and the amount of maltose equivalents in the sample was determined from a standard curve. A hydrolysis index (HI) for each sample was subsequently determined by calculating the area under the hydrolysis curve of each sample as a percentage of the corresponding area for white bread. In case of the starch/gluten mixtures, wheat starch suspended in water was used as the reference. Each chewing person was his or her own control.
Materials and Methods

From the HI it is possible to predict the GI for a test product by using the following equation: GI = 0.862 x HI + 8.198. This equation was based on data for 29 cereals and legumes (Granfeldt, 1994).

3.5 Microscopic studies

Pieces of reference and lactic-acid-containing wheat bread, respectively, were embedded into Historesin by the following procedure:

1) The sample was fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH=7) overnight (4°C).
2) The sample was washed and dehydrated (room temperature) with H₂O (3 x 30 min), 50% EtOH (3 x 30 min), 70% EtOH (3 x 30 min) and 95% EtOH (2 x 30 min), and left in a 3rd volume overnight.
3) The sample infiltrated (room temperature) for 2-3 days in a mixture (1:1) of 95% EtOH and an infiltration solution.
4) The sample was put in embedding medium 8 h overnight (room temperature) and then attached to an adapter using a mounting medium (10-15 min).

Using a microtome, 4 µm slices of the embedded sample were cut. The sample slices were put in water on an object glass and were left to dry in air. To stain the protein in the sample a light green solution (0.1%) was used and to stain the starch, Lugols solution (iodine) was used. The microscopic evaluations were performed at VTT (Helsinki, Finland) in collaboration with Prof. Karin Autio.

3.6 Meal studies in healthy volunteers

3.6.1 Subject characteristics

Healthy men and women, not undergoing any medical treatment and with normal BMIs (22±2) participated in the studies (Papers 1-3). The average age of the test subjects was 34±9 years and their fasting glucose and insulin levels were within the normal range; 4.6±0.3 mmol/l for blood glucose and 0.05±0.02 nmol/l for serum insulin. All values are presented as the mean±SD.

The subjects were told to abstain from alcoholic beverages and strenuous physical activity on the day before each experiment. Additionally, they were asked to eat a few, individually standardized, slices of white bread between 9 and 10 p.m. on the night before the experiment. For each subject there was approximately one week between the experiments and the breakfast meal, was commenced at the same time every morning. All meals were consumed steadily over a period of 12-14 min.
3.6.2 Postprandial effects

In Papers 1 and 2, the postprandial effects of fermented and non-fermented dairy, cereal and vegetable products are presented. The amount of available carbohydrates served in these studies varied from 25 g to 63.5 g and the reason for this was the difference in carbohydrate content of the tested food products. When the test subjects arrived in the morning a finger-prick capillary blood sample was taken to determine the fasting values of blood glucose and insulin. After breakfast, blood samples were taken for blood glucose analysis at 15, 30, 45, 70, 95 and 120 min and for serum insulin analysis at 15, 30, 45, 95 and 120 min. In the studies on milk products with added cucumber (Paper 1) and fermented barley gruel (Paper 2) an additional blood sample was taken at 180 min for determination of glucose. Blood glucose was analysed using a glucose oxidase peroxidase reagent and serum insulin was analysed with an immunoassay kit (Boehringer Mannheim GmbH, Mannheim, Germany).

3.6.3 Second meal effects

Barley bread or corresponding lactic-acid-baked barley bread were given to healthy subjects as a breakfast (Paper 3). The blood glucose and insulin responses were followed after a subsequent standardized lunch ingested 4h after the test breakfasts, i.e. at the second meal. Fasting blood samples were taken from each subject on the morning of the test. The test breakfast was then ingested during 12-14 min but blood sampling did not follow. However, the test subjects remained in the laboratory until 4 h after the breakfast, when they were served a standardized high-GI lunch consisting of fried meatballs and mashed potatoes. Prior to (0 min) and at 15, 30, 45, 70, 95, 120 and 180 min after the lunch, capillary blood samples were collected from each subject. Blood glucose was determined at all time points while insulin was analysed only at 0, 15, 30, 45, 95 and 120 min. Analysis procedures were the same as described above (Section 3.6.2).

3.6.4 Evaluation of glucose and insulin responses

For each subject and test meal, the glucose and insulin AUCs were calculated (GraphPad Prism, ver. 2.0/3.0; Graph Pad Software, CA, USA). All areas below the baseline were excluded from the calculations. Values are presented as mean±SEM and statistical calculations were performed in MINTAB Statistical Software (release 12/13 for Windows; Minitab Inc., State College, PA, USA). Significance was evaluated with the general linear model (analysis of variance), followed by Tukey’s multiple comparisons test. Values of \( P < 0.05 \) were considered significant. In the studies where GI and II were calculated (Papers 1 and 2) the glucose and insulin AUCs for each test meal at 95 min were divided by the corresponding areas for the white wheat reference bread. To evaluate the results presented in Paper 3, the AUCs following lunch were compared with each other.
3.7 Animal study

Semi-long-term metabolic effects of diets containing lactic acid were evaluated during a 14-day intervention study on obese rats (Paper 4). Zucker \((fa/fa)\) rats were chosen as the model because they are obese, hyperinsulinaemic and have several other characteristics linked to IRS. Forty male rats with an initial mean weight of 217 g (SEM=3) were used and the intervention diets were based on wheat bread with additions of either lactic acid (added prior to or after baking) or probiotic bacteria \((Lactobacillus plantarum 299v)\). On day 1 and 14 of the intervention period, OGTTs were performed on the rats, with blood sampling at 0, 8, 15, 30 and 60 min after glucose intubation. Urine was collected during the last five days of the study and when the rats had been killed with CO\(_2\), the livers were removed and the blood collected by cardiac puncture.

3.7.1 Evaluation of metabolic parameters

The blood samples were analysed for glucose \((Bruss et al., 1978)\), insulin \((Heading, 1966)\) and glucagon \((Ahrén et al., 1982; Panagiotidis et al., 1992)\). Glucose in urine was analysed by gas chromatography \((Hewlett Packard 6890 GC System)\) with flame ionisation detection \((FID)\), after derivatization according to AACC method 32-25 \((Theander et al., 1995)\). The total cholesterol in liver tissue was analysed with an enzymatic kit \((R-Biopharm no. 0139050, Germany)\), after extraction with potassium hydroxide \((1 M)\) in methanol. Serum cholesterol and triglycerides were analysed with enzymatic kits \((Sigma Diagnostics, USA; Infinity™ Cholesterol reagent, no. 401 and Triglycerides (UV), no 334-UV, respectively)\). The same statistical analysis as in the human studies was carried out on the analytical data from the rats. Values of \(P < 0.05\) were considered significant.
4 Results and Discussion

4.1 Effects of lactic acid on the rate of starch hydrolysis and microstructure in bread and bread-like systems

4.1.1 Barley bread baked with addition of lactic acid

Previously, Liljeberg et al. (1995 and 1996a) reported a glucose lowering effect of lactic acid in barley bread products, when added at a level corresponding to 1% of the dough weight. In Paper 3, an identical lactic-acid-containing barley bread was used as test breakfast to study potential effects on glucose tolerance on a subsequent lunch meal in healthy subjects (Section 4.3) In the mentioned studies by Liljeberg et al. it was shown that a lowered acute glycaemic response to lactic-acid-containing barley bread could be predicted from the in vitro rate of starch hydrolysis (equation presented in Section 3.4). That finding supported the relevance of using in vitro experiments to detect effects of lactic acid on glycaemia to bread products in vivo.

4.1.2 Rate of in vitro amylolysis

To investigate the mechanisms behind the glucose lowering effect of lactic acid in bread products, model systems containing water and starch (1 g available starch) were subjected to heat treatment at simulated baking conditions, with and without lactic acid (1.4% of mixture weight) and gluten (15% of total starch weight) (Paper 2). The in vitro rates of starch hydrolysis were measured and corresponding HIs were calculated.

Heat treatment of the starch/gluten mixture had no effect on HI compared with heating of starch alone. When starch was heated in presence of lactic acid there was still no lowering of the HI. However, when lactic acid was added to the starch/gluten mixture, a 20% reduction was noted in the HI (P < 0.05). This suggests that some interactions between starch and proteins take place in the presence of lactic acid, resulting in a lower rate of starch hydrolysis.

In experiments with wheat bread products baked with lactic acid (0.7% of dough weight), a significant lowering of the HI (-8%, P < 0.05) was obtained by adding wheat gluten (1.2% of total flour weight) to the dough, compared with a similar lactic-acid-containing bread without gluten enrichment (Paper 2). This finding agrees with the results from the model products described above.

Also in barley bread products with lactic acid (0.8% of dough weight), an enrichment of wheat gluten (8.8% of total flour weight) significantly lowered the HI (-9%, P < 0.05) (Table 3, unpublished data). It should be noted that the barley bread contained 20% wheat flour, thus making it difficult to distinguish to what extent the proteins in the barley were involved.
Table 3. The HIs for barley bread to which lactic acid and gluten was added (unpublished data). Values are means from 6 replicates. Values with different superscripts are significantly different ($P < 0.05$).

<table>
<thead>
<tr>
<th>Bread product</th>
<th>HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100$^a$</td>
</tr>
<tr>
<td>Barley/wheat (80:20) bread with lactic acid</td>
<td>85$^b$</td>
</tr>
<tr>
<td>Barley/wheat (80:20) with lactic acid and gluten</td>
<td>78$^c$</td>
</tr>
</tbody>
</table>

Taken together the results with gluten addition to a model system of starch and lactic acid suggest that the presence of protein is a prerequisite to achieve a lowered rate of starch hydrolysis. The results from gluten enrichment of wheat and barley bread products further strengthen the hypothesis that lactic acid enhances interactions between starch and cereal proteins.

The effect of gluten on postprandial blood glucose response to bread and other cereal products has been studied previously in other laboratories. Jenkins et al. (1987c) found that bread made from gluten-free wheat flour increased the blood glucose response by almost 30% ($P < 0.05$) in healthy subjects. In contrast, Packer et al. (2000) found no differences in GI between gluten-containing and gluten-free wheat and maize products in type 2 diabetic subjects. Few studies have assessed the effect of enrichment with gluten. The present work does, however, indicate that the simultaneous presence of lactic acid and gluten importantly reduce the rate of amylolysis in vitro, and in future development of low-GI sourdough and/or lactic-acid-containing bread products, the potential of adding gluten or the use of wheat varieties with high protein content may be exploited. Enrichment with gluten, by enhancing the lactic acid effect, may further facilitate the production of sourdough bread with high sensory characteristics.

Results from the kinetic studies using soluble starch as a substrate showed that lactic acid does not act as a classical inhibitor of alpha-amylase (Paper 2). This was also evident from the differences obtained with equivalent amounts of lactic acid whether added prior to or after the simulated baking of the starch/gluten mixtures. When lactic acid was added after the gelatinization, no lowering effect was seen on the rate of starch hydrolysis (HI=105), whereas when lactic acid was present during heat treatment, the HI of the starch/gluten mixture was significantly lowered to 80 ($P < 0.05$). The latter finding suggests that heat treatment in the presence of lactic acid is a prerequisite for creating a barrier to starch degradation.
The effect of homogenization on amylase availability with and without lactic acid was also studied in the model system, to investigate a possible enzyme obstruction caused by macromolecular interactions. The results showed that homogenization of the starch/gluten mixture, heated in presence of lactic acid, significantly increased HI compared with the corresponding mixture that had not been homogenized (HI=100 and 80, respectively, $P < 0.05$).

Homogenization of a wheat bread baked with lactic acid also tended to increase the HI (81 vs. 86), although the increase was not statistically significant. Factors that make wheat bread different from the starch/gluten mixture discussed above are the presence of components other than starch in the flour (e.g. fibre, protein etc.), the presence of monoglycerides in the dough and a difference in water content. In ordinary white wheat bread to which lactic acid has been added, the amount of starch per gram water is ten times higher than in the studied starch/gluten mixtures. Hence, the microstructure of bread is different from that of the starch/gluten mixture and the interaction between starch and gluten in bread is perhaps more resistant to homogenization. Similarly, when spaghetti was vigorously homogenized, the rate of in vitro starch hydrolysis increased compared with intact spaghetti, but did not reach the level seen with bread made from identical ingredients (Granfeldt et al., 1991). In fact, it was not possible by mechanical means to obtain a homogenous pasta slurry. The results for spaghetti and lactic-acid-containing bread suggest that the microstructure is important in the lowering of glycaemia, and that it is not readily destroyed by mechanical treatment.

When investigating the effects of lactic acid on amylolysis in cereal products, a possible formation of RS must be considered. According to Liljeberg et al. (1996c), lactic acid may promote retrogradation of starch. However, they only found an increased RS formation in rye bread baked at pumpernickel conditions, and not in ordinary baked lactic-acid-containing rye bread (Liljeberg et al., 1995). Furthermore, HI was not lowered in the pumpernickel-baked rye bread products, suggesting that the increased amount of RS per se was not associated with a lowering of GI.
4.1.3 Microstructure of lactic-acid-containing bread

Light microscopy was used to find visible evidence of a possible enzymatic barrier in lactic-acid-containing bread (0.8% lactic acid of dough weight, unpublished data). When comparing the loaf volumes of wheat bread with and without lactic acid it was obvious that the lactic acid bread had a more dense structure. The micrographs presented in Figure 2, indicate a tendency of a limited granule swelling, suggesting a lower degree of starch gelatinization in a wheat bread containing lactic acid, than in a similar bread with no acid. Furthermore, in the lactic-acid-containing bread the protein phase was more evenly dispersed than in the reference bread, suggesting a higher degree of interaction between starch and protein in the former.

When studying the same bread products in microscope at a higher resolution, it was evident that there was less amylose leakage from the starch granules in the lactic-acid-containing bread compared with the reference bread. This is in agreement with a limited degree of starch gelatinization. The limited degree of granule swelling may have been due to enclosure of the starch within a starch-protein network, similar to the situation in pasta products (Section 1.4.2), and/or due to a limited granule swelling caused by lactic acid per se.
Micrographs of white wheat bread baked with (right) and without (left) lactic acid (unpublished data). Green represents protein and the starch granules are brown (amylopectin) and blue (amylose) (Autio et al., 2001).
4.2 Impact of lactic acid on glycaemia and insulinaemia to cereal- and dairy products in healthy subjects

4.2.1 Fermented barley gruel

The glycaemic and insulinaemic responses to lactic-acid-fermented or non-fermented barley gruel were evaluated and compared with the corresponding responses to white wheat reference bread (Paper 2). The gruel was based on high-amylose barley flour, which has previously been recognized as having a lowering effect on in vitro starch digestion of bread products (Liljeberg et al., 1996c). Preparation of the gruel was done by mixing water and barley flour with malt flour and an amylolytic enzyme preparation to reduce starch viscosity. The mixture was then heated in two steps (55°C and 95°C) followed by cooling to ambient temperature. Finally, a starter culture was added and the gruel was incubated at 37°C for 16 h. The difference between the fermented gruel varieties lay in the isomeric forms of lactic acid; one type of gruel containing only L-lactic acid and the other containing both the L- and D-forms.

All kinds of gruel elicited glucose and insulin responses in the range of the reference bread, or higher (GI=154-165 and II=95-115). The lack of effect on glycaemia of the fermented barley gruel might have been due to the fact that the lactic acid was not present during heat treatment of the barley flour. In parallel in vitro experiments it was seen that mixtures of high-amylose barley flour and water, with lactic acid added either prior to or after heat treatment, gave significantly different HIs (81 and 89, respectively, \( P < 0.05 \)).

Considering the finding that addition of water can increase the glycaemic response to a solid meal (Torsdottir et al., 1989) it is likely that the GER was higher with the liquid gruel than with the solid reference bread. Consequently, a higher GER might explain the higher glycaemic and insulinaemic responses to the barley gruel products. The lack of effect on glycaemia might also have been due to that the high-amylose barley flour was partly or completely hydrolysed by the enzyme preparation added, which contained both starch- and protein-degrading enzymes. The protease and amylase activity in the enzyme preparation might thus have eliminated the possibility of the formation of starch-protein interactions.
4.2.2 Fermented vs. non-fermented dairy products

From the results presented in Paper 1, it was evident that milk products, whether fermented or not, led to low postprandial glucose responses (GI=15-30). Consequently, the presence of lactic acid did not cause any additional lowering of the glycaemia, compared with non-fermented, regular milk. It should be pointed out, however, that the GI for all milk products fell within a very low range, making it more difficult to detect any possible lowering effect of lactic acid.

In a previous study of ropy milk it was found to elicit a lower glucose response than regular milk (Strandhagen et al., 1994). The lower glycaemia was explained by a lower GER, which was suggested to be due to the higher viscosity of ropy milk. The same authors showed that the viscosity of filmjölk was about half that of ropy milk (553 mPas at a shear rate of 99.4 s⁻¹), while regular milk had a very low viscosity (3 mPas, at the same shear rate). Consequently, the tendency towards a lower glucose response to the fermented compared with the regular milk products as described in Paper 1, may be related to a lowering of the GER due to viscosity. However, there were no difference in glucose response between filmjölk and ropy milk in Paper 1, despite their reported difference in viscosity.

Unexpectedly, despite their low GIs, all milk products elicited high insulin responses (II=90-98). Pronounced hypoglycaemia occurred 45 minutes after intake of all milk products, probably as a consequence of over-regulation mediated by the high insulin response. In addition, the insulin response to the fermented milk products had not returned to the fasting level 120 min after ingestion. Since the pure lactose solution had a GI of 68 and an II of 50, the insulinotropic effect of milk could not be explained by the carbohydrate component alone. Inconsistencies between blood glucose and insulin responses to milk have been reported previously (Gannon et al., 1986; Schrezenmeir et al., 1989; Liljeberg et al., 1996b), although the mechanism has not been evaluated in detail. Evidently, some food factor in addition to lactose appears to contribute to the insulinotropic effect.

An interesting study concerning the insulinotropic effects of milk in mixed meals was published recently (Liljeberg Elmståhl et al., 2001). In that study, low-GI spaghetti meals were served with milk or water to healthy humans. Upon serving 400 ml milk with the low-GI meal, the insulin response increased by 300%. This finding highlights the fact that the intake of milk with a low-GI meal substantially increases the insulin response. It is not known whether or not this hyperinsulinaemic impact of milk share the negative metabolic features of glucose mediated hyperinsulinaemia. In fact, there are epidemiological evidence suggesting that this may not be the case. Consequently, an increased dairy consumption has been identified as beneficial in relation to the IRS (Mennen et al., 2000; Pereira et al., 2002) (see discussion below).
Results and Discussion

Another finding (Paper 1) was that the addition of pickled cucumber (acetic acid) and yoghurt to a white wheat bread meal lowered the glucose and insulin responses by 30 and 32% respectively, compared with the responses to a meal with white wheat bread, fresh cucumber and regular milk. Based on the lack of difference in glycaemia and insulinaemia in response to regular and lactic-acid-fermented milk, the lower responses to the acid-containing meal (acetic and lactic acid) could probably be ascribed to the presence of acetic acid in the pickled cucumber. These results are in accordance with those of a previous study in which acetic acid lowered postprandial glycaemia and insulinaemia to wheat bread meals in healthy humans by delaying the gastric emptying (Liljeberg et al., 1998). In contrast to the situation with lactic acid, the dipping of white wheat bread in vinegar reduced glycaemia and hormonal responses in healthy subjects by lowering the GER.

In a recent study (Granfeldt, Östman, Elmståhl and Björck, unpublished data), acetic acid was added (as a vinaigrette sauce) at three levels to high-GI bread meals. The results showed linear relations between the amounts of acetic acid added and the glucose peak \( (r = -0.47, P = 0.001) \) and the insulin peak \( (r = -0.44, P = 0.002) \), respectively, in healthy subjects. In addition, a linear relation was found between the acetic acid content of the meal and satiety \( (r = 0.41, P = 0.004) \), which was measured with a visual analogue scale. These findings add further evidence of metabolic merits of products or meals containing organic acids.

It is concluded that with milk products, no beneficial effect on glycaemia and insulinaemia was noted following lactic acid fermentation. The lowered metabolic responses to bread meals containing lactic-acid-fermented yoghurt and pickled cucumber were probably associated with the acetic acid content of the latter meal.

It can be questioned whether the GI concept is applicable or not to low-carbohydrate foods, such as milk. However, as long as milk products are listed as low-GI foods it is important to acknowledge that they also cause very high insulin responses, at least in healthy humans with normal insulin secretion. More research is needed to evaluate the metabolic effects of milk products in subjects with abnormal insulin secretion.

A scientific discussion is ongoing regarding the importance of studying the insulin response together with the glucose response to single foods and mixed meals. The results for milk in mixed meals indicate the importance of monitoring the insulin response. It remains to be seen whether insulinaemia mediated by non-carbohydrate food components is less detrimental from a metabolic perspective, and if, in fact, non-glucose mediated insulin secretion could facilitate glycaemic control in subjects with type 2 diabetes.
Interestingly, recent epidemiological data from a 10-year follow-up of more than 3,000 young adults in the USA show a strong inverse association between IRS and dairy consumption, suggesting that dairy foods have a protective effect (Pereira et al., 2002). The association was found only in subjects who were overweight, and the mechanisms discussed regarding this protective effect were the possible influence of single nutrients, such as magnesium, calcium and potassium, an intracellular role of calcium, or an enhanced satiety caused by the lactose, fat or protein content in the dairy products. Furthermore, a European study of almost 5,000 men and women has revealed an inverse relation between bread and dairy product intake, respectively, and the presence of IRS in men, but not in women (Mennen et al., 2000). Thus, men who ate more than 50 g bread per day or 1 or more portions of dairy products per day had a 40% lower prevalence of the metabolic syndrome than those who consumed less bread or dairy products.

4.3 Second meal effects

In order to evaluate possible additive metabolic effects of lactic-acid-containing bread, a second meal study was performed in healthy subjects (Paper 3). Information about the acute effects on glucose and insulin with the tested barley bread products was collected from a previous study (Liljeberg et al., 1996a) and the acute characteristics are presented in Figure 3. The AUC after the barley bread with lactic acid was 26% lower ($P < 0.05$) than after the barley bread with no added lactic acid. Also the insulin AUC was significantly lower (24%, $P < 0.05$) after the lactic-acid-containing barley bread.

The results from the second meal study (Paper 3), showed that a breakfast with lactic-acid-containing barley bread improved the glucose tolerance to a subsequent standardized high-GI lunch, compared with the corresponding barley bread breakfast without lactic acid. Consequently, the glucose AUC 95 min after lunch was reduced by 23% following the breakfast with lactic-acid-containing barley bread, compared with the corresponding area after the breakfast with barley bread without lactic acid. In addition, the insulin level 45 min after the lunch was significantly lower after the lactic-acid-bread breakfast. A suggested mechanism for the described second meal effect is that low-GI foods by prolonging the digestive phase, are capable of suppressing plasma FFA levels for a longer time period, leading to improved IS at the time of the subsequent lunch (Wolever et al., 1995).
Figure 3. Mean incremental blood glucose and serum insulin responses to barley bread with (▼) and without (▲) lactic acid (Liljeberg et al., 1996a). Values marked with * are significantly different.
Besides the lactic-acid-containing barley bread studied in the present work, other low-GI foods associated with beneficial effects on second meal glucose tolerance include lentils (Jenkins et al., 1982; Wolever et al., 1988), spaghetti, and a mixed barley breakfast consisting of pumpernickel-baked high-amylose barley bread and beta-glucan-rich barley flakes (Liljeberg et al., 1999). In case of the spaghetti and barley meals, the blood glucose levels just before the second meal had still not returned to the fasting level, indicating an on-going absorptive phase at the start of lunch (Liljeberg et al., 1999). Such a late blood glucose increment might also have been present in the case of the presently discussed lactic-acid-containing barley bread (Paper 3). However, no information was collected regarding the late glycaemic levels.

There is also evidence that low-GI foods differ in their capacity to improve glucose tolerance at a second meal ingested 4 h after the test meal. Thus, a meal of white wheat bread dressed with vinegar (acetic acid) did not improve the second meal glucose tolerance, in spite of a low GI (64) and II (65) (Liljeberg et al., 1999). Overall, it can be concluded that the 4 h second meal effect of low-GI foods seems to be independent of the content of indigestible carbohydrates (Wolever et al., 1988; Liljeberg et al., 1999), but instead associated with a prolonged absorptive phase, and a concomitant suppression of FFA levels when commencing the second meal.

In second meal studies performed overnight, it has been shown that certain low-GI evening meals can improve the glucose tolerance at a breakfast, served the next morning (after 10-11 h) (Granfeldt, Xiaomei and Björck, unpublished data). Consequently, whereas spaghetti had no beneficial overnight effect, boiled barley kernels lowered the postprandial glucose and insulin areas after breakfast by 23 and 29% respectively. These findings suggest that the higher amount of indigestible carbohydrates in the barley meal was responsible for the improved glycaemia at breakfast, probably by stimulating the production of SCFAs in the large intestine. Thus, SCFAs, particularly propionic acid, produced upon colonic fermentation of dietary fibre have been shown to beneficially affect hepatic glucose metabolism (Thorburn et al., 1993). It can thus be concluded that low-GI foods should preferably exhibit a prolonged absorptive phase and, in addition, be rich in indigestible fermentable carbohydrates.

As judged from the present work, the presence of lactic acid (1% of dough weight) during baking of barley bread reduces acute glycaemia by approximately 25%, and is capable of reducing the postprandial blood glucose area (23%) and the 45 min insulin response (18%) after a standardized high-GI meal ingested, 4 h later.
4.4 Effects of lactic acid on metabolic parameters in rats

The semi-long-term effects of lactic-acid-containing bread were studied in obese, hyperinsulinaemic Zucker (fa/fa) rats (Paper 4). An experimental design was chosen to enable separation of the potential influence of lactic acid per se, from that of probiotic lactic acid bacteria. The bacterium studied was *Lactobacillus plantarum* 299v, which has previously been shown to colonize the small and large intestines of the rat (Herias *et al.*, 1999). A daily dose of bacteria was administered through the feed. The calculated GL was approximately 87 for the diet based on lactic-acid-baked bread (lactic acid content was 0.7% of dough weight), and close to 100 for the diet based on bread without lactic acid. The GL for the diets based on bread with lactic acid added after baking, or the diet containing probiotic lactic acid bacteria was assumed to be similar to that with bread baked in the absence of lactic acid, *i.e.* 100.

Before and after a 14-day test period, the glucose tolerance was tested after an orally intubated glucose load. Besides glucose, the insulin and glucagon levels were also measured in the fasting and postprandial blood samples. At the end of the test period, the glucose tolerance had improved significantly in rats fed lactic-acid-baked bread. The total glucose AUC was significantly smaller (51%, *P* = 0.007) in the lactic-acid-bread group at the end of the experiment. The blood glucose level 30 min after the oral glucose load was significantly lower (*P* < 0.05) at the end of the test period for the lactic–acid-bread group, than in the other three groups. The results are in accordance with an improved insulin economy following the diet with lowered GL based on lactic-acid-baked bread. They are also in accordance with the finding of an improved second meal glucose tolerance with lactic-acid-baked bread in healthy subjects (Paper 3). The diet to which lactic acid was added after baking did not improve the glucose tolerance, which supports the hypothesis that lactic acid must be present during heat treatment in order to improve the glycaemic properties of bread. Besides a lowered insulin level at 15 min postprandially at the end of the intervention, the probiotic bacteria had no effect on glucose tolerance. All four groups had significantly lower fasting glucagon levels after, compared with prior to the intervention period. No differences in blood lipids, hepatic cholesterol level or urinary glucose excretion were seen during the 14-day intervention study with any of the four test diets.

In conclusion, the lowering of the GI of bread by adding lactic acid prior to baking, improves glucose metabolism in obese hyperinsulinaemic rats, as judged from an improvement in their glucose tolerance over a 14-day dietary period. The effect was not related to the presence of lactic acid per se. It was also concluded that adding probiotic bacteria (*L. plantarum* 299v) to the diet did not improve the glucose tolerance.
These results support the metabolic advantage of a low-GI diet, and suggest that beneficial effects may also arise from a modest lowering of the dietary GI, \textit{i.e.} from about 100 to 87. Furthermore, the results demonstrate that low-GI foods with metabolic merits can be designed by enclosure of lactic acid during the baking of bread.
5 Conclusions

- The effect of lactic acid on the rate of amylolysis of starch in cereal products differs depending on the food system. In order to reduce the rate of starch hydrolysis in cereal-based foods, lactic acid must be present during starch gelatinization (i.e. heat treatment).

- The presence of gluten was a prerequisite for a lactic acid effect in wheat starch systems subjected to heat treatment at simulated baking conditions. These results indicate that macromolecular interactions between starch and cereal protein is the responsible food mechanism for a blunting of glycaemic response in the presence of lactic acid. Moreover, gluten supplementation may be used for further lowering of the GI and II characteristics of lactic-acid-fermented bread, as judged from in vitro experiments with wheat and barley bread.

- No effect of lactic acid was seen in probiotic gruel products subjected to pre-treatment with enzymes to reduce viscosity, and with the gelatinization step prior to lactic acid fermentation with probiotic bacteria. This suggests, that the production of low-GI probiotic gruel products, based on the lactic-acid-related food mechanism, offers particular difficulties.

- Barley bread baked in the presence of lactic acid improves the insulin economy at a subsequent standardized lunch meal, as judged from a lowering of the second meal glucose response by 23% and the 45 min insulin response by 18%.

- A diet based on wheat bread to which lactic acid was added prior to baking improved the glucose tolerance after 14 days in hyperinsulinaemic rats, compared with wheat bread where the same amount of lactic acid was added after baking.

- Fermentation and presence of lactic acid in milk products did not significantly affect either GI or II. Interestingly, the GIs were surprisingly low for all milk products (GI=15-30), and lower than with a corresponding amount of carbohydrates in the form of pure lactose (GI=68). In contrast, the IIs were unexpectedly high (II=90-98), or almost twice that of lactose. The results imply that a component in milk, in addition to the lactose, stimulate insulin secretion.
Acknowledgements

Först och främst vill jag tacka alla på avdelningen som gör att det är roligt att gå till jobbet varje dag. Jag kan inte föreställa mig bättre kollogor!

Jag vill också rikta speciellt stora TACK till:

Inger - för entusiasm och humor och för att du delar med dig av allt.

Helena - för ditt stora engagemang i mig och mitt arbete.

Mikael & Yvonne – för att det är så roligt att samarbeta med er.

Ulla & Lisbeth - för att ni gör det lätt att hålla humöret uppe i labbet.

Marianne & Anna Themner-Persson - för all hjälp med rättorna.

Sopplaget – som gör varje torsdag till en fest.

LiFT-”familjen” – en härlig skara människor som kan utföra stordåd.

Barbro Smedberg, Tina Hansson & Inger C Larsson - för att ni tagit er tid och lärt oss mycket om ert arbete på Findus.


Karin Autio & Kati Katina – för samarbetet kring mikroskoperingen.


Alla försökspersoner – för tappert ätande av alla mer eller mindre goda måltider.

Nord Mills AB i Malmö - för ert praktiska kunnande och för bakningen till rättstudien.

Mina vänner – för att ni förtydligar min fritid och för att det inte är viktigt för er vad jag gör på jobbet.

Henrik & min övriga familj – som betyder allra mest och som alltid finns där.

This project was supported by grants from the Swedish Council for Forestry and Agricultural Research (SJFR).
References


References


References


