DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2004; **20**: 369–382.
Published online 21 June 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/dmrr.488

REVIEW ARTICLE

Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification

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Received: 8 October 2003
Revised: 26 January 2004
Accepted: 4 February 2004

Summary

The Maillard reaction is a process in which reducing sugars react spontaneously with amino groups in proteins to advanced glycation endproducts (AGEs). Although an elevated level of glucose had been thought to play a primary role in the Maillard reaction, on a molecular basis, glucose is among the least reactive sugars within biological systems. The formation of AGEs is now also known to result from the action of various metabolites other than glucose, which are primarily located intracellularly and participate in the non-enzymatic glycation reaction at a much faster rate, such as fructose, trioses and dicarbonyl compounds. In this review, we considered the glycation reaction with particular attention to the potential role of fructose and fructose metabolites. The two sources for fructose are an exogenous supply from the diet and the endogenous formation from glucose through the aldose reductase pathway. Despite its ~eightfold higher reactivity, the contribution of extracellular glycation by fructose is considerably less than that by glucose, because of the low plasma concentration of fructose (5 mmol/L glucose vs 35 μmol/L fructose). Intracellularly, fructose is elevated in a number of tissues of diabetic patients in which the polyol pathway is active. In the cells of these tissues, the concentrations of fructose and glucose are of the same magnitude. Although direct evidence is not yet available, it is likely that the high reactivity of fructose and its metabolites may substantially contribute to the formation of intracellular AGEs and may contribute to alterations of cellular proteins, dysfunction of cells and, subsequently, to vascular complications. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords fructose; glycation; advanced glycation endproducts; diabetes; Maillard reaction; polyol pathway

Introduction

The reducing free carbonyl group of fructose and glucose, in common with all reducing sugars, may react non-enzymatically with amino groups of proteins (Figure 1). Louis Camille Maillard first described the non-enzymatic glycation reaction in the early 1900s and it came to be known as the *Maillard reaction*. The Maillard reaction is a process in which reducing sugars react spontaneously with amino groups in proteins, lipids and nucleic acids to advanced glycation endproducts (AGEs). Although Maillard predicted that this reaction could have an important impact on medicine, at that time the chemistry was investigated extensively by food chemists and not by medical researchers [1,2]. It was not until 1980 that the pathophysiological significance of the Maillard reaction emerged in medical science, in particular in relation to diabetic complications and ageing [3,4]. The physiological consequences of the Maillard reaction in ageing and in the aetiology of a range of important diabetic complications has been described in excellent reviews, to

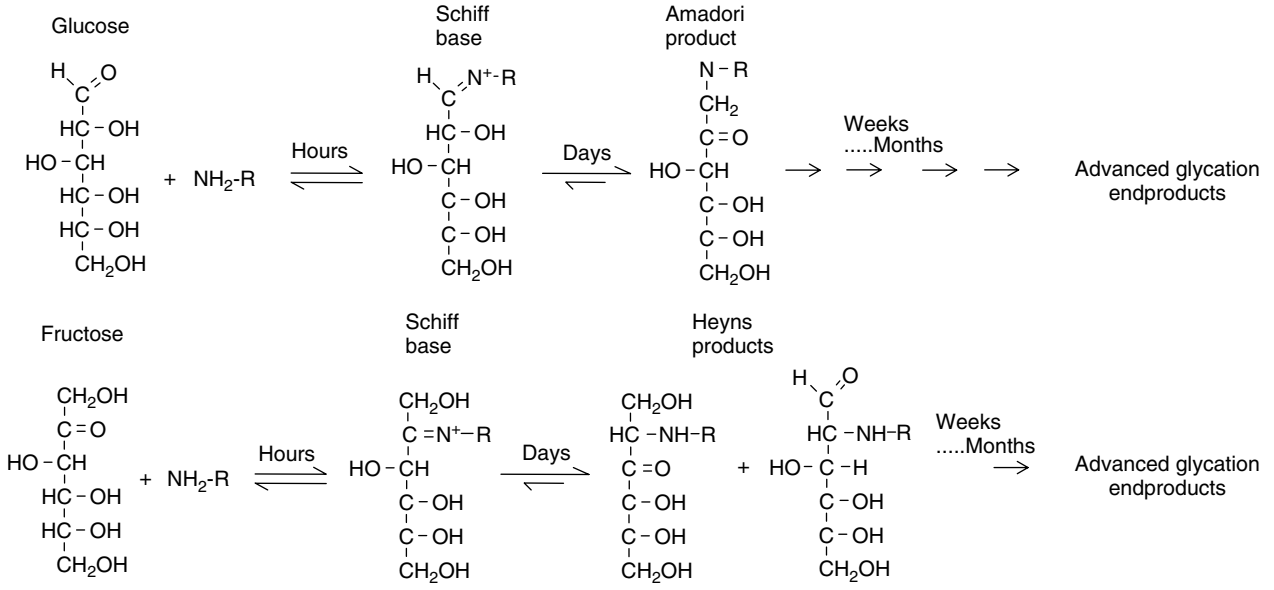

Figure 1. The initial Maillard reaction with D-glucose and D-fructose. The initial condensation reaction of the carbonyl group of glucose and fructose with amino groups leads to the formation of unstable Schiff bases. In the case of glucose, the Schiff base undergoes an Amadori rearrangement. With fructose, the reaction is similar, but the reaction is termed *Heyns rearrangement* and results in the formation of two separate Heyns products. Evidence also suggests some formation of Amadori product from fructose as formed in the Amadori rearrangement [28]

which the reader is referred [5–15]. In addition, a large body of evidence has been accumulating that the Maillard reaction is not only implicated in diabetic complications but also in the development of age-related diseases such as inflammation [16], atherosclerosis [17–20], neurodegenerative disorders [21–23], and in dialysis-related amyloidosis [24] and cancer [25–27].

Although an elevated level of glucose had been thought to play a primary role in the Maillard reaction, the formation of AGEs is now known to result also from the action of various metabolites other than glucose. The realisation that fructose and its metabolites, at elevated concentrations, can initiate potentially deleterious changes such as the non-enzymatic fructosylation of proteins has raised interest and concern about this property of fructose [29]. With respect to human health, the contribution of fructose as an effective glycating agent is of importance for three reasons. First, on a molecular basis fructose and fructose metabolites, which are primarily located intracellularly, may participate in the non-enzymatic glycation reactions at a much faster rate than glucose. Second, fructose consumption has increased steadily during the last two decades. An increased fructose intake from the diet might result in changes in tissue concentration of fructose and its metabolites, which can in turn potentiate the Maillard reaction. Third, in several organs, fructose is formed from glucose by the polyol pathway. Its concentration is elevated in those tissues of diabetic patients in which the polyol pathway is active, in particular the ocular lens, kidney and peripheral nerves [30,31]. This is especially relevant in that activation of the polyol pathway that has been proposed as being involved in the pathogenesis of diabetic neuropathy and other diabetic complications [32].

In this review, the biochemical mechanisms and significance by which fructose and its metabolites will contribute to protein glycation will be surveyed.

The metabolism of fructose

The two sources for fructose in the body are the exogenous supply from the diet, either as sucrose or free fructose, and the endogenous formation from glucose through the aldose reductase pathway. The contribution of these sources of fructose depends on many factors including the availability of exogenous fructose, its transport rate and on the activities of aldose reductase and sorbitol dehydrogenase [33].

Metabolism of dietary fructose

Fructose is the most common, naturally occurring monosaccharide of human diets [34]. In the 1970s, when high-fructose syrup manufactured from starch began to be used as a replacement for sucrose in beverages and baked foods, the daily intake of fructose in adolescents and adults in Western countries increased by 26%, from 64 g per day in 1970 to approximately 80 g per day in 1997 [35].

At least two carrier-mediated transport mechanisms for intestinal transport of monosaccharides exist: both D-glucose and D-galactose are transported by the Na^+-dependent cotransporter SGLT1 while D-fructose is transported across the brush-border membrane into the cytosol of the enterocytes by a Na^+-independent facilitated diffusion process involving the fructose transporter GLUT5

[36]. GLUT5 is abundantly expressed in the intestinal tissue, and also in liver, brain, kidney, testis, spermatozoa, in adipose and muscle tissue and, although in a low concentration, in pancreatic beta cells [37,38]. The monosaccharides glucose and fructose, once accumulated in the enterocytes, exit from the cell across the basolateral membrane by the Na^+-independent glucose and fructose transporter GLUT2 [39].

The tissue expression levels of the GLUT5 transporter are responsive to alterations in fructose in the diet. A high-fructose diet significantly increased the rates of intestinal fructose by increased GLUT5 mRNA levels, while rates of brush-border glucose uptake and the levels of SGLT1 are not dependent on the diet [36,40]. Furthermore, levels of the GLUT5 transporter in intestine are increased in experimental diabetes and type 2 diabetes [41,42]. In the small intestine of diabetic patients, GLUT5 and SGLT1 protein levels as well as GLUT2 mRNA were threefold increased as compared to control [42]. Although the molecular mechanism involved in the increased protein and mRNA levels of SGLT1, GLUT2 and GLUT5 in diabetic patients is unknown, the enhanced expression is independent of any changes in blood glucose or insulin levels [42]. The molecular basis of the increased levels of these monosaccharide transporters requires further investigation since the ability of the intestine to increase monosaccharide absorption may further enhance the occurrence of complications of the disease.

After oral feeding, the liver takes up at least 50% of the initial flux and as a consequence of the high rate of uptake of fructose by the liver and an active hepatic fructokinase enzyme system, a maximum concentration of 1.0 mmol/L fructose is recorded in peripheral blood [43]. Because the utilisation of fructose in the liver is highly efficient [44], a considerably smaller amount will be available for the kidney, adipose tissue, skeletal muscle and other tissues [45].

The two monosaccharides, glucose and fructose, are metabolised differently as indicated in Figure 2. In the context of AGE formation, the formation of glyceraldehyde from fructose as compared with that of glyceraldehyde-3-phosphate from glucose is an important difference between these two monosaccharides [46], since glyceraldehyde is very reactive in the Maillard reaction [47,48].

Fructose is phosphorylated by the enzyme fructokinase to fructose-1-phosphate, while glucose is phosphorylated by the enzyme hexokinase to glucose-6-phosphate. Fructokinase is the initial enzyme in the fructose metabolic pathway and is found in the liver, kidney and intestine, but not in other tissues. In adipocytes, fructose is phosphorylated to fructose-6-phosphate by hexokinase [45]. Fructose-1-phosphate is split by ketose-1-phosphate aldolase into glyceraldehyde and dihydroxyacetone phosphate. Fructose-1,6-diphosphate is formed by isomerisation of glucose-6-phosphate followed by the energy requiring phosphorylation by phosphofructokinase. The latter reaction seems to be the rate-limiting step in glucose metabolism. The cleavage of fructose-1,6-diphosphate by fructose diphosphate aldolase yields dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate.

Biosynthesis of fructose in tissues by the polyol pathway

The polyol metabolising pathway, which consists of two enzymes, aldose reductase and sorbitol dehydrogenase,

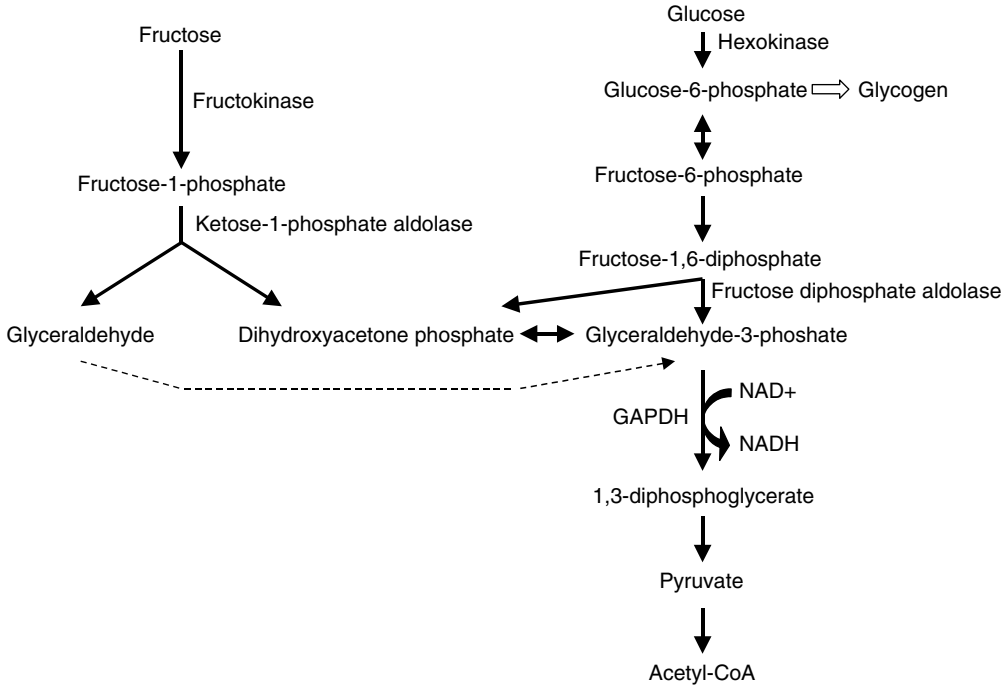

Figure 2. Metabolism of fructose and glucose in the liver, kidney and gut

converts glucose to fructose. Aldose reductase is present in all tissues examined [49]. In human tissues, aldose reductase is most abundant in the inner medulla of the kidney (29 μg/mg protein), followed by the sciatic nerve (5 μg/mg protein), lens (3 μg/mg protein), testis and heart (2 μg/mg protein each), and cornea (1 μg/mg protein) [50]. Among blood cells, monocytes are richest on a per cell basis (44 ng/10^6 cells), followed by neutrophils (2 ng/10^6 cells) and erythrocytes (0.3 ng/10^6 cells). Sorbitol dehydrogenase is present in virtually all tissues. In human tissues, high levels of sorbitol dehydrogenase transcripts were observed in lens and kidney, as judged from Northern blot analysis [51]. The polyol pathway is activated by hyperglycaemia in numerous cells and tissues, that express the enzymes aldose reductase and sorbitol dehydrogenase and in which glucose transport is not rate-limiting for overall glucose metabolism. Fructose as formed from sorbitol through the polyol pathway is found in ocular lens, kidney and peripheral nerves [52]. In these tissues, excess glucose is reduced to sorbitol by aldose reductase accompanied by increased oxidation of NADPH to NADP, and sorbitol is subsequently oxidised to fructose by sorbitol dehydrogenases accompanied by the reduction of NAD to NADH (Figure 3).

Under normoglycaemic conditions, the intracellular concentration of sorbitol is very low due to a low affinity of aldose reductase for glucose. An excess of glucose is metabolised to sorbitol by aldose reductase (AR), accompanied by increased oxidation of NADPH to NADP+. A putative decreased level of NADPH, which is a cofactor for glutathione reductase, may lead to a depletion of reduced glutathione and thus to enhanced oxidative stress and the formation of AGEs. Oxidation of sorbitol by sorbitol dehydrogenase (SDH) yields NADH and causes an increase in the ratio NADH/NAD+. Since in tissue hypoxia responses are also linked to an increased NADH/NAD+ ratio, the increased cytosolic ratio NADH/NAD+ by hyperglycaemia is called *hyperglycaemia pseudohypoxia* [53]. An important feature of an increase of the ratio NADH/NAD+ induced by the sorbitol pathway is that it inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and, subsequently, may lead to the accumulation of triose phosphates glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone-phosphate (DHAP). Both trioses are precursors of methylglyoxal [54] and these trioses are potent AGE-forming compounds [48]. In addition, the sorbitol pathway may further increase the levels of methylglyoxal by a decrease of NADPH and depletion of reduced glutathione (GSH) in the cell. GSH has a detoxification function in the glyoxalase pathway by facilitating the conversion of methylglyoxal to D-lactate [55]. Fructose and its derivative fructose-3-phosphate, which is produced by the phosphorylation of fructose by fructose-3-phosphokinase, are potent glycating agents. Fructose-3-phosphate is also hydrolysed to 3-deoxyglucosone, which rapidly reacts with protein amino groups to form AGEs.

Metabolism of endogenously formed fructose

The first step in the metabolism of fructose in peripheral tissues was generally thought to be the phosphorylation of fructose to fructose-6-phosphate by hexokinase [56]. However, data from studies of Szwergold and co-workers in diabetic rat lenses [57–59] and hearts [60], rat, pig, and rabbit lenses [33] and human erythrocytes [56,61],

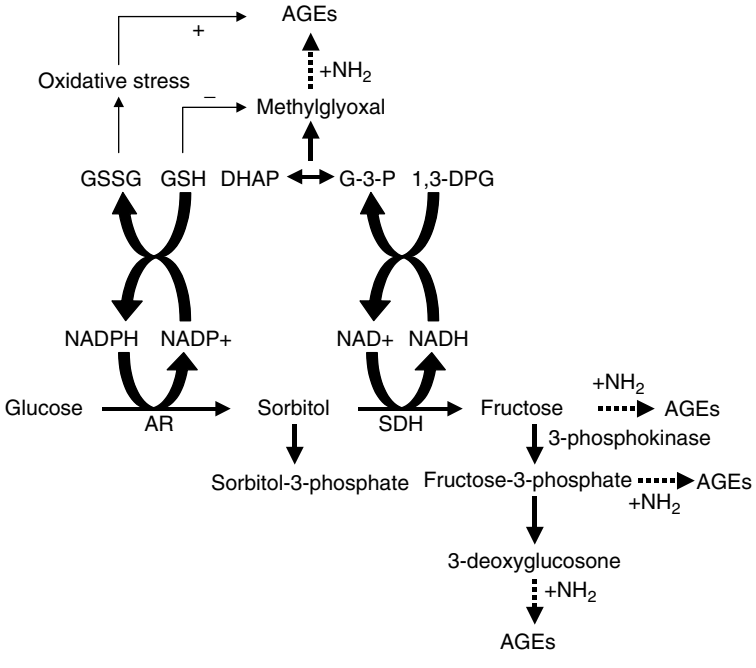

Figure 3. Hyperglycaemia-dependent activation of the sorbitol pathway in peripheral tissues and the formation of fructose and potential intermediates in the formation of AGEs

demonstrated that the first step in the metabolism of fructose is the direct phosphorylation of fructose at the C-3 position by fructose-3-phosphokinase (Figure 3). Fructose-3-phosphate is of particular interest since it is a potent glycating agent [62], which may contribute to the increased cross-linking and cataract formation in the lenses of diabetic patients. Fructose-3-phosphate can subsequently be degraded to 3-deoxyglucosone [58], which can radically accelerate the formation of AGEs [63].

Interestingly, it has recently been demonstrated that fructose-3-phosphokinase phosphorylates fructosamine with at least 300-fold higher affinity than fructose and this fructosamine-3-kinase was recently purified and cloned [64–66]. The phosphorylation of fructosamine by fructosamine-3-kinase to fructosamine-3-phosphate destabilised the fructosamine adduct and led to spontaneous decomposition, thereby reversing the glycation reaction at an early stage. Because of the ubiquitous distribution of this enzyme and the lability of fructosamine-3-phosphate, fructosamine-3-kinase may be involved in the control of intracellular non-enzymatic glycation by a 'deglycation' process, as was recently demonstrated in erythrocytes [67].

Fructose concentrations in blood and tissues

Fructose concentrations in blood

The normal systemic blood concentration in humans is in the low μM range when no fructose is being absorbed [68], and up to the low mM range after eating, depending on the fructose concentration in the diet [43].

Several studies have reported comparable serum and urinary fructose concentrations in diabetic patients and non-diabetic patients [69,70], while other investigators found increased levels in diabetic patients [68]. This discrepancy might be explained by an insufficient removal of glucose by the methods used, which interfered with the measurement of small amounts of fructose, or by an impaired precision of the measurements by complicated pre-treatment. In a recently described method, these problems were overcome by the use of $^{13}C_6$-fructose as internal standard [68]. Serum fructose concentrations in patients with diabetes (12 ± 4 μmol/L) were significantly higher than those in healthy subjects (8 ± 1 μmol/L) and were correlated significantly with HbA_{1c}. In patients with diabetes, the serum fructose concentrations decreased rapidly, concomitantly with an improvement in glycaemia [68]. Therefore, hyperglycaemia is associated with increased serum and urinary fructose concentrations in patients with diabetes.

Fructose concentrations in tissues

Since the dietary intake of fructose in humans leads to significant elevations of the serum fructose levels, it is likely that fructose in the diet could also lead to elevations of tissue fructose concentrations. Indeed, fructose accumulation in sciatic nerves of streptozotocin-diabetic rats maintained on a 72% fructose diet for four weeks, was significantly increased from 2.7 to 7.6 μmol/g wet weight, which was 30% greater than in rats maintained on a 72% glucose diet for the same period [71,72]. However, in control rats the concentration of fructose in sciatic nerves was not affected by fructose feeding [72]. This difference in fructose accumulation between diabetic and non-diabetic rats may be explained by an enhanced expression of fructose transporters in diabetes [40,41].

In addition to increased levels of fructose by dietary intake, different studies have demonstrated that the fructose content of several tissues is markedly elevated during hyperglycaemia. In some organs, such as the ocular lens and peripheral nerves, fructose is biosynthesised by the oxidation of sorbitol in a reaction catalysed by sorbitol dehydrogenase [52]. In diabetic subjects, the concentrations of fructose often approach and sometimes exceed those of glucose in the ocular lens [58,73–75] and in nerves [76,77]. The correlation of sorbitol and fructose with HbA_{1c} suggested that the lens could synthesise substantial quantities of sorbitol and fructose in response to the excess glucose available to the lenses of diabetic patients [74]. In diabetic lenses, Gabbay and Kinoshita have shown a 23-fold increase in fructose concentration, which is twice as high as that of glucose [52]. In cardiac ventricular tissue of diabetic rats, a 65-fold increase in fructose content with a fourfold increase in sorbitol content was demonstrated, which was completely normalised by insulin treatment [78].

In conclusion, in organs in which the polyol pathway is active, such as the kidney, ocular lens, peripheral nerves and cardiac tissue, the concentrations of fructose are of the same order of magnitude as that of glucose and are strongly increased by hyperglycaemia, making *in vivo* glycation by fructose a highly probable event. The question is whether changes in tissue concentration of fructose and its metabolites could potentiate the Maillard reaction *in vivo*.

Fructose and the Maillard reaction: fructosylation

Role of the Maillard reaction in hyperglycaemia-induced diabetic complications

Clinical trials in both type 1 and type 2 diabetes demonstrated that hyperglycaemia plays an important role in the pathogenesis of nephropathy, retinopathy, neuropathy and accelerated atherosclerosis and emphasised that hyperglycaemia is an independent risk factor for these vascular complications [79,80]. Several pathways have emerged to explain how hyperglycaemia causes these

diabetic complications [81,82] (Figure 4), including the formation of AGEs [6–11].

Various mechanisms have been proposed to explain how hyperglycaemia causes diabetic vascular dysfunction. Under normal conditions, glucose is metabolised through the glycolytic pathway. An increase in intracellular glucose will lead to an increase in the flux of glucose to sorbitol via the sorbitol pathway, an increase in fructosamine-6-phosphate via the hexosamine pathway, the activation of protein kinase C (PKC) via *de novo* synthesis of diacylglycerol (DAG) and the formation of advanced glycosylation endproducts. In several cell types, excess glucose can be metabolised in the *sorbitol pathway* to sorbitol and fructose by aldose reductase (AR) and sorbitol dehydrogenase (SDH). In the hexosamine pathway, fructose-6-phosphate is converted to fructosamine-6-phosphate by the enzyme glutamine : fructose-6-phosphate amidotransferase (GFAT) and subsequently into *N*-acetylglucosamine (GlcNAc). The mechanism responsible for the activation of PKC by hyperglycaemia is related to *de novo* synthesis of the PKC activator diacylglycerol (DAG) from a stepwise acylation of glycerol-3-phosphate (GP) and phosphatidic acid (PA). Recent evidence indicates that these four biochemical and metabolic mechanisms are the consequence of a hyperglycaemia-induced overproduction of oxidative stress in the mitochondria [83]. This part of the figure is adapted from ref [82]. Three of the major biochemical pathways implicated in the pathogenesis of hyperglycaemia-induced vascular damage (the hexosamine pathway, the diacylglycerol (DAG)-protein kinase C (PKC) pathway and the formation of AGEs) can be inhibited by the lipid-soluble thiamine derivative benfotiamine, by activating the pentose phosphate pathway (PPP) enzyme transketolase (TK) [84]. In addition to hyperglycaemia, fructose may lead to increased reactive oxygen species (ROS) by a fructose-induced activation of NADPH oxidase or inactivation and reduced expression of the antioxidant enzymes catalase and superoxide dismutase [85–88]. A fructose-induced reduction of antioxidant enzymes and uncoupling of eNOS [89] will contribute to an increase in intracellular superoxide production, which may reduce GAPDH activity. Furthermore, high fructose feeding was reported to reduce GAPDH activity [86]. By these mechanisms fructose may compromise the conversion of glyceraldehyde-3-phosphate and cause accumulation of glycolysis intermediates that, according to Brownlee's hypothesis, contributes to vascular dysfunctioning.

AGEs have different biological functions: some are protein cross-links, such as pentosidine, and some are recognition factors for specific AGE-binding receptors such as CML. Accumulation of AGEs in the extracellular matrix can interfere with tissue functioning by the disturbance of normal matrix composition and causing aberrant cross-linking, resulting in a decrease of elasticity of vessels. In addition to these direct interactions with matrix proteins, proteins modified by AGEs can bind to AGE-receptors on different cell types [90,91]. A rapid intracellular AGE

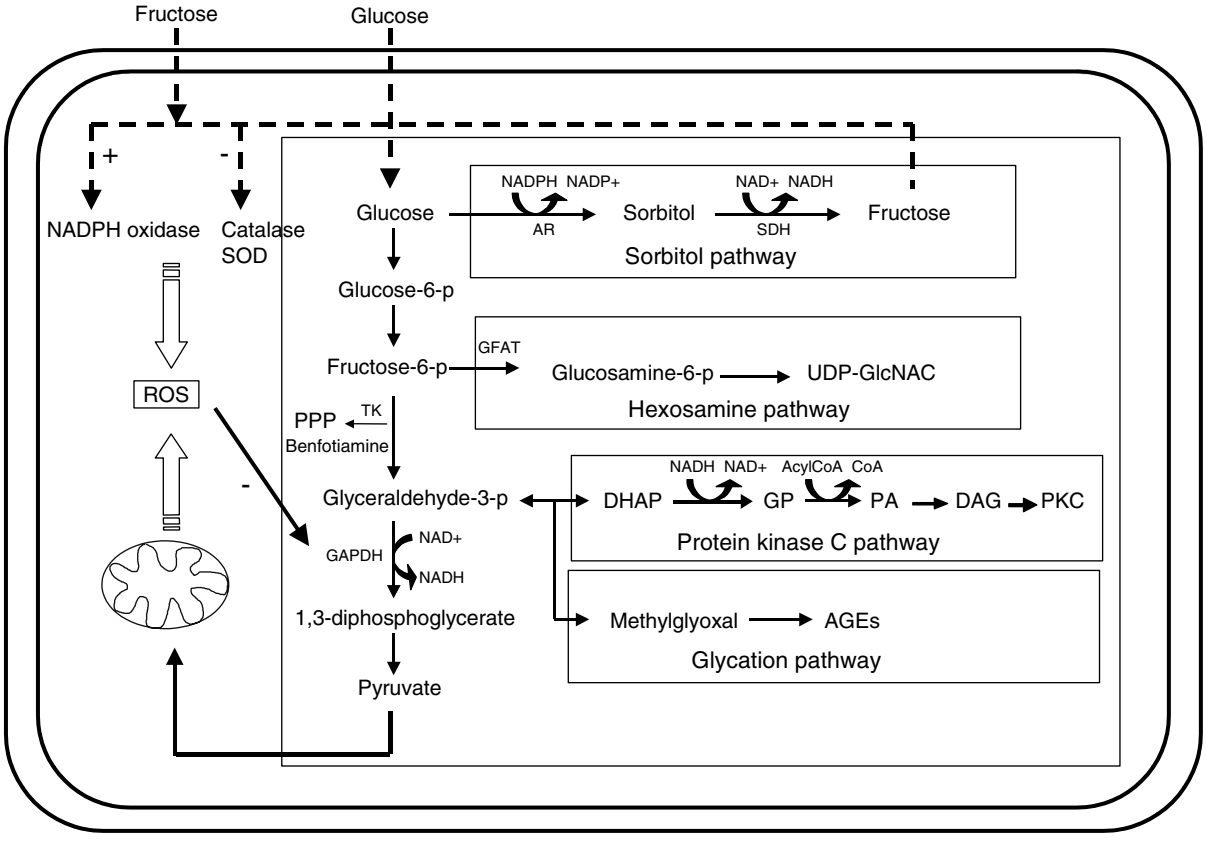

Figure 4. Hyperglycaemia and its immediate biochemical sequelae

formation by various intracellular precursors such as fructose and fructose metabolites has recently attracted attention. In addition to extracellular AGE accumulation, the intracellular AGE formation may also be an important contributor to diabetic angiopathy [92].

The Maillard reaction with fructose

So far, most studies have been focused on non-enzymatic glycation by glucose, because glucose is by far the most abundant sugar in blood. In principle, however, all reducing sugars [93] as well as certain molecules related to sugar, such as ascorbic acid [94] can initiate the Maillard reaction *in vivo*. Because of the slow rate of the reaction of glucose with proteins, AGEs have been thought to form only at long-lived extracellular molecules. In addition to the extracellular formation of AGEs, rapid intracellular AGE formation by various intracellular precursors has recently attracted attention [95,96]. In the context of intracellular glycation, it is important to emphasise that glucose has the slowest rate in the glycation reaction of any sugar in cells. The rate of glycation is directly proportional to the percentage of sugar in the open-chain form [97] and the rate for fructose is 7.5-fold faster than that of glucose (0.002% open-chain) and, most strikingly, the glycolytic intermediate glyceraldehyde-3-phosphate (100% open-chain) forms over 200-fold more glycated protein than do equimolar amounts of glucose [81]. Thus, of importance for the intracellular Maillard reaction are precursors other than glucose, such as fructose and its metabolites glyceraldehyde, dihydroxyacetonephosphate, glyceraldehyde-3-phosphate and the dicarbonyl compounds methylglyoxal and 3-deoxyglucosone. Because of the faster rate of these reactive glycolytic intermediates in the formation of AGEs, they are believed to be important precursors in the intracellular formation of AGEs *in vivo*. Indicative of the involvement of endogeneous fructose in the glycation process *in vivo* are data that show that 10 to 20% of the sugar moieties attached to human ocular lens proteins were via carbon 2 [98].

The initial phase of the Maillard reaction with fructose, that is, the condensation of the carbonyl group of fructose with the terminal α-amino group or the ε-amino group of lysine residues, is quite similar to that for glucose (Figure 1). In the case of glucose, the reaction leads initially to the formation of acid-labile Schiff bases, which undergo a rearrangement to early glycation Amadori-adducts such as fructosamine. Only a small part of these relatively stable Amadori-products undergoes rearrangements that lead to irreversible formation of AGEs. In the case of fructose, the rearrangement of the Schiff base is termed the *Heyns rearrangement*. Two separate Heyns products are then formed [28], which are obviously different in structure from the Amadori-adduct. Heyns compounds have been detected in liver extracts and in human ocular lens proteins [98]. The initial kinetics of glycation is dependent on temperature, the protein, the amount of the reducing sugar and the proportion of the reducing sugar existing in an open-chain form under the reaction conditions [93]. Fructose exists to a greater extent in the open-chain form than does glucose. Differences in local conditions at sites of glycation may explain the more rapid initial rates of fructose-adduct formation in haemoglobin and liver alcohol dehydrogenase [98,99], the similar reactivity glucose and fructose with ribonuclease A, and the eightfold higher reactivity of glucose with albumin than that of fructose [98,100,101]. On the basis of *in vitro* experiments under physiological conditions on the assumption that the plasma concentration of glucose is ~5 mmol/L and of fructose, ~35 μmol/L, the estimated rate of formation of the fructosamine derivative of albumin in blood plasma *in vivo* is 0.83 μmol/day, while the estimated rate of formation of fructose-derived Heyns products is only 0.007 μmol/day [102]. However, differences in the initial rate of reaction, as have been observed between glucose and fructose, do not reflect the overall kinetics of the Maillard reaction. Kinetically, fructose may differ from glucose predominantly by a much faster conversion of Heyns compounds as compared with Amadori groups, which may explain the much higher rate of fluorescence generation. *In vitro* studies demonstrated that fructose reacts with ribonuclease A and albumin with the development of non-tryptophan Maillard fluorescence at a constant rate that was about 3- to tenfold greater than glucose [28,62,98]. In the case of ribonuclease A, this was accompanied by the formation of cross-linked oligomers [98].

In contrast to studies performed in buffer systems, in studies done under dry or low-moisture conditions, D-glucose is more effective in causing protein cross-linking and in generating protein-bound Maillard fluorescence [103]. The discrepancies in the literature may, therefore, be related to differences in the conditions under which the Maillard reaction was conducted. Dry or low-moisture conditions are highly relevant in the application of glycated food proteins in the food industry, while the experiments that have been carried out in buffered *in vitro* incubations, in which non-enzymatic fructosylation results in the formation of highly fluorescent products and oligomerisation, might be of pathophysiological relevance. Studies performed under these latter conditions, revealed that fructose may serve as a precursor for pentosidine [104–106].

Fructose consumption and fructosylation

By contrast with the normal intake of a relatively low amount of fructose, long-term consumption of diets high in fructose had adverse effects on certain aspects of health. On the basis of animal studies it has been implicated as a contributor to the development of obesity and nearly all of the classical manifestations of the insulin resistance syndrome [35]. The data in humans are less clear. Fructose intake is associated with impaired

glucose tolerance, insulin resistance, hyperlipidaemia and hypertension [44,45]. Most importantly, fructose does not stimulate the production of two key hormones, insulin and leptin, which are involved in the long-term regulation of energy homeostasis. A decrease in leptin and insulin could lead to an increase in energy intake. It is, therefore, possible that the long-term consumption of diets high in energy derived from fructose contributes to weight gain, obesity and its metabolic consequences in humans [35].

Recently, the *in vivo* effects of long-term fructose consumption on the normal ageing process has been studied in rats [71]. Aortic endothelial cells, obtained from high-fructose-fed rats, produced fourfold more superoxide radicals than control rats, which was mediated through the activation to NADH/NADPH oxidase [85]. In addition, fructose may lead to increased oxidative stress by a fructose-induced inactivation and reduced expression of the antioxidant enzymes catalase and superoxide dismutase (see also Figure 4) [86,87]. Glycation, as measured by fructosamine and glycated haemoglobin levels, was significantly higher in fructose-fed rats compared to glucose- or sucrose-fed rats. However, in this study, higher glycated haemoglobin and fructosamine levels failed to reflect the 77% increase in fasting blood fructose, probably due to the fact that neither assay can accurately detect *in vivo* fructosylation. These assays were developed to detect glucose Amadori products of haemoglobin and albumin [107] and will underestimate the fructose-derived Heyns products. Fluorescence measurements at 335/385 nm (excitation/emission), characteristic of pentosidine and commonly used to measure the extent of advanced Maillard reaction cross-links, demonstrated a significantly greater fluorescence intensity in the cortical bones of fructose-fed rats compared to control and glucose- or sucrose-fed rats. Although the most physiologically relevant cross-links are colourless and non-fluorescent such as glucosepane [108–110], these data indicated that long-term fructose consumption potentiates the formation of both early and late glycation products and suggests that fructose consumption induces adverse affects on ageing. In contrast, long-term feeding of three-month old male Fischer 344 rats with various specific dietary carbohydrates, that is, cornstarch, glucose, sucrose, fructose or equimolar amounts of fructose and glucose, up to 26 months of age, had no effect on markers of glycaemic stress and collagen-associated fluorescence or on the accumulation of pentosidine in tail tendon collagen and trachea collagen [111]. In a study in type 2 diabetic patients, three 28-day isocaloric feeding periods where 20% of calories were either fructose, sucrose or starch, AGE accumulation reflected more closely caloric intake rather than dietary carbohydrate [112]. In agreement, Cefalu *et al*. reported that caloric restriction reduced the pentosidine content in skin collagen in ageing rats [113] and Teillet *et al*. found that a 30% food restriction in lean female rats prevented intrarenal and vascular accumulation of AGEs [114]. However, other investigators did not observe any effect of calorie restriction in reducing the accumulation of pentosidine in collagen from tail tendon, ear auricle or aorta [115–117]. These discrepancies in the literature may reflect differences in the methods of extraction and detection of pentosidine, differences in the animal models studied and/or, probably most importantly, differences in the susceptibility of specific tissues to the accumulation of AGEs.

In conclusion, diets high in fructose as well as caloric intake may effect the accumulation of AGEs, but the accumulation of AGEs is tissue-specific. This should be considered when effects of dietary carbohydrates and caloric intake on the accumulation of AGEs are studied.

Glycation by intermediate metabolites of fructose: role of trioses

In addition to extracellular AGE accumulation, intracellular AGE formation may be a contributor to diabetic angiopathy. The sorbitol pathway generates reactive intermediates such as fructose, fructose-3-phosphate, glyceraldehydes-3 phosphate and 3-deoxyglucosone and may substantially contribute to intracellular AGE formation by the reaction of these intermediates with proteins [48]. Indeed, long-term treatment with an aldose-reductase inhibitor prevented the accumulation of pentosidine in lenses from galactosaemic rats [118] and AGE-fluorescence and cross-linking of extracellular matrix in the skin collagen of diabetic rats [119]. In erythrocytes of type 2 diabetic patients, the aldose reductase inhibitor epalrestat reduces the levels of the CML protein-adducts along with those of fructose, triose phosphates and 3-deoxyglucosone. However, epalrestat also reduced lipid peroxidation in erythrocytes, independent of glycaemic control [120], and recent studies provided a direct link between the formation of CML and lipid peroxidation [121–124]. Therefore, the reduced formation of CML by aldose reductase inhibitors may be due to a reduced lipid peroxidation, rather than reactive intermediates from the sorbitol pathway. The positive correlation between 3-deoxyglucosone and sorbitol and fructose, but not with a marker of lipid peroxidation, indicated that an increased sorbitol pathway flux plays a substantial role in the intracellular formation of AGEs. Since the sorbitol pathway in erythrocytes is believed to be a surrogate of that in other tissues such as lens and nerve [124,125], the data observed in erythrocytes may be applicable to other tissues.

Glycation by glyceraldehyde

Fructose is phosphorylated in the liver and also in the gut, intestine and kidney by the enzyme fructokinase to fructose-1-phosphate, which is split by ketose-1-phosphate aldolase into glyceraldehydes and dihydroxyacetone-phosphate. Since fructokinase is found in the liver, kidney, intestine and gut, but not in other

tissues, glyceraldehyde might be expected in these tissues. Indeed, a fructose load has been found to increase the glyceraldehyde concentration in the liver [126]. Glyceraldehyde and its isomer dihydroxyacetone are major sources for glycation and protein cross-linking [127,128]. Among their products are argpyrimidine, triosidines and probably pentosidine. The cross-linking of glyceraldehyde was threefold higher than that of erythrose or threose, and eightfold higher than that of ribose [129].

Glyceraldehyde-derived AGEs may contribute to neuronal cell toxicity in diabetes [130] and induced apoptosis and overexpression of vascular endothelial growth factor in bovine retinal pericytes [131]. Although argpyrimidine is mainly produced by methylglyoxal, it appears as the major modification produced in proteins after an incubation of porcine cornea with glyceraldehyde [47,132,133]. The formation of pentosidine from glyceraldehyde is supposed to be a minor pathway [108]. Some investigators detected the formation of pentosidine from glyceraldehyde [132], while others were unable to detect it [47]. This discrepancy might be due to differences in the interpretations of the NMR spectra.

Four Maillard reaction pyridinium compounds named triosidines were reported recently, from the reactions between lysine, arginine and glyceraldehyde [47]. All of these newly identified Maillard products contain two glyceraldehyde molecules. Two of them are fluorescent, UV-active cross-links, the arginine–lysine cross-link arg-hydroxy-triosidine and the lysine–lysine cross-link lys-hydroxy-triosidine, while the two other triosidines, trihydroxy-triosidine and triosidine-carbaldehyde, are only UV-active lysine adducts [47]. Incubations with other sugars and oxoaldehydes indicated that the four triosidines are specifically derived from glyceraldehyde and its isomer dihydroxyacetone. Since the triosidines are glyceraldehyde specific, detection of these compounds might be used as specific markers of the glycation reaction by glyceraldehyde.

Glycation by fructose 3-phosphate

Fructose 3-phosphate and sorbitol 3-phosphate are novel metabolites that have been shown to associate with the polyol pathway in animal experiments. The levels of both metabolites were significantly higher in diabetic than in non-diabetic subjects [56–61]. Fructose 3-phosphate is of particular interest because of its potent glycation capability as compared with other glycolytic intermediates, for example, fructose [57]. Epalrestat, an aldose reductase inhibitor, induced significantly lower levels of fructose-3-phosphate in erythrocytes from diabetic patients as compared with those untreated [134], suggesting a possible explanation for the preventive effect of an aldose reductase inhibitor on non-enzymatic glycation. Surprisingly, the aldose reductase inhibitor sorbinil was found to have no effect on the levels of fructose-3-phosphate in diabetic rat hearts, which may indicate alternative routes for the production of fructose in rat hearts [61].

Glycation by 3-deoxyglucosone

The polyol pathway is associated with the production of 3-deoxyglucosone [135]. After conversion to fructose-3-phosphate, fructose is degraded to 3-deoxyglucosone [58]. The concentration of 3-deoxyglucosone in human blood plasma of healthy subjects is 60 nmol/L and increased two- to threefold in diabetes mellitus and, because of a decreased metabolism, three- to fourfold in uraemia [136,137]. The improvement of hyperglycaemia resulted in a significant decrease in 3-deoxyglucosone, indicating that plasma glucose levels are a predominant factor that determines the plasma 3-deoxyglycosone levels in diabetic patients. Recent data demonstrated that diabetic patients with relatively higher 3-deoxyglucosone levels were prone to suffer from more severe complications, indicating a possible association of 3-deoxyglucosone with diabetic microangiopathy [138].

3-Deoxyglucosone is a potent and rapidly acting glycosylating agent [63]. Administration of the aldose reductase inhibitor epalrestat reduced the increase of 3-deoxyglucosone in erythrocytes of diabetic haemodialysis patients and decreased imidazolone and CML [139], suggesting a role for 3-deoxyglucosone in the formation of AGEs. 3-Deoxyglucosone first attacks arginine residues and the adduct formed by this reaction has recently been characterised as imidazolone. The reaction is rapid, reaching a maximum imidazolone concentration within 24 h. Although pentosidine, CML and pyrraline [140] are also AGEs formed by the non-enzymatic reaction of 3-deoxyglucosone with lysine and arginine residues in proteins, imidazolone is the most specific AGE for the *in vivo* involvement of 3-deoxyglucosone in the modification of tissue proteins. Since the polyol pathway is associated with the production of 3-deoxyglucosone and imidazolone is the most specific AGE formed from 3-deoxyglucosone, intracellular detection of imidazolone might be used as an index of the non-enzymatic glycation by the polyol pathway. Thus far, imidazolone has been detected in the aortas and kidneys of diabetic patients [141].

Glycation by methylglyoxal

The formation of methylglyoxal is increased in hyperglycaemia associated with diabetes [142]. Methylglyoxal is mainly formed by conversion of the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate, which are derived from glucose and fructose metabolism [143,144]. Methylglyoxal is a potent glycating agent [145], which plays a key role in the formation of intracellular AGEs as demonstrated by the inhibition of intracellular AGE-formation by the overexpression of the enzyme glyoxalase I [92]. Methylglyoxal is detoxified

by the conversion to S-D-lactoylglutathione and D-lactate, catalysed in the cytosol of all cells by glyoxalase I and II and reduced glutathione. The concentration of methylglyoxal is also increased in the kidney cortex and medulla and lens of streptozotocin-induced diabetic rats [146]. The increases in blood, kidney medulla and lens, but not that in kidney cortex, were prevented by the aldose reductase inhibitor statil [146]. This points to an important contribution of the polyol pathway and fructose in the formation of methylglyoxal in these tissues. Although the inhibition of methylglyoxal by aldose reductase inhibitors is complex, this effect can be explained by the inhibition of the flux through the sorbitol pathway leading to impaired formation of triose phosphates and depletion of cellular glutathione.

Perspective

Fructose is a significantly more effective glycating agent than glucose is in the initiation of the Maillard reaction. However, in assessing the *in vivo* significance of fructosylation under physiological conditions in human health, it must be taken into account that glucose clearly dominates in the living organism, for example, the human plasma level of fructose is estimated in the low μM range, whereas the glucose concentration is 5 mmol/L. The very low extracellular concentrations of fructose raise the question about the actual role of fructose as an intermediate in the formation of AGEs under physiological conditions. As the use of fructose as a dietary sweetener has increased, the effect of long-term intake of fructose on tissue fructose concentrations and the formation of AGEs has to be monitored. Because of the high fructokinase activity in the liver, one may expect that an increased consumption of fructose does not substantially enhance plasma fructose levels and extracellular fructosylation and cross-linking.

Not only is fructose itself of importance in the Maillard reaction, but also the fructose derivatives fructose-1-phosphate and D-glyceraldehyde, as produced by fructokinase, have to be considered. Especially D-glyceraldehyde is a potent inducer of the Maillard reaction. In this regard, the detection of the specific D-glyceraldehyde-induced Maillard products triosidines would be valuable markers to estimate the contribution of D-glyceraldehyde in the Maillard reaction. In conclusion, fructose as well as carbohydrate intake in general may effect the accumulation of AGEs, but the accumulation of AGEs is tissue-specific. This should be considered when effects of dietary carbohydrates intake on the accumulation of AGEs are studied.

The polyol pathway is the second mechanism of fructose accumulation and is activated by hyperglycaemia in various tissues such as ocular lens, kidney and peripheral nerves. In these tissues the concentration of fructose is low, and only at glucose excess, glucose metabolised to reactive intermediates such as fructose, fructose-3-phosphate and 3-deoxyglucosone. The polyol pathway may substantially contribute, by the reaction of these intermediates with proteins, to intracellular AGE formation. In fact, the prevention of the accumulation of AGEs by long-term treatment with an aldose-reductase inhibitor demonstrated the importance of the polyol pathway in the Maillard reaction. Because the polyol pathway is associated with the production of 3-deoxyglucosone and is elevated in those tissues of diabetic patients in which the polyol pathway is active, detection of the specific 3-deoxyglucosone adduct imidazolone may be used to further elucidate the contribution of the polyol pathway to the Maillard reaction.

The impact of the Maillard reactions depends on the nature of the AGEs formed. The formation of AGE-derived cross-links has been established as an important factor in vessel and heart stiffness [147,148]. Furthermore, cellular activation by AGEs has been indicated as a potential noxious stimulus. In particular, vascular pericytes bind AGEs and are sensitive to these compounds, probably because they express the full-length receptor RAGE [149]. However, recent data suggest that the formation of AGEs per se, which involves the generation reactive oxygen species, may have more impact on retinal vessels than the AGEs themselves [84]. These observations point to the importance of the intracellular processes that are affected by fructose and the trioses derived thereof. Intracellularly formed AGEs may add to these effects.

To summarise, on a molecular basis fructose is a reactive monosaccharide that easily forms Heyns products and intermediate and advanced glycation endproducts. Despite its high reactivity, the contribution of extracellular glycation of proteins by fructose is considerably less than that by D-glucose, because of the much lower concentration of fructose in blood. However, intracellularly the higher reactivity of fructose and its subsequent metabolism may contribute to alterations of cellular proteins and dysfunction of cells. Further insight into these processes is needed to properly estimate the relevance of such alterations in healthy and diabetic people.

Acknowledgements

C. G. Schalkwijk is supported by a grant from the Diabetes Fonds Nederland. V. W. M. van Hinsbergh was supported by a grant from the University Stimulerings Fonds of the Vrije Universiteit Amsterdam. We would like to thank Dr T. Teerlink for helpful suggestions.

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