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Hansen, Max

Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

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Citation (APA):

Hansen, M. (2007). *Biological factors related to the possible genotoxicity of simple carbohydrates in the rat colon.*

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Biological factors related to the possible genotoxicity of simple carbohydrates in the rat colon

Ph.D. Thesis

Max Hansen

Department of Toxicology and Risk Assessment
Technical University of Denmark

Institute of Public Health
University of Copenhagen

Preface

The present study was performed between 2001 and 2006 as a part of my employment on the National Food Institute, Technical University of Denmark. The formal Ph.D. study was started in the autumn 2004. The work was supported by the project “Health Effects of Air Pollution and Diet (HEAPOD) granted from the Danish Research Council (grant number 9801314).

I would like to thank my supervisors, Associate Professor Peter Møller from Institute of Public Health, University of Copenhagen and Research Professor Lars Dragsted, Institute of Human Nutrition, University of Copenhagen

March 2007

Max Hansen

Abstract

In previous studies it has been shown that sucrose is mutagenic in the rat colon and affects the levels of bulky DNA adducts in rat liver and colon. The aim of the present work was to suggest biological factors behind these effects and discuss them in the context of sucrose as a potential cancer risk. Three papers describe the experimental studies included in this thesis.

The first study was designed to test the hypothesis that sucrose enhances the colonic genotoxicity of the known food carcinogen, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). In this study groups of 8 big blue rats were given 0 % 3.4 % or 13.4% sucrose with or without 70 ppm IQ for 21 days. Added sucrose replaced cornstarch. The mutation frequency in colon and the level of DNA adducts in colon were increased, and several markers for systemic and local oxidative damage, DNA repair, and cell proliferation in colon were determined. It was concluded that the effects of sucrose and IQ were additive and that the two substances were genotoxic by independent mechanisms. The experiment confirmed that sucrose does not affect markers of systemic or local oxidative stress. A small and statistically insignificant increase in colonic cell proliferation was observed. This might indicate an effect on insulin sensitivity.

Sucrose is a disaccharide and it is cleaved to the two monosaccharides glucose and fructose shortly after ingestion. Physiological effects of sucrose intake may therefore be caused by sucrose, glucose or fructose. In the second study, groups of 12 big blue rats were given either 30 % potato starch (control), 30 % sucrose, 30 % fructose or 30 % glucose in the diet for 35 days. The aim was to compare the effects of these three carbohydrates, to study their effects on markers of insulin sensitivity and to make a preliminary investigation concerning possible effects on the colon microflora. Therefore the pH of the caecum and the concentration of acetic acid, propionic acid and butyric acid (Short Chain Fatty Acids, SCFA) were also measured. The formation of bulky adducts and the colon mutation frequency was similar in magnitude in all dosed animals. The three carbohydrates seem therefore to have similar effect on colon mutation and genotoxicity. The weakly increased cell proliferation in the colon in the previous experiment might indicate an effect on insulin sensitivity. This could result in an increased insulin secretion, which is known to increase cell proliferation. A marker for mean insulin plasma concentration (C-peptide) was therefore included. This time we observed no indication of an increased cell proliferation seen in the previous experiment and also C-peptide was unchanged. We conclude therefore that insulin resistance does not seem to be involved in the rat colon mutagenesis caused by the sugars.

The three sugars decreased the concentration of acetic acid and propionic acid, whereas no effect was observed on the concentration of butyric acid. As acetic acid and propionic acid are important elements in the synthesis of the cell membrane, and in the supply of energy to the colon cells a decrease in the level of these substances may affect the function of the cell and the epithelial cell membrane making the cell more prone to damage. To investigate possible systemic effects on metabolism a metabonomic study on plasma and urine was also performed. This lead to the conclusion that acetate concentrations were also decreased systemically. The metabonomic part of this study also indicates that glucose and fructose affect lipoprotein metabolism differently, but this difference seems unrelated to their genotoxic actions in colon.

The changes in caecal pH and in the caecal concentration of acetic acid and propionic acid in the second experiment indicate that intake of simple carbohydrates may negatively affect the colonic microflora. This is opposite to the well-known effects of resistant starches and of fibres such as inulins and oligofructoses. In a third study, groups of 8 conventional Fischer 344 rats were given 15 % sucrose, 15 % potato starch, 15 % inulin og 15 % oligofructose. The added carbohydrates replaced cornstarch. The faecal profile of bacterial DNA and the faecal profile of bacterial RNA

were measured. The caecal pH was decreased in animals given potato starch compared to animals given cornstarch or sucrose. The faecal bacterial DNA profiles were different in the three groups. The faecal bacterial RNA profile in the potato starch group was different from that in the sucrose and the cornstarch. These results indicate that the composition of the microflora in the colon is changed following sucrose in a way which is known to be associated with increased colon cancer risk, however, any mechanistic relationship to colon mutagenesis still needs further investigation.

A further potential mechanism behind sucrose mutagenesis was also hypothesised as part of this work: The formation of small reactive aldehydes in the metabolism of carbohydrates may cause the genotoxicity. Aldehydes are very reactive towards amine groups, which are common in protein and DNA. Protein adducts of aldehydes are known to be the source of advanced glycation end products (AGE). A chemical identification of the DNA adducts found in rats given sucrose is crucial to test this hypothesis. An attempt was made to measure the reaction products between proteins and small aldehydes but no publishable results have been achieved yet, partly due to problems in the chemical synthesis of the reference products and partly to insufficient analytical sensitivity with the available LC-single quadropole MS equipment. A short description of this work is included.

In conclusion there is no indication that oxidative stress is involved in the genotoxicity of simple carbohydrates, limited support induction of insulin resistance. Changes in the microflora was observed but no mechanistic explanation is suggested. The role of AGE is still an open question.

Sammenfatning

I tidligere undersøgelser er det vist, at sakkarose er mutagent i tyktarmen hos rotter og at det inducerer højere niveauer af store addukter til DNA i både tyktarm og lever. Formålet med dette arbejde var at komme nærmere på en forståelse af hvilke mekanismer der ligger bag disse effekter. Andre studier har antydet, at sakkarose fungerer som et co-carcinogen i tyktarmen. Det første af de tre studier, der indgår i dette arbejde, var sat op for teste en hypotese om at sakkarose forstærker genotoksiciteten af det kendte fødevare-carcinogen 2-amino-3-methylimidazol[4,5]quinoline (IQ). I dette studie blev grupper på 8 rotter givet 0 %, 3,4 % eller 13,4 % sakkarose i foderet enten med eller ude 70ppm IQ. Det tilsatte sakkarose erstattede majsstivelse i dette forsøg. Ud over at bestemme mutationsfrekvensen og niveauet af store DNA addukter i tyktarmen blev der inkluderet markører for systemisk og lokal oxidativ stress, DNA repair og cellevækst i tyktarmen. Konklusionen blev at sakkarose og IQ har additive effekter og at de virker genotoksk ved uafhængige mekanismer. Der blev ikke fundet tegn på hverken lokal eller systemisk oxidativ stress. Der blev set en lille stigning i cellevæksten i tarmvævet, hvilket kunne antyde at insulin resistens kunne være involveret. Dette blev undersøgt yderligere i det næste eksperiment.

Formålet med det andet dyreforsøg var at undersøge om de effekter der er fundet af sakkarose bliver forårsaget af sakkarosen eller det er en af monosakkariderne, glukose eller fruktose, som sakkarose spaltes til, der giver de observerede virkninger. Der blev inkluderet markører designet til at give informationer om de kemiske forhold i tarmindholdet. Til dette blev en kapillarelektroforese-metode, der oprindeligt er designet til at måle, små organiske syrer (SCFA) i jord sat op. Også pH i caecumindhold blev målt. En markør for middelinsulinkoncentrationen (C-peptid) blev medtaget for at undersøge om fodring med simple carbohydrater inducerer insulinresistens, men der blev ikke fundet nogen effekt på denne parameter.. Designet i dette forsøg var, at grupper á 12 dyr blev givet enten 30 % kartoffelstivelse (kontrol), 30 % sakkarose, 30 % glukose eller 30 % fruktose i 35 dage. Resultaterne af forsøget viste at de tre simple carbohydrater udviste nogenlunde samme genotoksitet og at også næsten alle af de øvrige markører giver samme effekter for alle tre. Dette blev brugt til at konkludere at det ikke er den kemiske struktur af carbohydraterne der er årsag til deres genotokiske effekt. De simple carbohydrater sænker niveauet af acetat og propionat i caecum, hvorimod de ikke har nogen effekt på butyrat. Dette bevirket også en sænkning af pH i caecum. Eddikesyre og propionsyre er kendt for at være vigtige for enegesstofskiftet i tarmceller og byggesten i lipidsyntesen og dermed opbygningen af celle membraner. Det blev derfor foreslået at en sænkning af disse kan ændre funktionaliteten af cellemembranerne og muligvis påvire.

Formålet med det tredje studie var at undersøge om carbohydrater på kort tid kan ændre på sammensætningen eller aktiviteten af den bakterielle mikroflora i tyktarmen. Flere carbohydrater var inddraget i dette studie blandt andre majsstivelse, sakkarose og kartoffelstivelse. Også i dette studie blev de kemiske omgivelser i tyktarmen bestemt. Forsøget viste at både sammensætningen og aktiviteten af mikrofloraen kan påvirkes af de nævnte carbohydrater.

Der blev desuden gjort et forsøg på at opsætte en metode til at måle glycosyleringsprodukter fra simple carbohydrater eller deres metabolitter. På grund af utilstrækkelig følsomhed ved den anvendte metode var det ikke muligt at drage nogen konklusioner på dette område.

Papers included in this thesis

Paper 1

Hansen, M., Hald, M. T., Autrup, H., Vogel, U., Bornholdt, J., Moller, P., Molck, A. M., Lindecrona, R., Poulsen, H. E., Wallin, H., Loft, S., & Dragsted, L. O. 2004, "Sucrose and IQ induced mutations in rat colon by independent mechanism", *Mutat.Res.*, vol. 554, no. 1-2, pp. 279-286.

Paper 2

Hansen, M., Baunsgaard, D., Autrup, H., Vogel, U., Bornholdt, J., Moller, P., Lindecrona, R., Wallin, H., Loft, S., & Dragsted, L. O. 2006, "Genotoxicity of fructose, glucose and sucrose in the Big Blue™ rat colon", Submitted to *Food Chem.Tox.*

Paper 3

Licht,T.R., Hansen,M., Poulsen,M., and Dragsted,L.O. (2006) Dietary carbohydrate source influences molecular fingerprints of the rat faecal microbiota. *BMC Microbiol.*, **6**, 98.

Related papers not included in this thesis

Dragsted, L. O., Daneshvar, B., Vogel, U., Autrup, H. N., Wallin, H., Risom, L., Moller, P., Molck, A. M., Hansen, M., Poulsen, H. E., & Loft, S. 2002, "A sucrose-rich diet induces mutations in the rat colon", *Cancer Res*, vol. 62, no. 15, pp. 4339-4345.

Dragsted, L. O., Pedersen, A., Hermetter, A., Basu, S., Hansen, M., Haren, G. R., Kall, M., Breinholt, V., Castenmiller, J. J., Stagsted, J., Jakobsen, J., Skibsted, L., Rasmussen, S. E., Loft, S., & Sandstrom, B. 2004, "The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers", *Am.J.Clin.Nutr.*, vol. 79, no. 6, pp. 1060-1072.

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Moller, P., Hansen, M., Autrup, H., Bornholt, J., Vogel, U., Molck, A. M., Wallin, H., Dragsted, L. O., Risom, L., Poulsen, H. E., & Loft, S. 2003, "Dietary low-dose sucrose modulation of IQ-induced genotoxicity in the colon and liver of Big Blue rats", *Mutat Res*, vol. 527, no. 1-2, pp. 91-97.

List of abbreviation

8-oxo-dG: 8-oxo-deoxyguanosine

AAS 2-amino adipic semialdehyde.

AGE: Advanced glycation end products

ACF: aberrant crypt foci.

DGGE: Denaturing Gradient Gel Electrophoresis

IGF-1: Insulin Growth Factor 1

IGFBP-3: Insulin binding proteins

IQ: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)

LDL: Low density lipoproteins

MDA: Malon dialdehyde

SCFA: Short chain fatty acids

VLDL: Very low density lipoproteins

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Chapter 1

Introduction

Sucrose is a disaccharide consisting of the two monosaccharides, glucose and fructose. Previous experiments have indicated that sucrose is genotoxic to the rat colon but they only give limited suggestions concerning the mechanism behind this effect. The aim of this thesis is to suggest biological factors related to this effect and to discuss the possible carcinogenic effect of simple carbohydrates in relation to these factors. As genotoxic substances are potential carcinogens it is relevant to discuss the epidemiological data concerning the intake of sucrose and cancer. This is done briefly in the chapter 2. The conclusion is that there may be an epidemiological association between high intakes of sucrose and colon cancer. The carcinogenic process is a multistage process, which can be affected by sucrose at several stages. In chapter 3 these stages are described in details sufficient to suggest where sucrose may affect this process. This is discussed in relation to experiments described in the literature and to the experiments presented in this thesis. The most important of the experimental methods used in this word is described and discussed. Chapter 4 describes biological factors presented in this thesis concerning oxidative stress, insulin resistance, changes in microflora in the colon and the formation of glycosylation products. As oxidative stress is expected to be related to insulin resistance [1] and it is also a process involved in the formation of glycosylation products [2] the chapter begins with a brief introduction to oxidative stress. Also this chapter contain information and discussion of the most important experimental methods. Chapter 5 summarise the conclusions. The describtion of the methods to synthesis and isolation two AGE's is placed in an appendix.

Chapter 2

Epidemiological data concerning intake of sucrose and cancer

The aim of this chapter is to give a brief overview of the epidemiological data concerning colon cancer and pancreatic cancer in relation to intake of sucrose.

Colon cancer is very common disease in the western world and relative rare in the developing countries [3] This indicates an environmental or lifestyle influence on the risk for this type of cancer. The results presented in this thesis and in some of the previous works from this group [4;5] suggest that sucrose is genotoxic to the rat colon. Intake of sucrose may therefore be a risk factor for colon cancer. Some epidemiological studies have investigated the relation between intake of sucrose and colon cancer or cancer in the pancreas. Only few epidemiological studies have investigated cancer at other sites in relation to intake of sucrose. Genotoxic substances usually induce cancer at several different sites. If the epidemiological studies show that intake of sucrose is a risk factor for cancers at more than one site, it may be an indication that genotoxicity is involved in the process, and that the process has a systemic rather than a local origin.

Sucrose and cancer epidemiology

Whereas sucrose constituted only 1% of total carbohydrates in the diet before the 20th century, it presently constitutes about 14% of all carbohydrate consumed. It is difficult to estimate the sucrose intake per capita due to underreporting in epidemiological studies based on food questionnaires [6]. The estimated intake in Denmark at present based on sales figures is about 110-140 g daily per capita [6]. Similar intake levels have been reported for The United States [7].

Epidemiological studies suggest that high consumption of sucrose may be associated with increased risk of colon cancer. In a recent review, 16 of 18 studies reported an association of sucrose with increased risk of colon cancer [8]. Figure 2.1 illustrates the association between intake of sugar and colorectal cancer in eight different studies, most of them case-control studies. It has been published by the World Cancer Research Fund [9] and illustrates an apparent dose-dependent increase in colorectal cancer risk with a more than twofold increase for those consuming more than 60 grams of sugar per day compared to those eating less than 10 g sugar per day [9]. Some of the data in figure 2.1 seems a little strange. The reason for the low intake in the lowest group in the studies in the figure is probably that data in the figure represent the lowest dose in the lowest quartile. The overall

intake represented in the figure seems also to be rather low compared to the mean intake in Denmark. This might be caused by underreporting of the sucrose intake, which is frequently seen in case-control studies. Figure 2.1 suggest that intake of sucrose may be a risk factor for colon cancer as all data point in the same direction. In the report it is concluded that there is still insufficient evidence that sucrose is a causative factor in colon cancer. One reason for this may be the limited number of cohort studies included in the analysis. The estimates of specific food item intakes in case-control studies rely typically on interview of cases and controls. As it is well known that this exposure estimate may be imprecise due to recall bias, only more recent prospective studies are included here. In one of two recent prospective cohort studies, high intakes of fructose and sucrose were related to an elevated colorectal cancer risk among men, whereas these factors did not seem to increase the risk among women [10]. In the other study the risk for colorectal cancer was increased statistically significantly in women with a high intake of easily digestible carbohydrates, including these sugars [11]. The relative risks for men and women were 2.6 and 2.09 in the two studies, respectively. These studies as well as the older cohort study included in the figure support the case-control studies and indicate a possible association between intake of sucrose and risk of colorectal cancer.

In a case-control study a strong association with pancreatic cancer risk was reported for total carbohydrate intake and for added sugar [12]. Because of the high fatality rates, case-control studies on risk factors for pancreatic cancer are even more difficult to conduct because the information has often relied on proxy information for case subjects, which probably increases the errors in these studies. In two large prospective cohort studies with a total of 88794 women (Nurses health study) and 49364 men (Health professionals Follow-up study), carbohydrate and sucrose intake were not associated with overall pancreatic cancer risk. A statistically non significant 53% increase in risk of pancreatic cancer was observed among women with a high glycemic load and a similar 57 % increase was observed for women with high intake of fructose [13]. Women in the highest quartile of intake of sugar sweetened soft drinks did experience a significant increase in risk whereas no such association was seen in men [14]. Glycemic index is a measure of the extent to which a food, as compared to pure glucose, raises blood sugar. It does not incorporate information on the intake of the specific food. This is quantified in the glycemic load, which is defined as the index multiplied by the amount of carbohydrate consumed. The glycemic load of a serving consisting of several simple carbohydrates makes it possible to assess the amount of sucrose needed to give the same glycemic effect. Glycemic load is thereby a calculated measure corresponding to intake of simple carbohydrate and not a measurement of intake of sucrose. However, it seems

reasonable to expect that high glycemic loads often reflect a high intake of sucrose [10;15]. A recent prospective study reports an increased risk of pancreatic cancer in individuals with a high intake of sugar or soft drinks. It was concluded by the authors that foods with a high content of sugar may be associated with a greater risk of pancreatic cancer [16].

In conclusion, these studies indicate that high intakes of sucrose might be a risk factor for cancer in the colon. There is a very weak indication that intake of sucrose may also be a risk factor for pancreatic cancer but the data are insufficient. Consequently, no conclusions regarding the mechanism behind a possible carcinogenic effect of sucrose can be drawn based on these data.

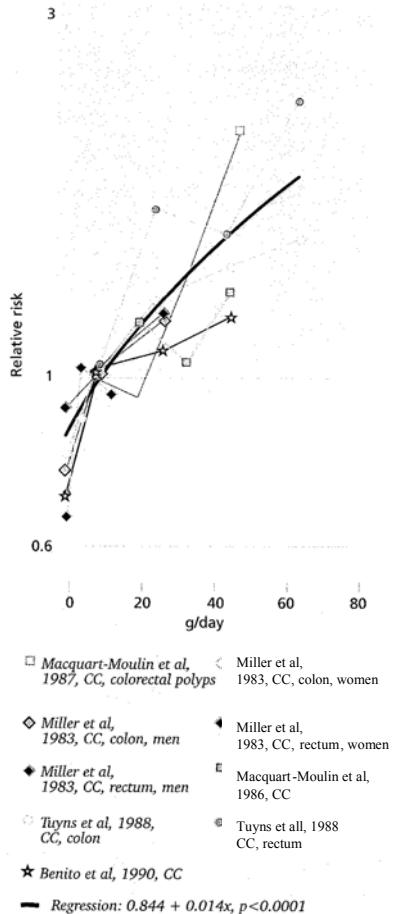


Figure 2.1. This figure suggests a steady increase in colon cancer, rectal cancer and, in one case, colorectal polyps in the presence of higher intakes of sugar and foods high in sugar. The fitted regression line is consistent with a greater than twofold increase in risk for those consuming about 60 g/day of sugar and sugar-rich foods compared with those consuming none. The figure shows that all studies are consistent above 30 g/day compared with those consuming less than 10 g/day.

Chapter 3

Chemical carcinogenesis in relation to intake of sucrose

Introduction

The aim of this chapter is to give a brief introduction to the major stages in the chemical carcinogenic process and relate these stages to intake of sucrose. In the following section the results obtained in this thesis are presented and discussed in relation to these stages. This is followed by a brief presentation of other studies in relation to these subjects. Most of the data are from animal experiments. Some limitations in the use of animal models in the study of macronutrients are outlined in the final section of this chapter.

General Introduction to chemical carcinogenesis

Chemical carcinogenesis is a complex multistage process. Depending on the chemical nature of a specific substance it may contribute to the carcinogenic process at different stages. The pathogenesis of neoplasia consist of at least three operationally defined stages beginning with initiation and followed by an intermediate stage of promotion, from which evolves the stage of progression [17].

Initiation may be defined as the heritable cellular change responsible for the formation of the first preneoplastic (initiated) cell in a tissue [18]. At this stage structural changes in the genome, such as formations of DNA adducts and mutations, can be observed [17]. At least three processes are important in initiation: Formation and binding of reactive metabolites to DNA, DNA repair, and cell division [17]. The initiating carcinogens have been shown to be strong electrophiles (direct-acting carcinogens) or to be metabolised into such compound (procarcinogens) [18]. Sucrose is certainly not a strong electrophile and this could explain why very few studies on cancer initiating effect of sucrose has been performed. However, reactive aldehydes like glyoxal and methyl glyoxal are formed either during the metabolism of sucrose or as a result of the degradation of carbohydrates in the non enzymatic glycosylation reactions. These compounds are mutagenic in the Ames test [19;20], a prokaryotic *in vitro* cell test often used to identify potential eukaryotic mutagens. The formation of reactive aldehydes may therefore explain an initiating effect of sucrose. These products will be discussed further in chapter 4.

DNA repair is important in the initiation process as efficient or increased DNA repair might reduce the possibility that the damage is fixed in the cells. A proliferative effect in the target tissue is required in initiation because the DNA damage requires cell division for the changes to be finally fixed as a gene mutation [21].

Promotion is used to describe the action of any compound that increases the tumor yield by expanding the pool of initiated cells [18]. Promotion depends on continued administration of the promoting agent [17] and the process is therefore operationally reversible. Reversal, loss or regression of preneoplastic lesions developing during the promotion stage has been observed in many instances in both animal and human carcinogenesis [21]. Unlike initiating carcinogens, there is no evidence that promoting agents or their metabolites directly interact with DNA or that metabolism is required for their effectiveness [17]. Some studies indicate that sucrose may act as a promoter.

Progression is an irreversible process characterised by increased karyotypic instability [17]. In this phase changes have been observed in growth rate, invasiveness, metastatic frequency, hormonal responsiveness and morphological characteristics [17]. The progression phase includes growth and development of a tumor up until visibility and its further development into a metastatic tumor [18]. The stage of progression usually develops from cells in the stage of promotion but may develop directly from normal cells, usually as a result of the administration of relatively high, usually cytotoxic doses of complete carcinogenic agent that are capable of inducing both initiation and progression [17].

Animal studies with simple carbohydrate in relation to cancer initiation

The aim of this section is to present data from the animal studies in relation to cancer initiation and to discuss this subject in relation to studies from the literature. The experiments presented in paper 1 and 2 were performed on the transgenic Big Blue® Rats. These rats have an incorporated vector in the DNA of all somatic cells, which allows the detection of mutations *in vivo* in all tissues by either a reverse mutation assay in the *lacZ* gene or a forward mutation assay in a temperature-sensitive promoter. Studies with these animals are suitable to screen for *in vivo* mutagenic and potential cancer-initiating effects of chemicals. In the determination of mutation frequency in the studies included here we used the forward assay. The DNA from the tissue of interest was isolated and packed with Transpack packing extract (Stratagene). This phage preparation was used to infect *Escherichia coli*. Phages with mutations at the cII locus were identified by plaque formation under

selective growth conditions at 24°C (see figure 3.1). total number of phages was determined by growth in non selective conditions (37 °C). The main problem with this method is that mutations resulting from clonal expansion in a subset of cells created during tissue development will be multifid (so-called jackpot mutations). Another concern is that compounds leading to cell proliferation in a certain tissue may increase the fixation of background mutations leading to an apparent mutagenic effect.

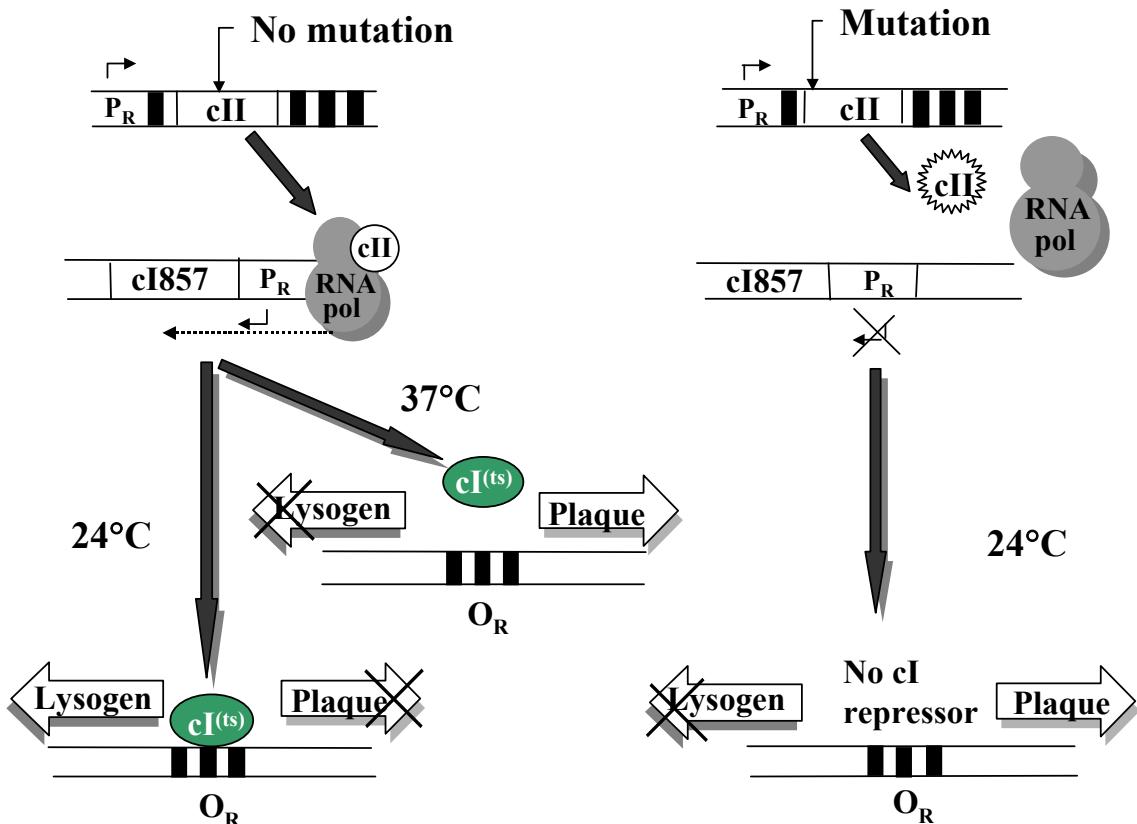


Figure 3.1 Detection of *in vivo* mutations in the Big Blue® rats. The cII gene encodes a protein that activates transcriptional promoters in lambda that are essential for lysogenization. Mutations in the cII region that lower the levels of cII protein result in a decreased ability of lambda to lysogenize. When grown under conditions that favour lysogeny (24°C), lambda prophages carrying such mutations (cII-) survive only by entering the lytic pathway of development, forming plaques. Prophages that are wild type for the cII region (cII+) integrate into the host genome and become part of the developing bacterial lawn. As a result, only mutated phage can form plaques greatly reducing the odds of obtaining *ex vivo* mutations. In addition, a dilution of infected cell is plated and grown under nonselective conditions (37°C) to determine the total number of rescued phages. (Source: Stratagene, cat no.# 720120)

The results from paper 1 confirm previous results [4;5] showing an increased mutation frequency in the colon mucosa with increased intake of sucrose (see figure 3.2). The increase in colon mutation frequency in paper 2 is less than the increase observed in paper 1. This was unexpected since the

doses were higher in the study reported in paper 2 (13,4 % in paper 1 and 30 % in paper 2) and the duration of the study was longer (3 weeks in paper 1 and 5 weeks in paper 2). The increase in the duration of the study gives the mutations more time to become fixed. In the study presented in paper 2, the simple carbohydrates replaced potato starch in contrast to the study presented in paper 1 where it replaced cornstarch. Potato starch is more resistant to digestion than the cornstarch and several studies have reported that resistant starch affect colon in a beneficial way and may reduce the risk for colon cancer [22-30]. Especially a reduced rate of cell proliferation has been observed in the colon of animals given potato starch [24;29;30]. A larger difference in the mutation frequencies between control and dosed animals was therefore expected in paper 2. The increased statistical power due the increase in the number of animals from 8 per dose group in paper 1 to 12 per group in paper 2 should increase the probability of finding statistical significant differences between control and dosed animals provided there is an effect of the test substance. The lower mutagenicity observed in study 2 is therefore not readily explained by other factors but biological variation although the Big Blue® is an inbred strain and the model is therefore less prone to random variation due to genetic variation. However, a lower mutation rate is also observed in the control group in study 2, indicating that some biological variable might cause the differences. The background rate of cell proliferation may be such a factor. In paper 2, the increase in the mutation frequencies in animals given simple carbohydrates is not statistically significantly different from control by ANOVA when each of the carbohydrate groups are compared to the control group. If fructose, sucrose and glucose are considered as one ‘simple carbohydrate’ group the mutation frequency in this group becomes statistically significantly different from the control group by Students t-test. This leads to the conclusion that the data presented in paper 2 confirms the mutagenicity of sucrose and also indicates a similar mutagenicity of fructose, glucose and sucrose although the evidence for this is not very strong.

The mutation spectrum of the individual mutations was determined in paper 1 and shows that sucrose feeding leads to an increase in the background mutation rather than an induction of specific mutations.

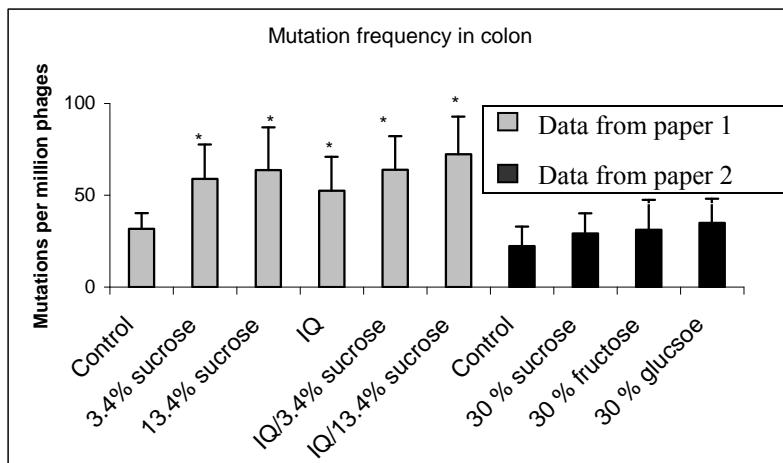


Figure 3.2 The mutation frequency in the colon of rats given different concentrations of simple carbohydrates. A clear dose response of increasing doses of sucrose is observed in the first 6 columns. The increase in the mutation frequency is less clear in last 4 columns. * P < 0.05

The level of bulky DNA adducts, determined by ^{32}P postlabeling, was increased in both experiments whereas no increase was observed in our previous experiments [4;31]. In the ^{32}P postlabelling method, DNA is digested with nuclease and phosphodiesterase and the resulting digest labelled using T4 nucleotide kinase and ^{32}P -labelled ATP, separated by TLC and visualised using photoimaging. Densitometry is used to quantitate the resulting spots. In order to increase the sensitivity for nucleotides carrying an adduct, two procedures are commonly used, nuclease P1 treatment or butanol extraction. The sensitivity of the procedures depend on the adduct in question, so each procedure may have advantages and draw-backs. For the current study the generally less sensitive butanol extraction procedure was used, and a different result might have been obtained using nuclease P1. The procedure is highly sensitive and picks up very low levels of adducts, even unknown adducts. The main problem with the ^{32}P postlabelling technique is that it is at best semiquantitative for known adducts and that only relative amounts can be determined for unknown adducts. There is a marked batch to batch variation, which is a hinder to comparisons between analytical batches. Linearity is limited by the dynamics of the densitometry, so that dense spots easily hit the maximum intensity, which is the upper limit of quantitation.

The differences between the present and the previous experiments may therefore rest in methodological issues but it is more probably caused by the differences in experimental design and composition of feeds. It is difficult to suggest a mechanistic explanation for the differences due the large differences in the composition of feed in the studies. In the previous studies sucrose was added to the feed at levels of 3, 13, and 30%, diluting all other ingredients, whereas sucrose

replaced cornstarch in paper 1, and sucrose, fructose and glucose replaced potato starch in paper 2. With increasing sucrose doses and increased dilution of other feed components the overall level of DNA-adducts decreased. In another former study, lard doses of 3, 13, and 30% were given in the same way, diluting the other feed ingredients [32]. An even steeper decrease in bulky adduct levels was observed in that study, indicating that sucrose as opposed to lard may have actually led to some increase in bulky adducts even in the previous studies. In figure 3.3 the data for the level of bulky DNA adducts in paper 1 and 2 are illustrated.

The level of bulky adducts was at the same level in rats given 30 % sucrose in paper 2 compared to rats given 13,4 % in paper 1. A possible explanation may be the upper level for the simple carbohydrate induced increase in the level of bulky adducts is reached at doses lower than the 13.4 % sucrose used in paper 1. The example illustrates very well the general problem of between-batch comparisons with the ^{32}P -postlabelling method and should not be over-interpreted.

These studies indicate that intake of sucrose result in the formation of bulky DNA adducts in the colon and in an increase in the mutation frequency. As sucrose is mutagenic and forms DNA adducts it may be considered genotoxic and thereby a possible cancer initiator. In some of the studies from the literature, which are discussed in the following section concerning promotion there are some data, which also concern initiating effects of sucrose. It can be noted here that a study indicating a possible initiating effect in the liver after feeding sucrose only was performed in 1972 [33]. In this study liver hyperplasia was observed without concomitant exposures to any known initiating carcinogen.

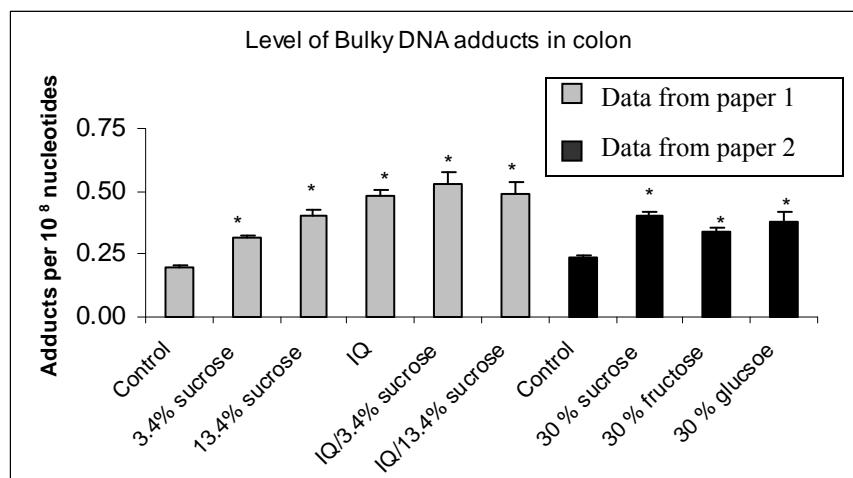


Figure 3.3. The level of bulky DNA adducts in the colon of rats given simple carbohydrates and/or IQ. The first 3 columns indicate a dose response in the increase in the level of bulky DNA adducts. When IQ is co-administrated it is only the 3,4 % sucrose, which increases the level of bulky adducts.

Studies with simple carbohydrate in relation to cancer promotion

It is well known that 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is a mutagenic chemical[34]. If sucrose acts as a promoter an enhancement of the genotoxicity of IQ could be expected when the 2 substances are co-administered. This was tested in paper 1, where the rats were given sucrose, IQ, or both. IQ increased the mutation frequency in the colon (see figure 3.2) as expected. When sucrose and IQ was administered together there was a dose dependent increase in the mutation frequency with respect to sucrose (see figure 3.2). A statistical analysis using two way ANOVA indicates that there is no interaction between IQ and sucrose. The effect of the two substances seems purely additive.

Almost the same picture was seen for the level of bulky DNA adducts (see figure 3.3). Other data from this experiment confirm that the effects of sucrose and IQ are additive (see paper 1). These results indicate that sucrose as well as IQ act through independent mechanisms. Moreover, a limited effect on cell proliferation by sucrose can be observed after 3 weeks of sucrose dosing. The proliferation in the colon crypt was determined in the experiments reported in paper 1 and 2 (see table 3.1). The cell proliferation was reported to be increased in paper 1. When the different groups of sucrose-dosed animals were compared with controls the increase was not statistically significant in any of them. But if all sucrose-dosed rats were considered as one group, there was a significant increase in cell proliferation in this group compared to control. This lead to the suggestion that increased cell proliferation, determined as proliferating cell nuclear antigen, could be involved in the increased mutation frequency and in the increased level of bulky DNA adducts. This was not confirmed in paper 2, where no change was observed on colon cell proliferation.

The higher proliferation of the colon epithelium observed in study 1 may in part explain the higher mutation rate observed in this study. Since the mutation spectrum in study 1 indicate that sucrose increases background mutation, the mutation rate would be expected to depend on the background cell proliferation rate. The lower cell proliferation rates in the control group in study 2 compared to the cell proliferation in the control group in study 1 supports this explanation.

When cell proliferation is fast, there is less time to repair replication errors and random damage to DNA. During fast replication tissues are consequently more prone to genetic damage. In paper 1 the expression of three DNA repair enzymes was determined but none of them were increased in animals given sucrose indicating that increased DNA damage caused by the increased proliferation will not be repaired.

In conclusion there is only limited evidence from study 1 and 2 that sucrose might affect cell proliferation, however differences in the background levels of proliferation and in the proliferative response to feeding with simple carbohydrates in the two studies may have caused the observed quantitative differences in mutagenic response to sucrose.

A.

PCNA	Control	3.4 % sucrose	13.4 % sucrose	IQ	IQ 3.4 % sucrose	Sucrose 13.4 % sucrose
Lower third	12 ± 8	18 ± 13	17 ± 8	13 ± 9	18 ± 8	17 ± 13
Middle third	5 ± 3	11 ± 10	7.3 ± 4.7	4.6 ± 6.5	5.2 ± 3.9	7.1 ± 5.3

B.

PCNA	Control	30 % Sucrose	30 % Fructose	30 % Glucose
Lower third	4.6 ± 4.8	2.9 ± 2.3	4.0 ± 4.0	1.4 ± 1.7
Whole crypt	2.6 ± 2.5	1.4 ± 1.3	1.8 ± 1.6	0.7 ± 0.8

Table 3.1 The cell proliferation in the colon epithelium was determined. Part A is from paper 1 and show that IQ has no effect on the proliferation. Sucrose seems to increase the proliferation but the increase is not statistically significant. The proliferation in the colon was also determined in paper 2 but there was no change in this study even though the doses of simple carbohydrates was higher.

The literature contains some studies on the potential role of simple carbohydrates as cancer promoters. The cancer process is chemically initiated in these types of study. This is usually done by giving the rats one or more intubations with chemicals like 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), or injections with 1,2-dimethylhydrazine or azoxymethane (AOM). The endpoint in many of these experiments has been putative preneoplastic lesions in the colon determined as the number and/or size of aberrant crypt foci (ACF). The ACF is regarded as a group of cells at increased risk of developing into an adenoma and consequently a cellular change with an increased likelihood of housing initiated cells. Consequently, a tumor promoter would be expected to primarily increase the size and complexity of the foci, whereas an initiator or co-initiator would increase the number of foci [35]. Rats given AOM develop colon tumors in 10 month but very few studies on sucrose have been of this length. The studies on the effect of simple carbohydrates on the number and complexity of ACF are seen in table 3.2. In 5 of groups of the studies in table 3.2 an increase in the number of ACF in rats initiated by one of the chemicals mentioned above was observed [35-39] indicating a initiation effect of sucrose. In one study there was no increase in the number of ACF in rats given sucrose [40]. There is also a tendency towards formation of more small ACF in sucrose dosed animals. This does rather support an initiating effect than a promoting effect of sucrose. There is some controversy about the use of ACF as predictors for tumour development. Several studies indicate that increase in the number of ACF is reflected in an increase

in the number of tumours but the number of ACF does not correlate with the number of tumours induced in carcinogenic studies. When AOM initiated rats were dosed with sucrose in 10 month after initiation with AOM, there was no statistically significant increase in the number of colon tumors and no correlation between ACF and colon tumor of animals fed simple carbohydrates [37-40]. This is a strong indication that ACF has limited value as a predictor of tumor development in animals given simple carbohydrates.

Initiator	Control	Carbohydrate	Time (weeks)	Number of ACF	Multiplicity of ACF	Colon Tumours	Reference
AOM	Cornstarch	Sucrose	9	No effect	Less large crypt	No effect	Jacobsen et. al. [40]
		Sucrose	32	No effect	No effect		
	Potato starch	Sucrose	9	No effect	Less large crypt		
		Sucrose	32	No effect	No effect		
IQ	Cornstarch	Sucrose	25	No effect	Less large crypt	No effect	Lindecrona et al [38]
IQ	Cornstarch	Sucrose	14 46	Increased Increased	No effect No effect	No effect	Mølck et al [39]
AOM	Cornstarch	Sucrose (bolus)	4	Increased ¹	No effect		Luceri et al [35]
IQ	Cornstarch / potatostarch	Sucrose / dextrine	10	Increased	More small		Kristiansen et al 1996[36]
DMH	Cornstarch / potatostarch	Sucrose / dextrine	10 20 31	No effect No effect Increased	More small	No effect	Kristianse et al 1995 [37]

Table 3.2. Studies of the effect of sucrose on the number and complexity of ACF and tumour development in cancer initiated animals. These studies indicate that sucrose increase the number of ACF. None of the studies show a statistical significant increase in the number of tumours in the colon. Sucrose was given continuously in these experiments. In the study by luceri there was also a group where sucrose was given in bolus.

¹The increase in the number of ACF was only observed in rats given sucrose as bolus.

Increased cell proliferation is generally considered to increase cancer risk [41;42] and is also an indicator for cancer promotion [17]. The cell proliferation in colon mucosa was determined in three of the studies mentioned above. Increased proliferation was observed in one study [40], whereas no change was reported in another study [38]. Increased proliferation has been observed in rats given sucrose as bolus compared to rats given either starch as bolus or sucrose continuously [35]. The eating pattern of the rats may therefore additionally determine whether a proliferative response may be observed.

It is interesting in this context to note that a high intake of sucrose as a bolus in humans has been shown to increase the proliferation rate in the colorectal epithelium, and to expand the proliferative zone from less than 60% of the colonic crypt to the entire crypt [43]. Sucrose seems to increase the proliferation rate more than fructose and glucose [35] indicating a more rapid absorption of sucrose compared to glucose and fructose.

In conclusion, the results from paper 1 and 2 confirm that sucrose is genotoxic and indicate that fructose and glucose have similar effect. This means that the simple carbohydrates have the potential to be cancer initiators. The studies presented in this thesis does not support that a promoting effect of sucrose would be expected. The literature gives some support for a promoting effect of sucrose on preneoplastic lesions as some studies indicate an increased cell proliferation in animals given simple carbohydrates as bolus. On the other hand, the lack of tumour development in the AOM initiated animals does not support a cancer promoting effect of sucrose. If sucrose has a cancer promoting effect it is probably weak and related to short-term high intakes. Further studies would have to be conducted in order to clarify this.

To test for a tumor promoting effect of sucrose there is a need for long-term experiments where rats are given sucrose daily in bolus doses with and without chemical initiation.

The use of animal models in the study of macronutrients has some important limitations, which makes it difficult to draw conclusions about the absence of effects. These will be described in the following section.

Limitations in animal studies with macronutrients

There are at least two major difficulties when animal studies are performed to assess effects of macronutrient and estimate the potential implications on human populations of a change in macronutrient intakes or composition.

First, the statistical power to pick up weak effects is very low in animal studies due to the limited number of animals in each dose group. An increase in the number of animals is obviously a possibility to increase the statistical power but for practical reasons this is only possible to a limited extent. The human intake of most chemical carcinogens is very low compared to the total intake of food. This makes it possible to test these chemicals in animals at doses much higher than the expected human intakes and then extrapolate or use uncertainty factors. This does not increase the statistical power but the high doses makes it possible to observe an effect in most of the dosed animals and study the mechanism behind the effect. This strategy is obviously not possible in studies of macronutrients due to the high intake in a normal diet. A reliable animal study designed to prove a minor carcinogenic effect of a macronutrient would demand a large number of animals. This problem is clearly relevant in the three studies presented in this thesis because the number of animals was only 8-12 in each dose group in study 1 and 2. That was partly due to the high prices of

transgenic animals, partly because the mutagenic effects observed in previous studies from this group indicated a sufficient power to observe a mutagenic effect of sucrose feeding using a limited number of animals.

Second, the composition of the feed is changed significantly by addition of a macronutrient. In our previous studies up to 30 % sucrose was added to the feed diluting all other components in the feed. This design obviously has flaws making it very difficult to draw conclusion concerning the mechanistic causes of the effects because the outcome of the experiments may be caused by a decreased intake of one or more of the other component in the feed. By adding a control group, which is dosed with the same amount of an inert compound might solve the problem. We performed an experiment with the same design as the previous experiments but where the rats were given 30 % lard instead of 30 % sugar. In this experiment there was no changes in the colon mutation frequencies or in the level of bulky DNA adducts in the colon indicating no genotoxic effects of diluting the feed [32]. Of cause, there is a theoretical possibility that lard precisely counteract the genotoxicity caused by diluting the feed but this may be considered as unlikely as there is no evidence in the literature indicating an antimutagenic effect of lard or other dietary fats. However, lard can hardly be called an inert dietary compound in general and as a consequence of this, substituting lard for sucrose does not give an optimal study design.

In the studies presented in paper 1 and 2 the added carbohydrates replaced another carbohydrate in the feed, namely the polyglucose, starch. This makes it possible to keep the concentration of all other ingredients constant. In this type of experiments the effects of the two carbohydrates are compared. This method is scientifically useful because there are fewest possible differences between the composition of the feed but it is not relevant in relation to humans as it seem more likely that we reduce our intake of all other substances when we increase the intake of sucrose. However, the interpretation of the differences observed will depend on the eye of the beholder. We might for instance argue based on paper 1 and 2, that sugar is the reference and that cornstarch and potato starch are anti-mutagens. The consequence for dietary advice would not be changed much but the search for explanations might be altered. Curiously, in some animal studies showing anti-carcinogenic effects of fibers the authors have used sucrose as the reference compound for the control group [44].

In conclusion, the results from animal studies on macronutrients should be interpreted with great caution.

Chapter 4

Biological factors relating high intake of simple carbohydrates and cancer risk

Introduction

The aim of this chapter is to introduce possible biological factors relating intake of simple carbohydrates and colon cancer risk and to set the data obtained in this thesis in relation to these processes. An additional subject is whether the mutagenic effects of simple carbohydrates are induced systemically or through processes in the colon or other parts of the intestine. This will also be discussed briefly in this chapter. All sections begin with a short introduction to the subject followed by a presentation of the relevant data obtained from the experiments presented in this thesis. Before the conclusion an overview over the results reported in the literature is presented. As oxidative damage is considered an important consequence of both insulin resistance[1] and the formation of AGE[45] the chapter begins with an introduction to this subject.

As outlined previously, there are four partially independent biological effects, which are considered particularly relevant in an attempt to understand the mutagenic action of sucrose in the rat colon. These are oxidative stress, insulin resistance, carbohydrate fermentation in the gut, and the formation of advanced glycation end products.

Oxidative stress might be linked with the initiation process as oxidative products, such as malondialdehyde formed in the lipid peroxidation, form adducts to DNA [46]. Oxidative stress may also be related to promotion as many of these oxidation products are cytotoxic. This might result in increased proliferation [47], including proliferation of initiated cells.

Insulin resistance: This process has previously been linked with increased risk of colorectal cancer in relation to diet [1]. It is therefore relevant to discuss if this process is additionally involved in the genotoxicity of simple carbohydrates. Insulin resistance is characterised by an abnormal increase in the secretion of insulin as a result of intake of a bolus amount of sucrose and is thereby a systemic process.

Fermentation of carbohydrates: When considering effects of simple carbohydrates it is important to remember that they are absorbed early in the gastrointestinal tract, well before they reach the colon. A direct effect of simple carbohydrate on the colon is therefore unlikely. However, an indirect effect through changes in total feed composition, as more complex carbohydrate is replaced with simple carbohydrates, may change the microflora in the colon. Another possibility is that changes in the colonic microflora reflect changes in the more dilute flora of the upper part of the intestine. A local effect on the colon as a consequence of a high intake of simple carbohydrate can therefore not be excluded.

Formation of AGE: This is a large subject in relation to diabetes but it has never been related to intake of carbohydrates in healthy animals. AGE is able to explain the induction of DNA adducts and the generation of mutagenic substances and might therefore represent a plausible mechanistic explanation for the genotoxicity of simple carbohydrates. Therefore, an attempt was made to synthesise two of these products and to measure these products in biological samples from rats. The procedure and the results will be presented although the results are still far too limited for a scientific paper. Formation of AGE as a mechanistic explanation for the genotoxicity of simple carbohydrates has some limitations. Some of the AGE's are formed very slowly and AGE is also formed during processes unrelated to intake of carbohydrates. Finally AGE is present as such in some foods. These subjects are presented and discussed in relation to the possibility of measuring changes in the AGE concentration as a consequence of intake of simple carbohydrates.

oxidative stress

Oxidative damage is caused by reactive oxygen species, which include radicals like the superoxide anion or the hydroxyl radical. Also peroxides are considered reactive oxygen species although they are not radicals. Oxidative damage is usually measured as oxidative modifications to lipids, protein or DNA. This has been done in paper 1 and 2. The natural defences against oxidative damage include the enzymes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase. An increased activity of these enzymes in tissues may indicate that the tissue is exposed to an increased amount of reactive oxygen species.

Simple sugars and oxidative stress

Only two markers for systemic oxidative damage were included in this thesis. Protein oxidation in plasma was determined by the concentration of 2-amino adipic semialdehyde (AAS), a marker of oxidised lysine residues, and lipid oxidation in plasma by the concentration of malondialdehyde (MDA), a secondary lipid oxidation product. There were no observed changes in these markers (see

table 4.1 A) in blood plasma after increased sucrose dosing. Local oxidative effects in the colon were also investigated. The protein oxidation in the colon, as AAS, was not affected by intake of simple carbohydrates (see table 4.1. B) It was the first time this analyses was used to analyse samples from the colon epithelium. Two markers for DNA oxidation in colon were measured. The oxidation of DNA expressed as the concentration of 8-oxo-deoxyguanosine (8-oxo-dG) (see table 4.1 A) was not affected. The level of oxidative damage in colon epithelium was also assessed indirectly. Oxidation of DNA may be repaired by several repair enzymes. In this process the DNA strand is broken. The number of strand breaks in the DNA may thereby reflect the DNA repair activity, which reflects the amount of DNA damage. This was measured by the sensitive comet assay and the result confirms that there were no changes in the DNA oxidation in the colon epithelium cells in rats given simple carbohydrates (see table 4.1 A). The only effect on oxidative markers was an increase in the number of strand breaks in the animals given IQ. This was expected and confirms the sensitivity of this assay.

A.

Oxidative markers	Control	3.4 % sucrose	13.4 % sucrose	IQ	IQ 3.4 % sucrose	Sucrose 13.4 % sucrose
AAS (plasma) (Pmol/mg protein)	144 ± 14	152 ± 40	143 ± 17	148 ± 12	143 ± 9	144 ± 12
MDA (plasma) (Pmol/mg protein)	87 ± 16	77 ± 20	90 ± 18	73 ± 5	78 ± 11	72 ± 11
8-oxodG (colon) (per 10 ⁵ dG)	1.2 ± 1.2	0.9 ± 0.4	1.4 ± 0.7	0.8 ± 0.4	0.8 ± 0.4	1.4 ± 0.9
Strand breaks (%DNA in tail)	15.4 ± 6.0	15.1 ± 8.2	14.9 ± 5.8	26.6 ± 8.8*	23 ± 4.6*	21.9 ± 9.1*

B.

Oxidative markers	Control	30 % Sucrose	30 % Fructose	30 % Glucose
AAS (colon) (Pmol/mg protein)	51 ± 27	45 ± 35	52 ± 50	45 ± 28

Table 4.1 Systemic as well as local markers for oxidative damage. Part A is data from paper 1 and part B is from paper 2. Sucrose did not induce systemic oxidative damage to protein (AAS in part A.) or lipid (MDA). The markers for protein oxidation in colon (AAS part B) and DNA oxidation in colon (8-oxodG and strand breaks) were also unaffected by sucrose. Only the expected increase in the DNA oxidative damage in colon (strand breaks) in IQ induced animals was observed. * p<0,05

The lack of oxidative damage confirms the results obtained from the two previous studies related to this thesis [4;5]. The activity of the enzymes responsible for the scavenging of reactive oxygen species, superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase were measured in red blood cells in paper 1. This was confirmed in a study where rats were given sucrose for 7 days [48]. However, other studies suggest a prooxidative effect of simple carbohydrates. In rats given apple juice, orange juice or water containing a high concentration of glucose, fructose, or sucrose an increase in protein oxidation (as AAS) has been observed [49]. It was suggested that the carbohydrates present in the respective fruit juices might be fully or in partly responsible for the observed prooxidant effect of juice on plasma proteins. Increased concentrations of lipid oxidation products measured as thiobarbituric acid reactive substances (TBARS) have been found in plasma and urine of rats given 20 % sucrose for two weeks compared to rats given the same amount of starch [50;51]. In one of these studies there was also a decreased activity of Cu-Zn dependent superoxide dismutase [51]. The same enzyme has been measured in the lung of rats given a diet high in fructose and in this study the activity of this enzyme was increased especially in Zn deficient animals [52].

There are some limitations in the measurement of oxidative damage. Oxidative damage occurs at specific sites in the cells and in the tissues but most of the analyses are performed on whole tissue samples or in plasma and red blood cells as a measure for systemic oxidative damage. It is therefore possible that local oxidative damage is not detected in these assays. This was probably observed in a study where levels of oxidative damage in plasma proteins and plasma lipids were determined in hepatitis C patients. Several studies show that hepatitis C is a disease, which involves increased levels of oxidative damage in the liver [53-55]. However, no changes in the activity of the enzymes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in red blood cells, or in plasma MDA or AAS was observed in these patients [56]. It has been suggested that standard blood oxidative stress markers probably do not accurately reflect intrahepatic oxidative stress [57]. This may also be the case for oxidative damage in other tissues.

There are also limitations in the analytical methods. In the studies presented in this thesis, MDA was separated from other thiobarbituric acid reactive substances by HPLC. This gives an increase in the specificity but it does not solve the fundamental problem with the assay. One of the steps in the assay includes heating to 96 °C. At this temperature lipid oxidation may occur giving large

uncertainties in the determination. The assay may therefore only be able to measure very large differences in lipid peroxidation levels. There have also been large differences in the measurement of DNA determined as 8-oxodexyguanosine (8-oxodG) between different laboratories. These problems were reduced substantially following the work of The European Standards Committee on Oxidative DNA Damage (ESCODD), which was set up in 1997 to resolve methodological problems and to reach agreement on the basal level of 8-oxo-2'-deoxyguanosine (8-oxodG) in biological samples [58-62]. Only few assays exist to measure protein oxidation. The HPLC method used to measure AAS in this thesis may be considered as one of the most reliable because the assay does not allow the measurement of oxidation products created after the first step in the assay.

In conclusion, the studies presented in this thesis do not indicate an increase in oxidative damage in rats given simple carbohydrates. However, other studies indicate an increased level of oxidative damage locally as well as systemically in animals given sucrose. As the literature gives some evidence for an increase in oxidative damage in animals given sucrose and the measurement does not seem to be very sensitive it can not be excluded that high intakes of sucrose induce oxidative damage.

Insulin resistance

Insulin resistance is defined as impaired biological response to the action of insulin and is characterised by compensatory hyperinsulinemia [63]. The risk factors for colon cancer and insulin resistance are remarkably similar and include a typical Western diet, obesity, physical inactivity etc. [64;65]. Several epidemiological studies support an association between insulin resistance and colon cancer [64-67]. Also variables associated with increased insulin resistance are associated with increased risk of death from colorectal cancer [68-70]. The excess mortality from colon cancer in patients with type-2 diabetes supports this association [71;72]. In animal studies, injection with insulin increases the number of tumours in chemically initiated rats [73]. Insulin and proinsulin also increase the growth of ACF in azoxymethane initiated rats indicating a tumor promoting effect of insulin [74] [73;75].

In rodents, a high dose of sucrose (>60 % of total energy) or fructose (>35 % of total energy), has consistently decreased insulin sensitivity, and increased fasting plasma insulin levels [76-78], whereas dosing with glucose did not impair insulin sensitivity [79].

Insulin resistance exposes colonocytes over prolonged periods to hyperinsulinemia, hyperglycemia, elevated levels of triglycerides, nonesterified fatty acids, and insulin-like growth factor-1 (IGF-1) [63]. The increased level of free IGF-1 is partly caused by a decrease in the IGF binding proteins IGFBP's. IGFBP's binds IGF, which inhibit the binding of IGF-1 to IGF receptors. Binding leads to increased proliferation, an effect which is increased by the decreased level of IGFBP-3 [63]. Indeed, studies have shown that subjects with high IGF-1 and low IGFBP-3 have increased risk for colon cancer [8]. An attempt was made to measure IGF-1 using a IGF-1 kit but the quality of the data was too low.

As the plasma insulin concentration is fluctuating, one insulin measurement is not sufficient to give clear conclusions regarding the mean plasma insulin concentration over a longer period.



Figure 4.1 The structure of proinsulin. The C-peptide (depicted as open circles) is released in the final stage of insulin synthesis. This peptide is stable in serum and is a valid indicator of insulin production [80].

In the final step of the bio-synthesis of insulin from proinsulin, C-peptide is released from proinsulin (see figure 4.1). This peptide is more stable in plasma than insulin, and it is considered a valid indicator for insulin production [80]. The serum concentration of C-peptide was determined in paper 2 and although mean C-peptide concentrations were 22-35 % higher in the groups of animals given simple carbohydrates, the increase was not statistically significant compared to the control group.

As mentioned in chapter 3 (see table 3.1), the cell proliferation in the colon was measured but no consistent increase was found.

Finally, a metabonomic study was performed on plasma and urine in paper 2. The most notable results from this analysis with regard to insulin resistance was an increase in the plasma concentration of triglycerides and very low density lipoprotein (VLDL). High plasma triglyceride levels as well as high levels of VLDL (rats) or LDL (humans) concentrations is a risk factor for diabetes [1;71;79;81-84]. The increase was largest in animals given fructose, lower in rats given sucrose, and lowest in rats given glucose, indicating that the increase is caused almost entirely by fructose.

Two epidemiological studies indicate that proinsulin, and thereby also insulin, is a risk factor for colon cancer in humans [69;85]. In rat experiments, proinsulin stimulates growth of intestinal crypt cells through specific binding and may play a physiological role in the regulation of intestinal epithelial cell proliferation [86]. Determination of proinsulin would therefore have been particularly interesting in studies 1-3, and should be determined in future studies.

In conclusion, the increased level of triglycerides and VLDL may be a weak indication that insulin resistance is induced in animals given simple carbohydrates. But since this effect seems to be related only to intake of fructose and the increase in the level of bulky DNA adducts and mutation frequency was found in all animals given simple carbohydrate it is unlikely that insulin resistance is involved in a genotoxic mechanism. The lack of effect on other markers related to insulin resistance support this.. I am aware that there exist more sensitive markers for determination of insulin resistance and that this may explain the differences between the results in these experiments and the literature.

Fermentation of complex carbohydrates

The aim of this part of the work was to investigate differences in the composition or in the activity of the microbial flora in rats given cornstarch, potato starch or sucrose in the feed and to investigate effect of sucrose on the chemical environment in the colon compared the two starches. This chapter will present a short introduction to this subject and also outline the results of the experiments presented in paper 2 and 3. In the three experiments presented in this thesis simple carbohydrate replaced either cornstarch or potato starch in the rat feed. The outcome of these experiments may therefore be caused by the lack of complex carbohydrate rather than the presence of simple carbohydrates. This gives 2 different approaches for the assessment of changes in colon as a consequence of ingested food or feed. The chemical conditions can be determined or the

composition of the microbiological flora may be determined. Both approaches have been used in this work.

Starches reaching the caecum and colon are fermentation substrates for the anaerobic microflora. This fermentation results in the formation of small organic acids, short chain fatty acids (SCFA). These acids account for approximately 90-95 % of the organic acids produced by the fermentation [87]. Cornstarch and potato starch are not considered as resistant carbohydrates although they are more resistant than simple carbohydrate. We know from other studies that potato starch, which is the more resistant of the two, can significantly affect caecum pH and caecum wet weight, indicating that at least this starch has resistant properties in our rat model [28]. Part of the administered potato starch may therefore reach colon affecting the chemical environment and the microflora.

The concentration of the acetic acid, propionic acid and butyric acid was measured in the rat caecum using capillary electrophoresis. The method is a modification of a method, which has been validated and used to determine the concentration of these substances in soil [88;89]. This work was an important part of these and is therefore described in further detail below. It is a common problem when working with capillary electrophoresis that the injection volumes may fluctuate considerable. This is usually solved using internal standards. In the analysis performed in paper 2 trichloroacetic acid was chosen as internal standard and added to caecum contents shortly after it was sampled from the rats. From a theoretical point of view it is an ideal internal standard as it is not produced in caecum, but it gave some analytical problems because it almost co-migrates with butyric acid. Sufficient separation was obtained by adding β -cyclodextrine to the sample prior to analysis and by diluting samples 60 times. The analytical inter day variation was determined to be about 2 % and the intra day variation to be less than 5 %. Before the experiment reported in paper 3 was performed the method was improved by changing the internal standard to malonic acid. Malonic acid, which was shown not to be present in the rat caecum in the experiment reported in paper 2. Malonic acid migrates fast and gives a very sharp peak.

The pH in caecum was determined using a microelectrode. The pH and probably also the concentration of SCFA vary in different parts of the caecum. This problem was solved by taking samples to SCFA analysis and to determine pH as near as possible to the place where caecum is connected to colon. It may be argued that it is not logical to use SCFA in caecum to determine the

condition in colon, but as the caecum content determines the availability in colon this is fully justified.

The results from the experiment performed in paper 3 shows that addition of 30 % fructose, sucrose or glucose in the feed decreases the concentration of acetic acid and propionic acid whereas the concentration of butyric acid was unchanged (see figure 4.2). This is also reflected by the increase in pH from 5.5 ± 0.3 in the control rats to 6.7 ± 0.2 in the sucrose group, 6.8 ± 0.2 in glucose group and 6.7 ± 0.2 in rats given fructose.

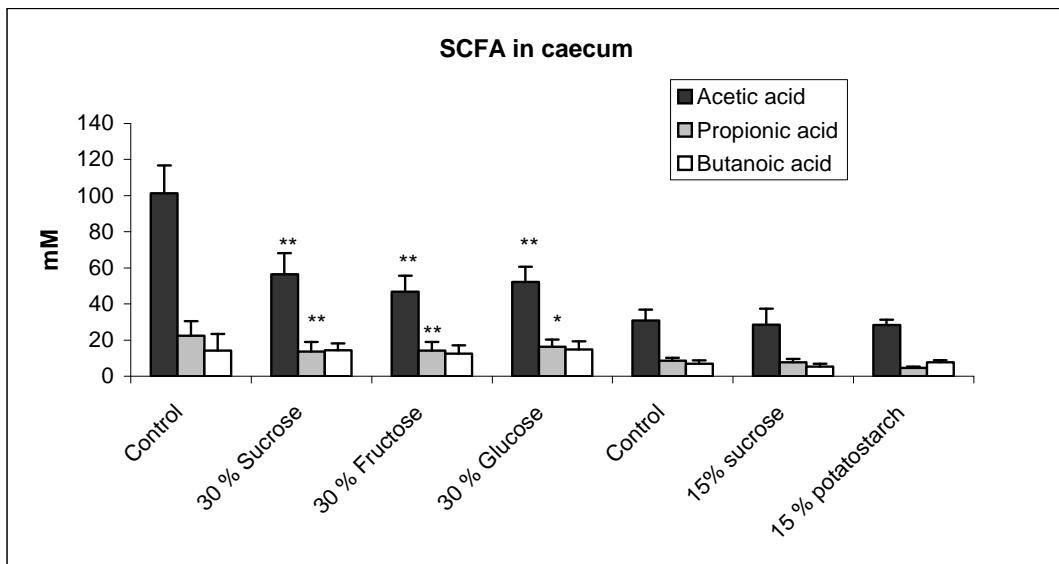


Figure 4.2. The concentration of SCFA in caecum in the experiments presented in paper 2 (first 5 groups) and paper 3. The concentration of acetic acid and propionic acid were decreased by simple carbohydrates whereas no effect was seen in paper 3 (3 last groups).
** $p < 0.05$, * $p < 0.01$

In paper 3 where the rats were given only 15 % of the carbohydrates, the changes in SCFA concentrations were not significant. The pH in caecum was increased from 6.5 ± 0.1 in the potato starch group to 6.8 ± 0.3 and 6.9 ± 0.2 in the cornstarch group (control) and the sucrose group respectively indicating only minor changes in the chemical composition in caecum. These very small changes in pH and SCFA concentrations in the caecum of rats given cornstarch or sucrose have subsequently been reproduced (unpublished data). The similarity in the concentration of SCFA in cornstarch and sucrose dosed animals is not unexpected because it is unlikely that the linear cornstarch will reach colon.

This has been confirmed in many experiments [90;91] and it is shown that the total amount and the profile of the SCFA can be altered by the consumption of different amounts and types of

carbohydrates [26;92;93]. The intestinal SCFA concentrations are highest where the bacterial flora is most abundant, which is in the caecum of rats. The formation of SCFA together with lactate causes a lowering of the pH. This has been observed repeatedly in rodents following administration of diets high in fiber, fructans or resistant starch [91;94;95]. A lowering of pH in colon is supposed to be beneficial as it creates a bacteriocidal environment for putative enteropathogens such as *Escherichia coli* and *Clostridium perfringens* [96]. A low pH is furthermore supposed to be beneficial as it inhibits the transformation of primary bile acids to secondary bile acid. Secondary bile acids may damage colon epithelium, cause hyperproliferation in the colon crypts, and possess toxic, comutagenic, and cocarcinogenic activity [97]. The influence of the microflora on development of colon cancer is far from being understood. Intake of complex carbohydrates in rodents are associated with an increase in the number of *Bifidobacteria* [90;98] indicating that intake of complex carbohydrate may alter composition and activity of the rat intestinal microbiota. There are many studies on the beneficial effects of increased growth of *bifidobacteria*. Higher *Bifidobacterium* population may result in changes in the microbial ecology of the colon that are detrimental to other anaerobic bacterial species[96]. The accepted mechanism by which bifidobacteria are thought to be inhibitory is related to the higher production of acetic and lactic acids during fermentation [90]. Furthermore, *bifidobacteria* stimulate immune function, particularly against malignant cells, produce B-complex vitamins, restore the normal intestinal flora during antibiotic therapy and reduce blood ammonia levels [99] A complete discussion of the effects of the microflora on colon is out of the scope of this thesis.

The SCFA are important substrates for the epithelial cells and/or intermediates in metabolism of the large intestine [100] and is also considered beneficial since they are able to increase the special glutathione transferase present in colon cells [101]. Butyrate is also inducing apoptosis in damaged colon cells [102;103]. However, there is some controversy about the chemopreventive effects of butyrate. When butyrate is given alone as slow-release pellets it does not modify azoxymethane induced intestinal carcinogenesis in rats [104]. It has been suggested that the chemopreventive benefits of butyrate depend in part on amount, time of exposure with respect to the tumorigenic process, and the type of fat in the diet [105]. Acetic acid and propionic acid serve as substrates for colonocytic lipogenesis [106] and are also important as energy supply to the cells. A decrease in the level of these substances may affect the permeability of the epithelial cell membrane

The effect on the microbial flora of giving rats a diet where 15 % potato starch or 15 % sucrose replaced cornstarch was determined in paper 3. A fingerprint of the composition of bacterial

population in faeces was determined by Denaturing Gradient Gel Electrophoresis of PCR-amplified rRNA genes (DNA-DGGE). These data gives only information the composition of the microbial population but no information on the activity of the different subpopulations. To this end profiles based on amplified ribosomal RNA sequences (RNA-DGGE) were determined. The DNA-DGGE profiles were different between the three groups indicating differences in the fingerprint of the composition of the bacterial population. There were no differences in RNA-DGGE profiles between the sucrose-based diet and the cornstarch-based control diet but the RNA-DGGE profiles in the potato starch-based diet was different from the other two. The result from the DNA-DGGE profiles indicates that although cornstarch is not resistant to digestion in the small intestine, a part of it may escape digestion and reach the bacterial population in the large bowel. Cornstarch consists uniquely of glucose subunits while the subunits of sucrose is glucose and fructose, and it can be speculated that the presence of fructose stimulates gut bacteria other than those stimulated by glucose. Animals given potato starch show differences in the composition as well as in the activity of in the micro flora.

In conclusion, compared to potato starch and cornstarch, the simple carbohydrates induce a different effect on the chemical composition of the caecum contents and on the composition and activity of the microflora.

Advanced Glycation End products

The formation of advanced glycation end products (AGE) is the results of chemical reactions between the amino group in a macromolecule like DNA or a protein and the aldehyde group in a simple carbohydrate or one of its metabolites. Reaction between DNA and aldehydes might explain the formation of bulky DNA adducts in rats given simple carbohydrates and is therefore of potential importance. Two well known reaction products between aldehydes and protein was synthesised and isolated but it was not possible to detect them in the biological samples. As the results of this work have not been published elsewhere it will be described in details in appendix. The aim of the theoretical part of this section is to provide sufficient background information on AGE to discuss the relevance in relation to the genotoxicity of simple carbohydrates. To this end it is important to understand: The processes of formation of AGE, the dietary sources of AGE and the bioavailability of AGE..

Formation of AGE

The class of reactions leading to formation of AGE is named Maillard reactions after a French chemist. Maillard reactions were already described in 1912 [107]. Maillard products are formed in foods during heating [108] but also *in vivo* where the reaction occurs slowly with normal aging and at an accelerated rate in diabetic patients [109]. Different pathways in the formation of AGE are illustrated in figure 4.3 and will be described below. As the figure shows there are different pathways, which all result in the formation of the important intermediates, the small reactive molecules glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG)(for chemical structure, see figure 4.4) followed by the formation the complex AGE [110]. In the pathway illustrated in the right side of figure 4.3, the first reversible step is the reaction between an amino group and the carbon atom, which binds the keto oxygen resulting in the formation of a Schiff base. This step is reversible, which means that the Schiff base may hydrolyse and disassociate if the concentration of one of the reactants is lowered. A re-arrangement may follow resulting in the formation of Amadori products. This is a slow process, but the equilibrium for the reaction is shifted towards the Amadori product. For instance, a high level of plasma glucose, hyperglycaemia, results in a slow but steady build-up of Amadori products. These steps are well understood and they can happen endogenously as well as exogenously for example during heating of food. The Amadori product may either rearrange in several less well described steps to the final AGE or they may release the intermediates mentioned above in degradation reactions. The left arrows from glucose in figure 4.3 shows a pure endogenous process. If the normal metabolism of sugar is overloaded or inhibited, glucose may be

metabolised through the polyol pathway, which results in the build-up of fructose. This may result in the formation of the reactive intermediates via fragmentation of triose phosphates or via catabolism of ketone bodies. Finally glyoxal might be formed during lipid peroxidation reactions, as indicated on the left side of figure 4.3.

These different pathways in the formation of AGE show that there are several different sources of AGE. The pathways include internal as well as external sources.

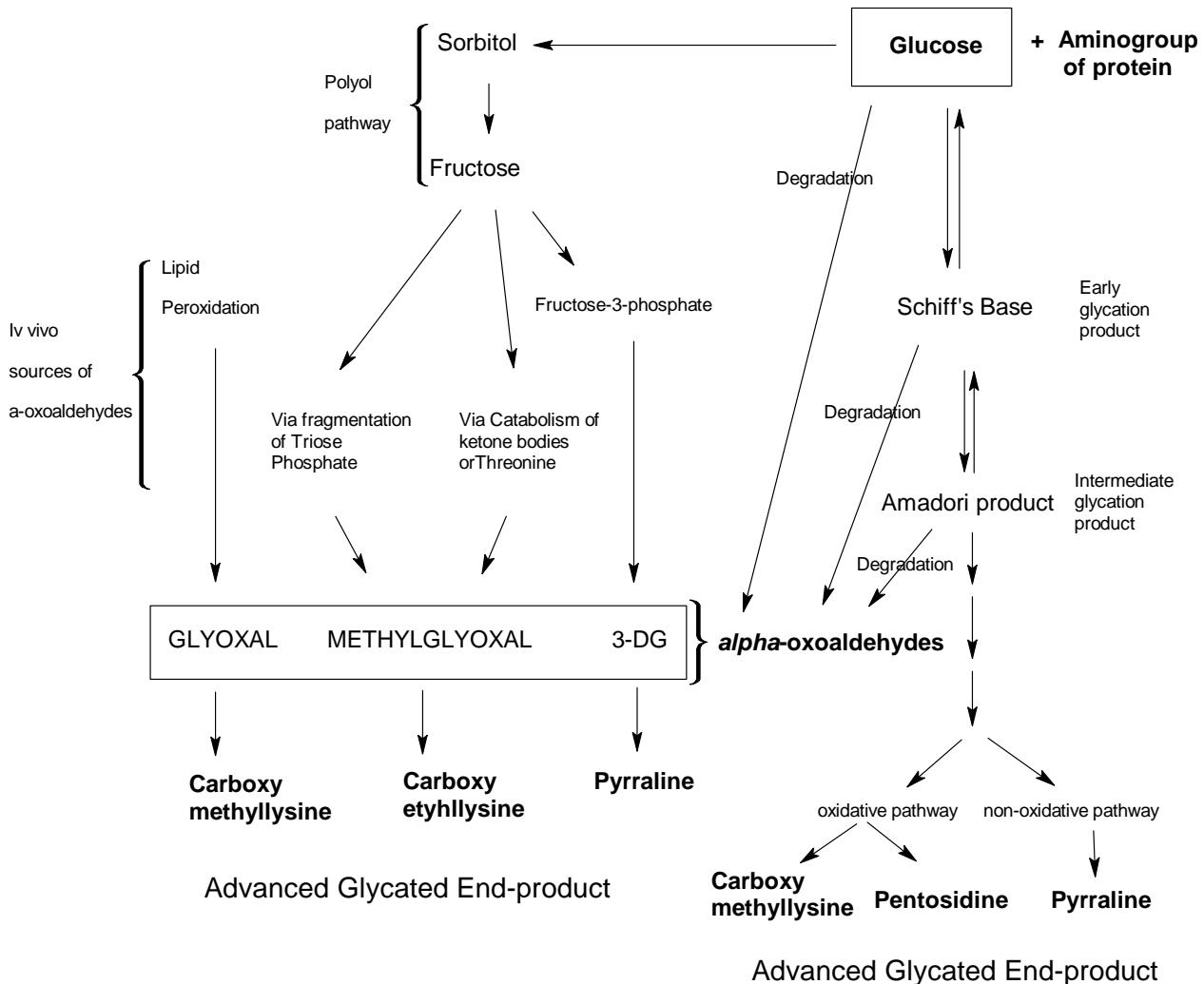


Figure 4.3 Formation AGE. The figure illustrates that there are several possible routes for the formation of AGE's. Glucose may either react directly with amino groups in protein or it may be degraded to the very reactive α -oxoaldehydes, which then react with amino groups in proteins. The relative importance of the pathways is presently not known. The left part of the figure shows that oxidative pathways are sources of the formation of glyoxal and thereby also sources for the formation of some of the AGE's. The three AGE's are only examples. More than 15 different AGE's have been identified. (Figure modified from Singh et. al., 2001 [110])

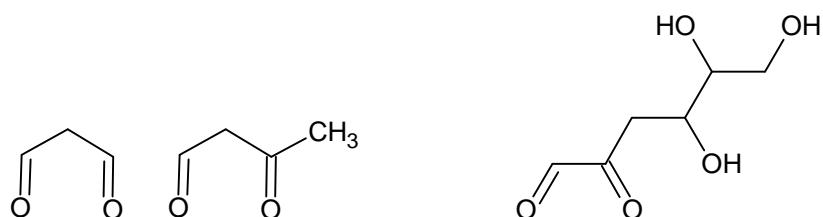


Figure 4.4 Chemical structure of the very reactive oxoaldehydes glyoxal, methylglyoxal and 3-deoxyglucosone

Dietary sources and bioavailability

The concentration of several different AGE's have been determined in different food sources and the highest concentration is found in heated food [111]. As an example carboxymethyllysine has been determined in milk and cola. In raw milk the concentration was 337 ± 94 nM increasing to 877 ± 94 nM in pasteurised milk (heated to 63 oC for 30 min) and further increasing to 2066 ± 497 nM in sterilised milk (heated at 115 oC for 15) [112]. In contrast there was only found $0,34 \pm 0,10$ nM carboxymethyllysine in cola [112] probably from caramel containing food additives. The concentration of carboxymethyllysine has been determined in 250 food items representing foods and culinary techniques typical of a multiethnic urban population [111]. The food items were divided into groups with high concentrations of fats, proteins or carbohydrates, respectively. The lowest concentration of AGE was found in food items with high concentration of carbohydrates. Only heated carbohydrates contain significant amount of AGE. These studies indicate that dietary AGE can be a major source of the circulating AGE.

Based on two studies the bioavailability of dietary AGE's has been estimated to about 10 % [108;113]. The renal excretion of dietary AGE in normal individuals is about 30 % in 48 hours and in diabetes patients only 5 % [108] indicating a build-up of dietary AGE's in organs. It was concluded that diet-derived AGEs are absorbed into the bloodstream representing a major source of chemically and biologically active toxins. If humans are given a diet with a very low concentration of AGE the excretion decreases [114] indicating that a significant part of circulating age is of dietary origin.

In a previous chapter it was mentioned that sucrose in some studies have induced more marked effects when it was given in a bolus. Hyperglycaemia is supposed to be responsible for the formation of AGE in diabetic patient ([115]). It could be speculated that a high intake of simple carbohydrate may result in an increased plasma concentration of simple carbohydrates affecting the concentration of AGE in blood or other compartments. One experiment has been performed in mice indicating that long-term administration of simple carbohydrates does not change glycemic stress measured as glycated hemoglobin, serum glucose or fructosamine levels [116].

It has previously been mentioned that sucrose has larger effect when it is given in bolus. This could lead to the suggestion that the polyol pathway in the formation of AGE may be involved in these effects. Although AGE is in general very stable compounds the intermediates in their formation, the α -oxoaldehydes glyoxal, methylglyoxal are reactive and, as mentioned earlier, known to be

genotoxic *in vitro*[19;20], whereas 3-deoxyglycosone has not been tested. These substances among other dialdehydes are possible reagents in the formation of the DNA adducts. However, as the ^{32}P assay is optimised for larger adducts it is unlikely that adducts are the results of single reactions between proteins and small aldehydes like glyoxal or methyl glyoxal. It is possible that polymerisation reactions or addition of other chemicals to free keto group in the dialdehydes may result in more bulky adducts. To elucidate this it is important to identify the precise chemical structure of the bulkys adducts induced by high intake of simple carbohydrates in rats.

Chapter 5

Conclusion and perspective

The aim of this thesis was to suggest biological pathways and biological factors involved in the genotoxicity and possible carcinogenicity of sucrose and other simple carbohydrates in the rat colon. A brief review of the epidemiological studies indicated that an association between high intakes of sucrose and colon cancer in humans cannot be ruled out.

Four biological factors were suggested in this thesis to be involved in sucrose mutagenicity: Oxidative stress, induction of insulin resistance, changes in the microbial flora in colon and formation of AGE. The possible influence of simple carbohydrates in the induction of colon cancer was also discussed. There was no clear evidence for any of the biological factors. None of the measured markers for oxidative stress was changed; therefore it was concluded that oxidative stress is probable not an important factor in the genotoxicity of simple carbohydrates. This is supported by paper 1 where there a lack of synergy between the genotoxic pro-oxidant IQ and sucrose was observed. Few of the markers related to insulin resistance were affected, indicating that it is also unlikely that insulin resistance is important in the genotoxicity of simple carbohydrates. Changes in the microbial flora in the colon were observed as changes in the prevalence and in the activity of certain groups of bacteria in rats given potato starch and sucrose. These changes were reflected in changes in the chemical environment in the colon where increased pH and a lower production of SCFA were observed as a consequence of high intake of sucrose. The DNA and RNA profiles of the microbial flora did not give any information on the quality of these changes. More studies on sucrose and also the other simple carbohydrates on the microbial composition are warranted to elucidate this. It was speculated that the more resistant starches favour the growth of beneficial microbial species or that sucrose increases the growth of deleterious species in the upper part of the intestine and that these are transported to and multiplied in the colon. The major part of the epithelial colon cells supply of energy and nutrients used to synthesise cell membranes come from the lumen. Acetic acid and propionic acid are important in these processes. It might be speculated that the decrease in these substances result in less optimal conditions of the colon epithelial cell metabolism and that lack of building blocks for the build-up of cell membrane makes the cells more prone to chemical modifications. Also in this area more studies are warranted. It could be interesting to investigate this in an experiment where Big Blue® rats are given either high doses of cornstarch (control), high doses of sucrose or sucrose combined with slow release tablets containing acetic acid and propionic acid. This experiment could elucidate the influence of these acids on

mutagenesis. The results indicate that the genotoxicity of simple carbohydrates is most probably caused by changes in the lumen rather than by systemic changes. The absence of systemic oxidative stress and the weak support for insulin resistance support this.

This project gave no information on the role of AGE in the process. From a theoretical point of view this process seems very plausible because reactive aldehydes are formed, which are known to form adducts with DNA. To test the hypothesis that AGE is included it is necessary to identify the chemical composition of the DNA adducts formed in the colon of rats given high doses of sucrose in the feed. The bulky adducts found in the experiments presented in this thesis are probably not the simple reaction product between a nucleotide and an aldehyde because the ^{32}P postlabelling assay was optimised to measure larger adducts. It could be more complex products like polymerisation products or cross-binding with peptides. It would be interesting to identify the result of an *in vitro* reaction between DNA and some of these reactive aldehydes and compare the products with adducts found in the colon of rats given sucrose or other simple carbohydrates. Two important factors make it difficult to measure changes in the concentration of AGE caused by high intake of simple carbohydrates. The existence of bioavailable dietary AGE and the fact that the concentration of glucose is controlled by excretion of insulin.

This theory is complicated by the fact that endogenous formation of AGE depends on the blood glucose level. Insulin secretion rather than intake of glucose control the level of blood glucose. The

In conclusion, at this point it seems more likely that the genotoxic effect of simple carbohydrate is caused by changes in the lumen than systemic effects.

The other part of this thesis concerns the stages in the carcinogenic process, which might be affected by dietary sucrose. The genotoxic effect found in these studies indicates that sucrose worked in the Big Blue® rat as an initiator. This is supported by studies, which show that sucrose increased the number of small ACF in the colon of rats. There is a limited support for a promoting effect in the study, for instance by increased proliferation. This indicates that sucrose could be a complete but very weak carcinogen. Life time studies in rodents, or in animal models more prone to colon cancer, where sucrose is fed throughout by bolus doses might be a way to elucidate this.

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Appendix

Preparation and isolation of selected AGE's

An attempt was been made to test changes in the plasma or red blood cell concentrations of some of these AGE's as a result of high intake of sucrose in rats. The chemical synthesis or the purification of most of these products is complex. The aim was experiment was first to synthesize and purify selected AGE's. It was hoped that it would be possible to measure these products in plasma or red blood cells in rat given high doses of sucrose. Pyrraline and pentosidine were successfully synthesised and isolated.

Synthesis of pyrraline

The synthesis of pyrraline (figure A.1) was a little more complicated than expected because it was very difficult to obtain the product by the procedure of Henle and Bachmann [117]. Some steps seem to be very delicate. The problem was solved with some modifications of the published procedure. The most important modification was the introduction of an extra purification step in the synthesis of the intermediate 3-deoxyglucolose. The chemical synthesis contains 4 steps and the following procedure was used. All chemicals were from Aldrich if other is not stated.

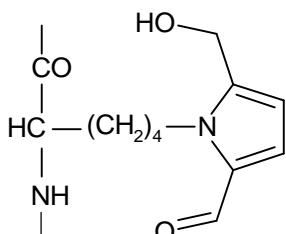


Figure A.1 Chemical structure of pyrraline

1. Synthesis of 3-deoxyglucosulose-benzoylhydrazone:

Amounts of 10 g glucose and 5,5 g *p*-toluidine were suspended in a mixture of 225 ml 96 % ethanol and 11 ml acetic acid. The mixture was boiled under reflux for 30min whilst being stirred continuously. To the solution, 16,5 g benzoylhydrazine was added, and the reflux was continued for 7 h. After cooling slowly to room temperature under continued stirring, the solution was kept at -20 °C overnight. 3-deoxyglucosulose-benzoylhydrazone was collected by filtration, washed with three portions of 20 ml ice-cold ethanol and diethylether, and dried on air. The yield was 10.6 g.

2. Synthesis of 3-deoxyglucolose

To a suspension of 10 g 3-deoxyglucosulose-benzoylhydrazone in 300 ml 96 % ethanol, 500 ml distilled water, 22 ml acetic acid, and 16 ml benzaldehyde were added. Under continuous stirring the mixture was boiled under reflux for 2 h. Then, the ethanol was evaporated while simultaneously 500 ml water was added within 30 min. The solution was cooled in an ice bath and precipitated benzaldehydebenzoylhydrazone was removed by filtration. After adding 60 g ion-exchanger Serdolyt MB2 (mixed-bed H⁺/OH⁻ Serva, Heidelberg, Germany) to the filtrate, the mixture was stirred in the dark for 15 min and filtered. The filtrate was concentrated under reduced pressure to about 100 ml, washed 6 times with 50 ml diethylether and evaporated to about 6 ml. The resulting syrup was dissolved in 50 ml water and added to a bond elut coloum (MEGA BE –C18 60 ml, Varian, Harbor city, USA). The column was eluted with 150 ml methanol. The methanol eluate was evaporated *in vacuo* at room temperature. The resulting yellow syrup was dissolved in a mixture of 5 ml water and 50 ml 96 % ethanol. After adding 10 g serdolyt MB2 and stirring in the dark for 10 min the solution was filtered to remove the ion exchanger, evaporated to about 5 ml, frozen and freeze-dried to give an amorphous yellow powder of crude 3-deoxyglucosulose. The yield was 3.1 g.

3. synthesis of 6-(2-formyl-5-hydroxymethyl-1-pyrrol)-2-(N-t-butyloxy-carbonyl-amino)-hexanoic acid (N^{α} -Boc-pyrraline)

Amounts of 738 mg (3mmol) Boc-Lys-OH and 2400 mg (\approx 13 mmol) 3-deoxyglucosulose were dissolved in 250 ml 0.1 M sodium acetate, pH 5.0. To the solution, 1.2 g cellulose (100 μ m particle size) was added and the suspension was freeze dried. The dry mixture was powdered and heated for 2 h at 70 °C in a drying oven. After cooling to room temperature, the brown powder was extracted five times, each time with 120 ml 96% ethanol. The pooled ethanol extracts were evaporated *in vacuo* at room temperature. The oily dark brown residue was dissolved in 20 ml water, membrane filtered and evaporated under vacuum at room temperature.

4. Synthesis of pyrraline

A solution of 274 mg (660 μ mol) N^{α} -Boc-pyrraline in 100 ml of 10 % acetic acid was heated for 4 h at 70 °C whilst being continuously stirred. After cooling to room temperature, 100 ml water was added and the solution was freeze-dried.

The identity of the product was verified using HPLC with a DAD as well as a MS detector. The chromatograms are illustrated in figure A.2. The spectrum of the UV signal with a peak at 7.005 min is identified as pyrraline (see figure A.3). The identity was verified by the MS spectrum of the

correspondent signal at 7.034 in the chromatogram from the mass detector (parent ion 255.2). In the chromatogram from the DAD signal there are other signals indicating that the product is not clean. The separation of the signals indicates that it is easy to purify this product further by changing the condition for the preparative HPLC runs. This was not done because it became obvious that the substance could not be analysed by quadropole LC/MC (see below).

Protein from plasma and red blood cells from rats given 30 % sucrose in the diet was isolated.

Pyralline was released from the protein by either enzymatic digestion as or by hydrolysis in hydrochloric acid. The analysis was performed on a quadropole LC-MS but no signal was obtained. Also protein digested from plasma and red blood cells from diabetes patients was analysed. Patients suffering from diabetes are known to have increased levels of pyrraline but since it was not possible to measure pyrraline in these samples it was decided that the sensitivity of the instrument was far too low to perform these analysis. There is a need for tandem mass spectrometry or an ion trap to analyse pyrraline in biological samples.

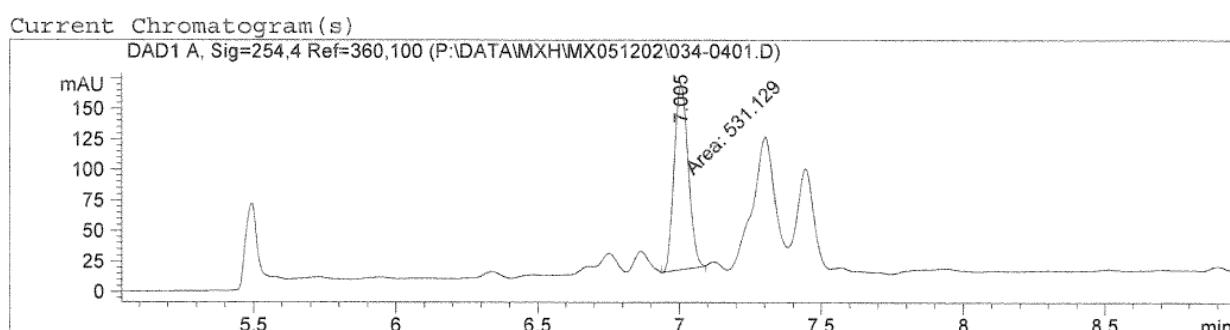


Figure A.2a The chromatogram from the DAD detector of the final synthesis product of pyrraline. The peak at 7.005 is identified as pyrraline by its UV spectrum. The other peaks in the chromatogram are impurities.

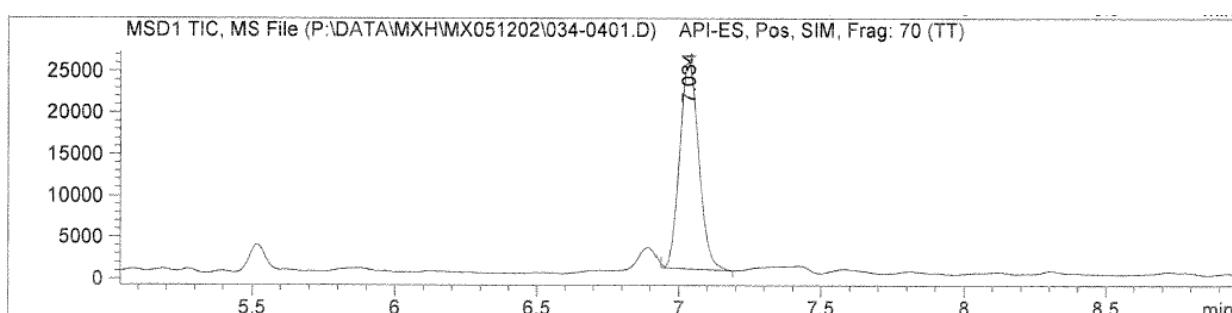


Figure A.2b The chromatogram from the MS detector of the final synthesis product of pyrraline. The identity of the peak was determined by the mass spectrum which was in accordance with the mass given in the literature.

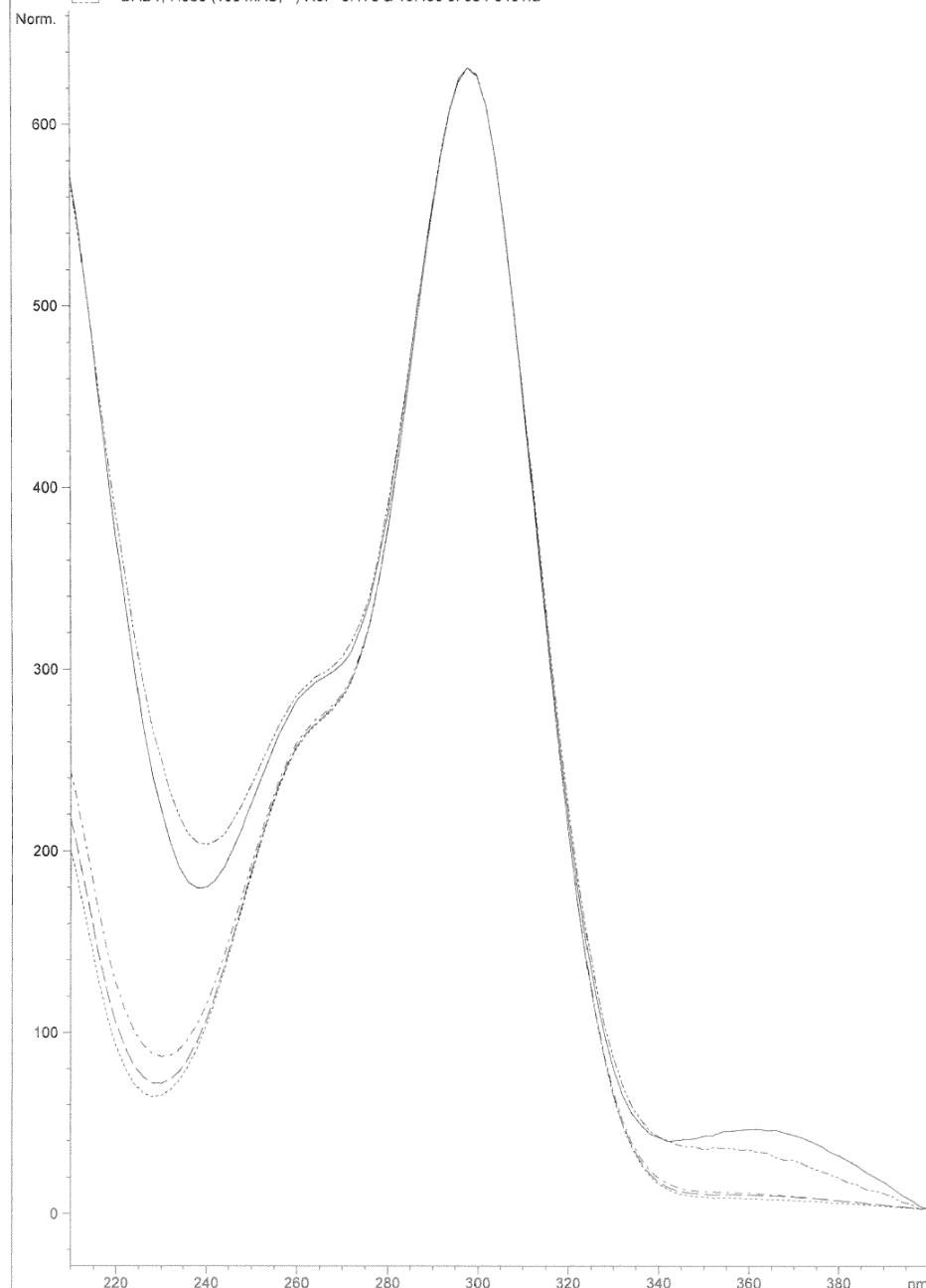
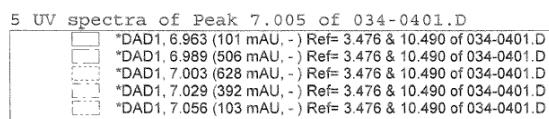


Figure A.3. UV spectrum of the signal at 7.005 in the DAD chromatogram of pyrraline. The maximum absorbans is at 298 nm where the litterature indicate a maximum absorbans at 297nm (Henle and Bachmann [117]). The shoulder at 265 nm is also important in the identification of this substance.

Preparation of pentosidine

Pentosidine (see figure A.4) was prepared according to a procedure described by Henle et. al. [118]. The purification was done by preparative HPLC using a standard C18 column.

3243 mg (15 mmol) Na-acetylarginine and 941 mg (5 mmol) Na-acetylysine were dissolved in 100 ml 0.2 N phosphate buffer, pH 9. The solution was heated at 65 °C for 48 h under continuous stirring. During incubation, 10 portions (75 mg each) of ribose were added to the reaction mixture periodically. After each addition of ribose, the pH was adjusted to 9.0. After incubation, 30 ml 12 N HCl were added and the mixture was heated at 110 °C for 2 h. The HCl was then removed under reduced pressure at 60 °C. The dried residue was dissolved in 20 ml water. Preparative HPLC using a Dionex Acclaim® 120 column (C18, 5 µm, 120 Å 4.6 x 250 mm), was performed to purify the product.

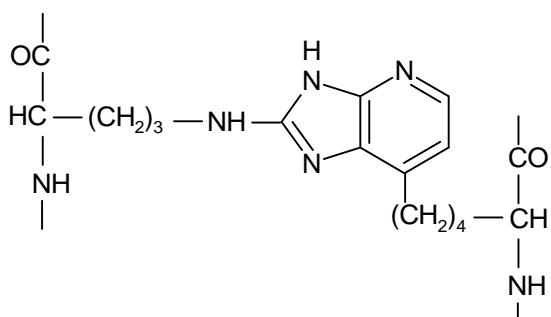


Figure A.4 Chemical structure of pentosidine

Also the final product in the preparation of pentosidine was verified using HPLC with a DAD as well as a MS detector. The chromatograms are illustrated in figure A.5. The spectrum of the UV signal with a peak at 5.482 min is identified as pentosidine (see figure A.6). The identity is verified by the MS spectrum of the corresponding signal at 5.511 min in the chromatogram from the mass detector (parent ion 255.2). This product could be used as an internal standard in the analysis of pentosidine in biological samples. Unfortunately, it was not possible to identify any product with this retention time and mass from the biological samples.

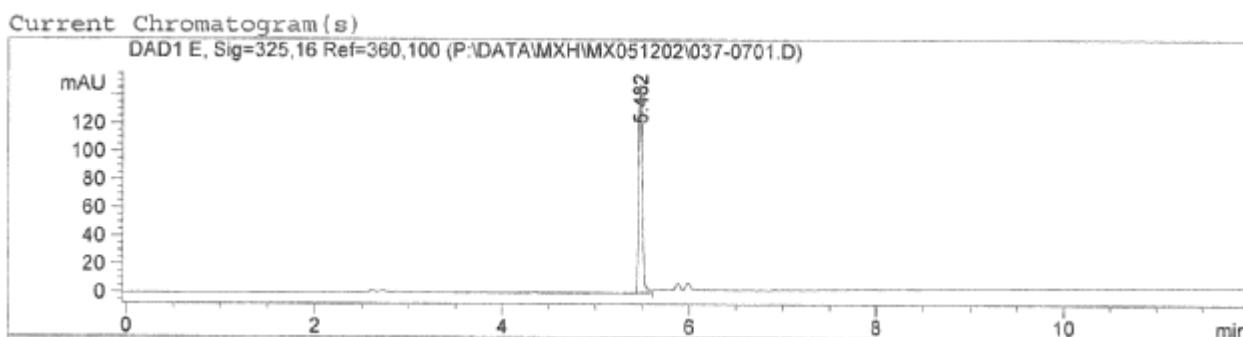


Figure A.5a The chromatogram from the DAD detector of the final product in the preparation of pentosidine. The peak at 5.482 is identified as pyrraline by its UV spectrum (see figure 3.9).

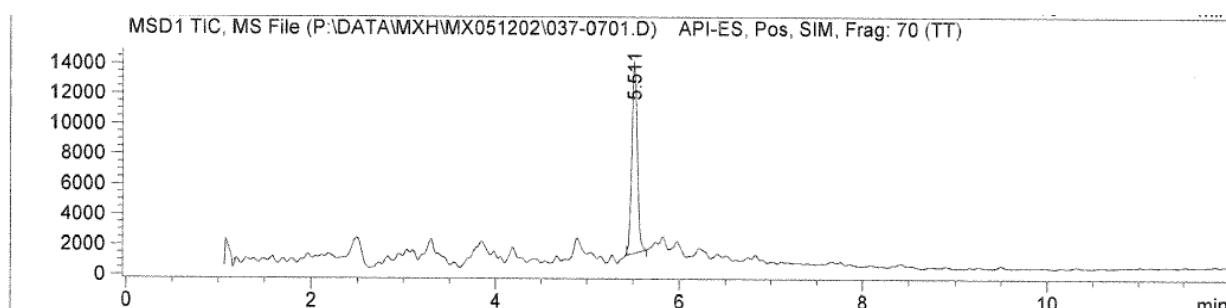


Figure A.5b The chromatogram from the MS detector of the final synthesis product of pentosidine. The identity of the peak was determined by the mass spectrum which was in accordance with the mass given in the literature.

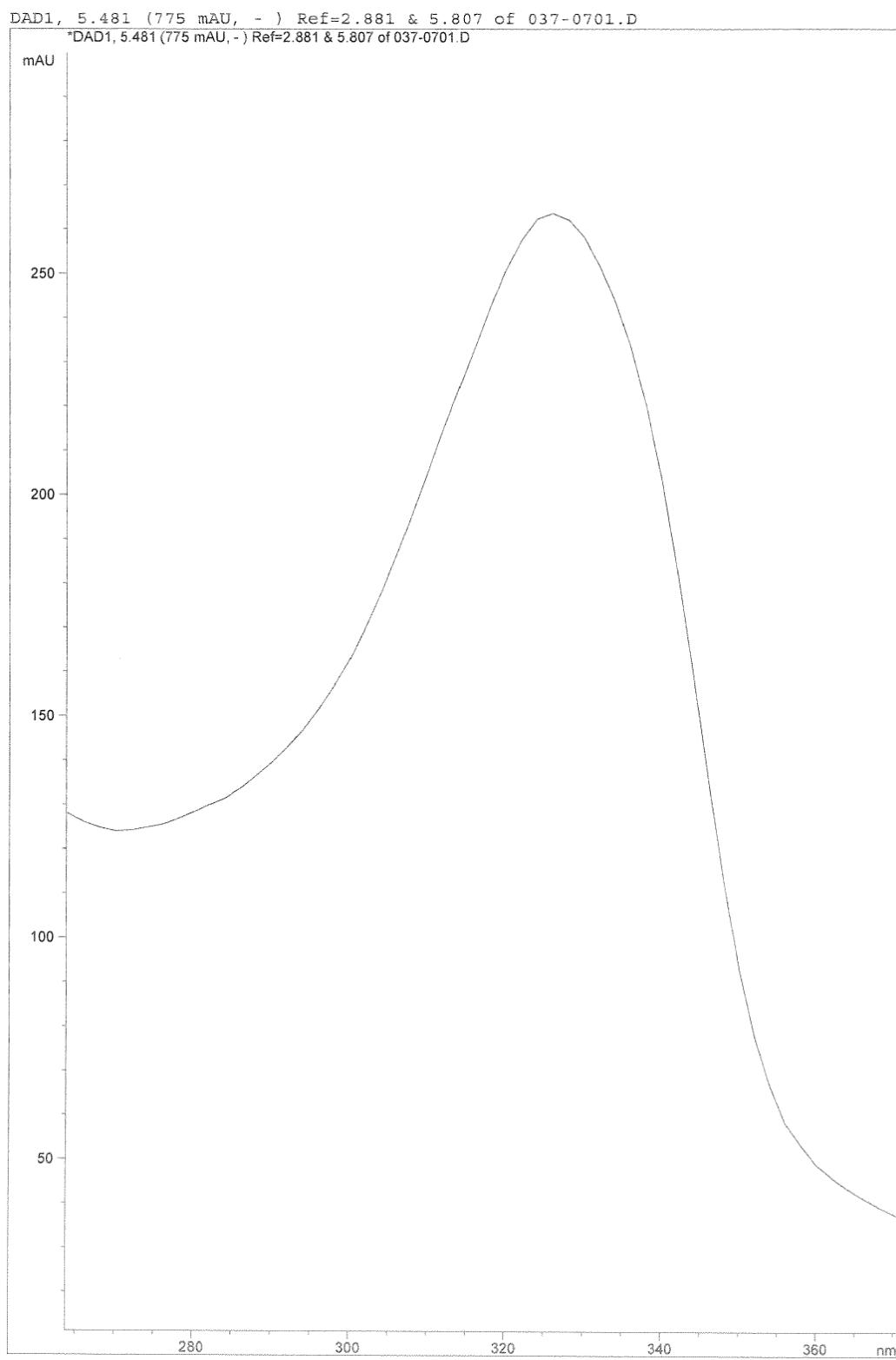


Figure A.6. UV spectrum of the signal at 5.481 in the chromagram of pentosidine. The maximum absorbance is at 326 nm. A maximum absorbance at 325nm has been published (Henle et al [118]).