

# Effect of Enzyme-resistant Starch on Formation of 1,*N*<sup>2</sup>-Propanodeoxyguanosine Adducts of *trans*-4-Hydroxy-2-nonenal and Cell Proliferation in the Colonic Mucosa of Healthy Volunteers<sup>1</sup>

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## Abstract

The effect of enzyme-resistant starch (RS) on the development of colon cancer was reported to include both chemopreventive activity in humans and tumorigenic activity in animals. A study was performed to detect the influence of enzyme-RS on lipid peroxidation-induced DNA damage and cell proliferation. During two 4-week periods, 12 volunteers consumed a controlled diet in which starchy foods were enriched with a highly resistant amylo maize starch (Hylon VII) in the high-RS period and with an available corn starch in the low-RS period (second period). At the end of each test period, biopsy specimens of the rectosigmoidal mucosa were obtained from each subject and analyzed for *trans*-4-hydroxy-2-nonenal-1,*N*<sup>2</sup>-propanodeoxyguanosine-3'-monophosphate adducts using a <sup>32</sup>P postlabeling assay, and cell proliferation was determined by bromodeoxyuridine labeling. The *trans*-4-hydroxy-2-nonenal-1,*N*<sup>2</sup>-propanodeoxyguanosine-3'-monophosphate adduct level of DNA from colonic mucosa of eight evaluated volunteers was significantly higher in the high-RS period (mean adducts/10<sup>7</sup> nucleotides ± SD, 3.83 ± 0.60) than in the low-RS period (2.69 ± 0.35; *P* < 0.05). There was no evidence for an increased cell proliferation in the upper crypt in the high-RS phase, compared with the low-RS phase. There are indications now that enzyme-RS induces oxidative stress that is not correlated with increased cell proliferation. If it is accepted that the formation of DNA adducts reflects oxidative stress, which in turn accelerates the process of carcinogenesis, then certain forms of RS

may have a tumor-enhancing effect rather than a tumor-protective effect.

## Introduction

There is convincing evidence that dietary factors play an important role in colonic carcinogenesis (1). Epidemiological studies show a reduction in risk for individuals and populations consuming high amounts of vegetables. The protective effect of vegetables may be due to their content of complex carbohydrates such as dietary fiber and starch (2). Whereas previous research has focused on dietary fiber as the predominant carbohydrate reaching the colon, it is now recognized that more starch than fiber may pass the ileocecal valve, even on an average Western-style diet (3).

Due to the lack of food composition data, the epidemiological evidence relating enzyme-RS<sup>4</sup> with cancer risk is currently insufficient. Whereas an inverse association between starch consumption and large bowel cancer incidence has been found in an international comparison in 12 populations worldwide (4), Italian authors have reported an increased cancer risk with high-starch intake (5, 6). Experimental studies concerning starch and colon cancer are similarly inconsistent. In animal models of colonic carcinogenesis (dimethylhydrazine-induced or genetically determined tumorigenesis), enzyme-RS, which is only broken down by anaerobic bacteria in fermentation processes, did not alter the number of tumors/rat (7, 8). In experimental animals, the number of aberrant crypt foci was elevated (9), unchanged (10), or diminished (11) by consumption of RS. Indirect evidence for a protective role of starch was presented in human intervention studies; the fermentation of starch increased the fecal concentration of *n*-butyrate (putative protective factor) and suppressed the conversion of primary to secondary bile acids (putative detrimental factor; Refs. 12 and 13).

In this intervention study (high-RS versus low-RS diet), two intermediate biomarkers of cancer risk were investigated in the rectal mucosa of healthy volunteers: (a) the formation of propano adducts of HNE as a marker of oxidative stress; and (b) the kinetics of crypt proliferation. HNE is, beside MDA, the main product of lipid peroxidation (14), and it is formed by oxidation of  $\omega$ -6 polyunsaturated fatty acids such as arachidonic acid or linoleic acid (15, 16). HNE is a specific marker for oxidative stress and is found in increased concentrations after postischemic reperfusion, glutathione depletion, and induction of lipid peroxidation (17–19). The cyclic DNA adducts

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<sup>4</sup> The abbreviations used are: RS, resistant starch; LI, labeling index; BrdUrd, bromodeoxyuridine; HNE, *trans*-4-hydroxy-2-nonenal; dGp, ; MDA, malondialdehyde; BME, Eagle's medium with Earle's salts, FCS, and antibiotic-antimycotic solution; NP1, nuclease P1; dGp = deoxyguanosine-3'-monophosphate.

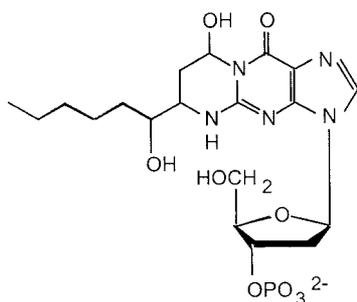


Fig. 1. Structure of the 1,*N*<sup>2</sup>-propanodeoxyguanosine adduct of HNE, a specific biomarker for lipid peroxidation-induced DNA damage.

that are formed either directly by HNE or during degradation of fatty acid peroxides are also assumed to be biomarkers for oxidative stress (20, 21). The exocyclic 1,*N*<sup>2</sup>-propanodeoxyguanosine adducts of HNE are highly specific markers for genotoxic interaction of lipid peroxidation products because the hydroxyhexyl side chain originating from Michael addition of HNE allows an unequivocal attribution of these adducts (Ref. 22; Fig. 1). We developed a new <sup>32</sup>P postlabeling method for the detection of HNE-dGp adducts that involves one-tube sample workup and two-directional TLC for adduct separation (23). Because 1,*N*<sup>2</sup>-propanodeoxyguanosine adducts in general highly contribute to the endogenous DNA damage in human and rodent tissues (23) and represent promutagenic DNA lesions (24–26), quantitation of HNE-dGp adducts was performed in human colon biopsy specimens to investigate DNA-related effects of enzyme-RS in this trial.

The second biomarker that has been studied in this trial is the proliferation kinetics within colonic crypts (27). Normal proliferation of colonocytes occurs in the basal 60% of the crypts. In the upper 40% of the crypts, however, proliferation stops, and colonocytes become fully differentiated, to be extruded after approximately 7 days of upward migration. The expansion of the proliferative compartment to the crypt surface (hyperproliferation, involving the upper 40% of the crypt length) is considered a preneoplastic biomarker and has been described in association with colorectal adenoma and carcinoma (28).

## Materials and Methods

**Subjects and Study Design.** Twelve healthy volunteers (5 females and 7 males; age, 25.2 ± 0.5 years; age range, 23–28 years) were recruited for this clinical trial. Volunteers did not receive any medication (including antibiotics or laxatives) 4 weeks before and during the study. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Wuerzburg (Wuerzburg, Germany). During two 4-week periods (with a washout phase of 6 weeks between runs), the volunteers consumed a controlled diet (percentage of energy intake: 47% carbohydrate, 38% fat, and 15% protein). Starchy foods (bread, pasta, cake, and biscuit) were enriched with a highly resistant amylo maize starch (Hylon VII; National Starch & Chemical Company, Bridgewater, NJ) in the high-RS period (first period) and with an available corn starch (Maizena; Knorr Caterplan GmbH, Heilbronn, Germany) in the low-RS period (low-RS period, second period). Women received 9,423 kJ/day, *i.e.*, 50.7 g RS/day during the high-RS period and approximately 2.8 g RS/day during the low-RS period. Men received

10,971 kJ/day, *i.e.*, 59.7 g RS/day during the high-RS period and approximately 3.2 g RS/day during the low-RS period.

An example of a day's menu included a breakfast of 120 g of toast, 10 g of butter, 25 g of jam, 2 slices of salami, 1 yogurt, and 100 ml of milk (3.5% fat). Lunch was supplied in the form of five different menus, *e.g.*, beef, pasta, and 200 ml of apple juice. Supper consisted of 120 g of bread, 10 g of margarine, 1 portion of cheese, and 1 portion of country-style liver sausage. Daily fiber content was restricted by 1 piece of fruit and 100 g of vegetable with either lunch or supper. Fifty g of cookies and 1 piece of cake were supplied daily between meals. Beverages included free mineral water, dietary soft drinks, coffee, and tea. Alcoholic beverages were restricted to 0.5 liter of beer or 0.25 liter of wine, respectively, per week. Refrigerated and portioned fast-food menus of highly steady compositions (*e.g.*, fatty acids and proteins) were used. All volunteers collected their food daily from the metabolic kitchen; volunteers ate meals at 8 and 10 a.m. and at 1, 4, and 7 p.m. Regular contact with the nutritionist (S. H.) in the study was important to ensure compliance. Additional details of this study are reported elsewhere (13).

At the end of each test period, four biopsy specimens of the rectosigmoidal mucosa (15 cm from the anal verge) were obtained from each subject with 3.4 mm forceps under endoscopic guidance with a flexible Olympus CF 20M rectoscope (Olympus, Hamburg, Germany). Rectosigmoidoscopy was always performed in the morning between 8 and 10 a.m. Bowel preparation was performed with tap water enemas, which have been found not to interfere with cell proliferation. Two of the samples were snap-frozen in liquid nitrogen, stored at –70°C, and transferred on dry ice to the Department of Toxicology for analysis of DNA adducts. The other two biopsy specimens were placed in BME culture (Life Technologies, Inc., Paisley, Scotland) for cell proliferation studies.

**Reagents for Postlabeling Analysis of DNA Adducts.** Micrococcal nuclease (168 milliunits/μg) from *Staphylococcus aureus* was provided by Sigma (Deisenhofen, Germany). Phosphodiesterase from calf spleen (spleen phosphodiesterase; 4 units/ml) was purchased from Boehringer Mannheim (Mannheim, Germany). NPI (7 units/μl) from *Penicillium citrinum* was bought from Fluka (Deisenhofen, Germany). Cloned T4 polynucleotide kinase (30 units/μl) was obtained from USB Amersham (Braunschweig, Germany), and [γ-<sup>32</sup>P]ATP (>7,000 Ci/mmol, 167 μCi/μl) was obtained from ICN (Eschwege, Germany).

**Instrumentation.** The sample DNA was quantitated on a Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). Thin-layer chromatograms of <sup>32</sup>P-labeled adducts were measured with an InstantImager (Packard, Meridan). A gas mixture of 1% isobutane and 2.5% carbon dioxide in argon was used for counting.

**Synthesis of HNE-dGp Adduct Standard.** The procedure for the synthesis, purification, and quantitation of adduct standard as a reference substance for the postlabeling assay has been described elsewhere (23).

**DNA Isolation and Quantitation.** Two biopsy specimens of colonic mucosa were obtained from each subject. The two specimens were pooled before the DNA isolation to yield enough DNA for the postlabeling assay. The DNA was isolated using the QIAamp kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). The concentration of the DNA was quantitated spectrophotometrically using the absorbance at 260 nm, and the purity of the DNA was determined by the absorbance ratios of  $A_{230\text{ nm}}:A_{260\text{ nm}}$  and  $A_{260\text{ nm}}:$

$A_{280\text{ nm}}$ , respectively. The colonic mucosa of the subjects (5–14 mg of tissue) yielded DNA amounts of 10–54  $\mu\text{g}/\text{sample}$ . The DNA preparation was found to be free of proteins and RNA, which was indicated by mean values of 0.43 for the  $A_{230\text{ nm}}:A_{260\text{ nm}}$  ratio and of 1.86 for the  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratio according to Ref. 29.

**DNA Hydrolysis and NP1 Treatment.** Polypropylene microtubes of 1.5 ml (Sarstedt, Nümbrecht, Germany) were used for the DNA hydrolysis and all subsequent steps. Each tube was prepared with 50 fmol of 1, $N^2$ -propanodeoxyguanosine-3'-monophosphate adducts of *trans*-2-hexenal as internal standard before the sample DNA was added. Samples of 10  $\mu\text{g}$  of DNA were incubated for 4 h at 37°C with 2.50  $\mu\text{l}$  of micrococcal nuclease solution (0.2 unit/ $\mu\text{l}$ ; 1  $\mu\text{g}/\mu\text{l}$ ) and 2.50  $\mu\text{l}$  of spleen phosphodiesterase solution (0.002 unit/ $\mu\text{l}$ ; 1  $\mu\text{g}/\mu\text{l}$ ) and 2  $\mu\text{l}$  of DNA digestion buffer [25 mM  $\text{CaCl}_2$  and 50 mM sodium succinate (pH 6.0)] in a total volume of 20  $\mu\text{l}$  (0.5  $\mu\text{g}/\mu\text{l}$  DNA hydrolysate). A volume of 6  $\mu\text{l}$  of NP1 mixture was added to the solution, consisting of 1.2  $\mu\text{l}$  (8.4 units) of NP1 solution, 1.8  $\mu\text{l}$  of 0.3 mM  $\text{ZnCl}_2$ , and 3  $\mu\text{l}$  of 250 mM sodium acetate (pH 5.0). The mixture was incubated for 45 min at 37°C, and the reaction was stopped by adding 2.4  $\mu\text{l}$  of 0.5 M Tris base. The solution was desiccated to dryness and redissolved in 10  $\mu\text{l}$  of water before the postlabeling reaction.

**$^{32}\text{P}$  Postlabeling of HNE-dGp Adducts.** A volume of 2.0  $\mu\text{l}$  of labeling mixture was added to the sample solution containing 10  $\mu\text{g}$  of NP1-enriched DNA. The labeling mix was made of 1.5  $\mu\text{l}$  of kinase buffer [100 mM DTT, 100 mM  $\text{MgCl}_2$ , 10 mM spermidine, and 400 mM bicine/NaOH (pH 9.5)], 0.3  $\mu\text{l}$  of 23  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (>7000 Ci/mmol; 1.9 MBq, 50  $\mu\text{Ci}$ , 6.9 pmol), and 0.2  $\mu\text{l}$  (6 units) of T4 polynucleotide kinase. The sample was incubated for 45 min at 37°C, and the reaction was stopped by application of the entire sample solution to a prewashed PEI-cellulose sheet (Macherey & Nagel, Düren, Germany). This sheet was developed in two directions for the determination of the adducts, as given under TLC conditions. From this chromatogram, the amount of HNE-dGp adducts/sample was determined.

**TLC Conditions.** Ammonium formate buffer (1.7 M; pH 3.5) was used for the development from bottom to top after attaching 4-cm wicks (Whatman No. 1) to 16  $\times$  20-cm (height  $\times$  width) prewashed PEI-cellulose sheets. The first 7 cm from the bottom of the plate and the wick at the top of the plate were excised after the first development and discarded. The plate was soaked in running water for 4 min, air dried, and turned around in an angle of 90° for chromatography in the next direction. The development from left to right was carried out in 2.7 M sodium phosphate buffer (pH 3.8) into a 6-cm wick (Whatman No. 1), which was excised and discarded after the development. The resulting plate was air dried. To ascertain that no other substance but HNE-dGp adducts is under this adduct spot, we have examined cochromatography of the substance isolated from this spot with the adduct standard by several chromatographic systems and have confirmed that the substance isolated from this spot consisted only of the HNE-dGp adducts (23).

**Quantitation of HNE-dGp Adducts.** Each chromatogram was visualized and counted by an InstantImager with an exposure time of 5–20 min. The relative counting error of a spot was <3% at the end of the counting period. The unspecific radioactive background was subtracted with background spots that were placed adjacent to the adduct spot. A template was saved for all further determinations. The net cpm value of the spot was given by the software. The mean value of all signals of internal standard was calculated, and all samples were adjusted to have

the same labeling efficiency. The HNE-dGp signals were quantitated by spiking amounts of 1–10 fmol of HNE-dGp adduct standard to several samples. The analytical variation of the method was <5% under these conditions.

**BrdUrd Immunohistochemistry.** Immediately after obtaining the biopsy specimens, they were immersed in BME (Life Technologies, Inc.) and then distributed on cell culture dishes containing 3 ml of BME. Then 200  $\mu\text{M}$  BrdUrd (Sigma, St. Louis, MO) and 20  $\mu\text{M}$  fluorodeoxyuridine (Sigma) were added to the reaction mixture, and the specimens were incubated for 2 h at 37°C in a modular incubating chamber (Billups-Rothenberg Inc., del Mar, CA), which was continuously gassed with carbogen gas (95% oxygen, 5% carbon dioxide) at 1 liter/min. During the incubation period, the culture dishes were gently rotated at 10 cpm on a rotary shaker (Heidolph, Kehlheim, Germany). The biopsy specimens were then fixed in Primafix (Camon, Wiesbaden, Germany), embedded in Para-Plast (Monoject Scientific, Athy, Ireland), and section-cut into 2- $\mu\text{m}$  slices using a Leitz microtome (Leitz, Wetzlar, Germany). Denaturation of DNA was achieved by incubation with 2 N HCl for 30 min. Mouse anti-BrdUrd (number 7580; Becton Dickinson, San Jose, CA) was applied as the first antibody (1: 1 00 dilution, 1-h incubation), followed by the second antibody biotinylated antimouse immunoglobulin (1:100 dilution, 30-min incubation; RPN 1001; Amersham, Little Chalfont, Buckinghamshire, England). After a 30-min incubation with biotinylated streptavidin (1:100; Amersham RPN 1051), BrdUrd-labeled cells were visualized using diaminobenzidine solution (Serva, Heidelberg, Germany) with 0.3 g of nickel chloride ( $\text{NiCl}_2$ ) and 0.3 g of cobalt chloride ( $\text{CoCl}_2$ ) as intensifier. Finally, the biopsy slides were counterstained with nuclear fast red.

The histological slides were viewed under a Laborlux S microscope (Leitz) at  $\times 625$  magnification. In each patient, proliferation of colonic crypt cells was evaluated by counting the number of BrdUrd-labeled cells and total number of cells in 20 longitudinally sectioned crypt columns according to the criteria set up by Lipkin *et al.* (27). An average LI per individual was calculated from the number of labeled cells divided by the total number of cells. The LI was computed for five different longitudinal crypt compartments (compartment 1, crypt base; compartment 5, crypt surface) to assess the distribution of labeled cells within the crypt. This calculation was also done for the crypt column as a whole (total crypt LI) and for the upper 40% of the crypt column (upper crypt LI). In addition, the  $\phi$ h value was assessed (number of labeled cells in the upper 40% of the crypt column divided by the number of labeled cells in the entire crypt column).

**Statistical Analysis.** According to the crossover study design, comparisons between the two test periods were made by nonparametric Wilcoxon's signed rank test for paired data. Formation of HNE-dGp adducts was compared with daily fecal excretion of MDA by linear regression. Comparisons between male and female volunteers were made by nonparametric Mann-Whitney *t* test for unpaired data. Values are given as mean  $\pm$  SD.  $P < 0.05$  was considered significant.

## Results

**HNE-dGp Adduct Levels after Receiving the High-RS and the Low-RS Diet.** Two biopsy specimens were obtained from the colon of each volunteer after both test periods and analyzed for the HNE-dGp adduct levels. Representative chromatograms of those that were evaluated show the HNE-dGp adducts of a male volunteer after receiving the high-RS diet and the low-RS

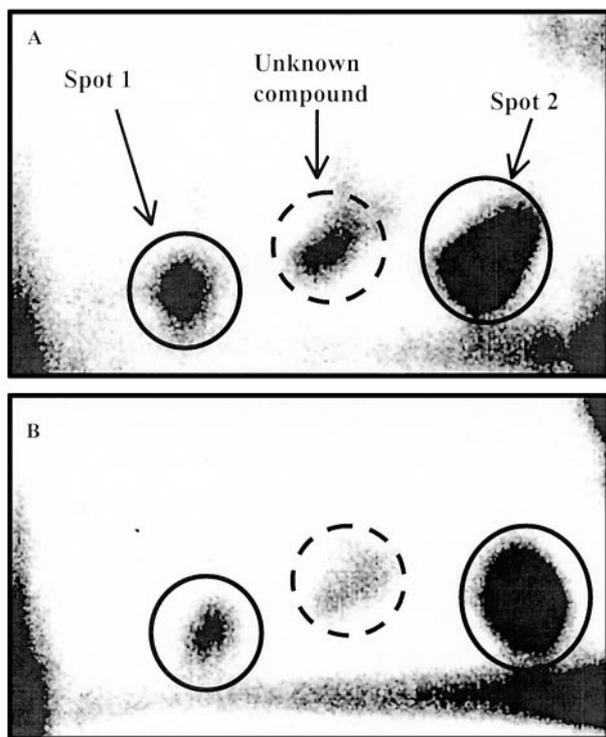


Fig. 2. Postlabeling analysis of human colon biopsy specimens of a male volunteer (A) after the high-RS period and (B) after the low-RS period. Higher contents of enzyme-RS in the diet increased HNE-dGp adduct levels (Spot 1); an unknown compound that is indicated by the dotted lines was also influenced by the diet and might therefore be another marker for lipid peroxidation-induced DNA damage. The stability of the analytical determination was monitored by internal standard (1,N<sup>2</sup>-propanodeoxyguanosine-3'-monophosphate adducts of *trans*-2-hexenal, Spot 2), which was added to the DNA samples before DNA hydrolysis.

diet, respectively (Fig. 2, A and B). The correct adduct spot was indicated by cochromatography after spiking with HNE-dGp adduct standard (data not shown). Some chromatograms were found to be insufficient for evaluation because of chromatographical problems in one set of determinations in which four samples were involved. Because DNA amounts did not allow us to repeat the determination, the comparison between the two test periods was carried out with the set of eight paired data out of twelve volunteers. This comparison showed a significant increase of DNA adduct levels in the high-RS period ( $P = 0.0117$ , Wilcoxon's signed rank test; Fig. 3). The mean  $\pm$  SD adduct levels (given in adducts/ $10^7$  nucleotides) are significantly higher in the high-RS period ( $3.83 \pm 0.60$ ) than in the low-RS period ( $2.69 \pm 0.35$ ;  $P < 0.05$ , Wilcoxon's signed rank test). Increases in adduct levels ranged from 1.11 to 1.94 (mean factor, 1.42) and indicated that RS revealed a similar effect on all individuals and that different susceptibilities can be assumed. The comparison of males and females within each test period indicated that higher levels of HNE-dGp adducts are present in the colon of male volunteers than in the colon of female volunteers. The mean adduct levels of male volunteers were  $4.19 \pm 0.44$  adducts/ $10^7$  nucleotides but only  $3.46 \pm 0.53$  adducts/ $10^7$  nucleotides in female volunteers after the high-RS period ( $P = 0.08$ ). The mean adduct levels after the low-RS period were  $2.75 \pm 0.29$  adducts/ $10^7$  nucleotides (male) versus  $2.63 \pm 0.43$  adducts/ $10^7$  nucleotides (female;  $P = 0.56$ , Mann-Whitney  $t$  test).

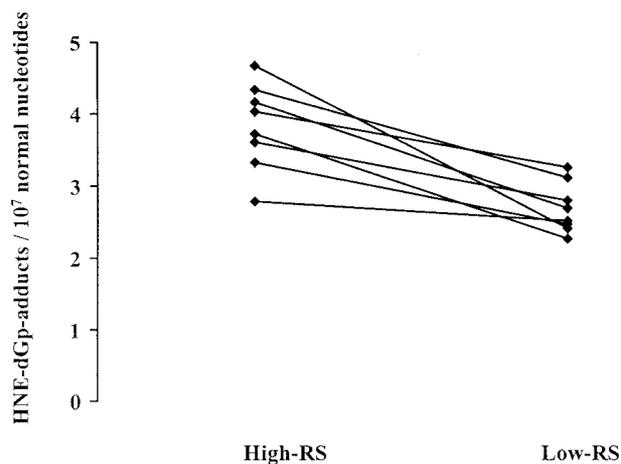


Fig. 3. Levels of HNE-dGp adducts in colon biopsies of human volunteers after receiving high- and low-RS diets ( $P = 0.01$ , Wilcoxon's signed rank test).

Previous results with this study showed that fecal wet and dry weight and daily fecal excretion of RS and MDA increased significantly with consumption of the high-RS diet (13). There was also higher excretion of stool fat with consumption of the high-RS diet (13). Significant changes in daily fecal excretion of MDA between the high-RS and low-RS periods were observed ( $524.4 \pm 42.4$  and  $348.6 \pm 38.2$  nmol/day, respectively;  $P < 0.005$ ). A huge difference between oral intake and fecal excretion of starch was observed; during the high-RS and the low-RS diets, 89% and 88%, respectively, of the RS consumed was fermented in the colon. One volunteer, however, excreted high amounts of RS during both the high-RS (31.0 g/day, *i.e.*, 44% of RS fermented) and low-RS (4.2 g/day, *i.e.*, 45% of RS fermented) periods.

HNE-dGp adduct formation was significantly associated with daily fecal MDA excretion ( $P = 0.008$ ,  $r = 0.66$ ). Excretion of RS did not correlate with either excretion of MDA or formation of DNA adducts; no effects were seen with the subject who fermented RS to a low extent. A higher consumption of diet by male volunteers in each test period when compared with female volunteers (16%) may have caused higher adduct levels in male volunteers than in female volunteers (21%, high-RS; 5%, low-RS).

**Cell Proliferation Data.** The distribution of proliferating epithelial cells over five colonic crypt compartments was parallel in both study periods, with the majority of proliferating cells found in the lower 60% of the crypt (compartments 1–3). There was no evidence for a decreased label in the upper crypt (upper crypt LI,  $\phi$ h value) in the high-RS phase, compared with the low-RS phase (Table 1).

## Discussion

The dietary fiber hypothesis postulates a protective effect of complex carbohydrates with regard to colonic carcinogenesis. Beside fiber (chemically non-starch polysaccharides), enzyme-RS enters the colon, possibly in larger quantities than fiber. These polysaccharides are broken down in the colon by anaerobic bacteria in a process called fermentation, which releases short-chain fatty acids as major end products. Starch fermentation seems to generate more *n*-butyrate than fiber (30). Numerous *in vitro* studies have demonstrated the effects of *n*-butyrate on colon cancer cells (*e.g.*, inhibition of prolifera-

**Table 1** Distribution of proliferating epithelial cells over five colonic crypt compartments after the high-RS period and after the low-RS period

No significant differences between groups.		
LI <sup>a</sup>	High-RS period	Low-RS period
Compartment 1 <sup>b</sup>	0.123 ± 0.008	0.110 ± 0.009
Compartment 2 <sup>b</sup>	0.177 ± 0.006	0.169 ± 0.005
Compartment 3 <sup>b</sup>	0.104 ± 0.005	0.105 ± 0.006
Compartment 4 <sup>b</sup>	0.037 ± 0.004	0.030 ± 0.003
Compartment 5 <sup>b</sup>	0.006 ± 0.002	0.005 ± 0.001
Whole crypt LI <sup>c</sup>	0.089 ± 0.002	0.084 ± 0.003
Upper crypt LI <sup>d</sup>	0.021 ± 0.003	0.017 ± 0.002
ϕh value <sup>e</sup>	0.087 ± 0.009	0.076 ± 0.009

<sup>a</sup> LI (number of labeled cells divided by number of labeled plus unlabeled cells) of colonic mucosal crypts after consumption of a diet high or low in RS (mean ± SE).

<sup>b</sup> Crypts are divided into five equal compartments and numbered from base to apex.

<sup>c</sup> Whole crypt LI, LI in compartments 1–5.

<sup>d</sup> Upper crypt LI, LI in compartments 4 and 5.

<sup>e</sup> ϕh value, number of labeled cells in compartments 4 and 5, divided by the number of labeled cells in compartments 1–5.

tion, induction of differentiation and apoptosis), which are compatible with protection against cancer (31). Therefore a high intake of enzyme-RS should theoretically confer greater protection than that of dietary fiber.

Recently, the concept of mucosal protection by dietary fiber and RS has been questioned by epidemiological and human intervention studies. In the latest evaluation of the Nurses' Health Study, a high-fiber diet over a 16-year period did not protect against the formation of adenoma or cancer (32). In two large-scale intervention trials, fiber did not prevent the recurrence of adenomas after endoscopic polypectomy within 3–4 years (33, 34). The consumption of at least 30 g dietary fiber/day is still recommended by the American Gastroenterological Association, but the controversy over conflicting epidemiological and experimental data has clearly been stated (35). The database linking RS with colorectal tumorigenesis is not firm either. There is no clear picture from epidemiological studies concerning risk of disease. Experimental data from animal studies have yielded conflicting results; this is true for the study end points of tumor occurrence, aberrant crypt foci, and cell proliferation.

In this study using a strictly controlled basal diet, Hylon VII (a highly enzyme-resistant amylo maize starch) increased the amount of DNA adducts in the colonic mucosa significantly. Higher DNA adduct levels have been detected in the nontumoral colonic mucosa of patients with adenocarcinoma, compared with tissues from healthy controls (36). Thus, the presence of DNA adducts may be a useful biomarker of colorectal carcinogenesis. The 1,N<sup>2</sup>-propanodeoxyguanosine adducts of HNE measured in this study provide a clear indication of the genotoxic interactions of oxidative stress and lipid peroxidation because of the C-9 substitution that stems from HNE (Fig. 1). Levels of HNE-dGp adducts also increased after application of carbon tetrachloride in rats (37). The elevated levels of HNE-dGp adducts with consumption of the high-RS diet therefore suggest lipid peroxidation as a consequence of oxidative stress in this study.

Butyrate is released during starch fermentation in the colon and absorbed by colonocytes (13), but the study design did not indicate whether interactions of butyrate with mitochondria or nuclei were involved in the enhanced cellular lipid peroxidation and the formation of HNE-dGp adducts. The miss-

ing effect with one person who fermented RS to a low extent and did not show outlying results in MDA output or DNA adduct formation might be explained by previous investigations; it was shown that HNE-dGp adducts reach steady-state levels under normal conditions and a higher steady-state level by a factor of 2 under chronic oxidative stress (37). These steady-state levels are due to DNA repair and, possibly, loss of cells by necrotic or apoptotic pathways, e.g., induced by butyrate; these events could be followed by a slight increase in cell regeneration. Therefore, high fermentation of enzyme-RS is possibly not associated with a linear increase in formation of DNA adducts, in comparison with low fermentation of RS; in other words, increases in MDA excretion or formation of HNE-dGp adducts by a factor in the range of 8 would have been expected for all volunteers with a basically high starch fermentation. Conclusively, enzyme-RS could have influenced MDA excretion and DNA adducts by bacterial fermentation in all volunteers.

Intestinal absorption of food ingredients was not investigated with this study. The diets of both test periods were not significantly different with regard to available energy (not including energy from RS), protein, fat, available starch, saccharides, and dietary fiber (13). Possible events might be decreased absorption of food ingredients with the high-RS diet, due to malabsorption or decreased neutral sterols that were detected in the feces after the high-RS period; however, these hypothetical effects show an unclear context with systemic influences on the colonic mucosa.

Colonic crypt hyperproliferation (expansion of the proliferative zone to the crypt surface) has been considered a preneoplastic biomarker because this phenomenon is regularly encountered in the normal mucosa of patients with adenomas or cancer (28). In this study in healthy volunteers, proliferation was not affected by the consumption of RS, as compared with available starch. Our negative finding is in agreement with the data of Grubben *et al.* (38) and van Gorkom *et al.* (39); both groups reported no effect by RS on mucosal proliferation in adenoma patients. On the contrary, Caderni *et al.* (40) and van Munster *et al.* (41) observed a decline of colonic epithelial proliferation after consumption of a high-starch diet. In experimental animals, proliferation was either decreased (42) by a high-starch chow or unchanged by Hylon VII (43), which was also used in the present study. The formation of HNE-dGp adducts was not paralleled by changes in cell proliferation. The adducts might therefore be an early direct marker of dietary habits that involve endogenous processes such as lipid peroxidation. The increase that was found in this dietary study with healthy volunteers was low but significant. The adduct levels were high in general, with several femtomoles of HNE-dGp adducts isolated from small DNA amounts of human colonic biopsies. It can be assumed that this type of adduct, which is considered a promutagenic lesion, represents a new DNA-based marker that can be measured before hyperproliferation occurs, and studies that correlate such early markers with tumor end points would be desirable to establish the role of HNE-dGp adducts.

It can be concluded from this study that Hylon VII, a highly resistant amylo maize starch, increases DNA adducts in the normal colonic mucosa of healthy volunteers without affecting cell proliferation. If it is accepted that the formation of DNA adducts reflects oxidative stress, which in turn accelerates the process of carcinogenesis, then certain forms of RS may have to be considered detrimental rather than protective. It cannot be generalized from this study that all forms of RS behave similarly. This finding also emphasizes the dilemma

that we have no definitive biomarkers of early colorectal carcinogenesis that are clearly validated as preneoplastic lesions. Currently, the only accepted (late) precursor of the carcinoma is the adenoma; intervention studies are in progress that examine the role of RS in the transition from adenoma to carcinoma (44).

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## Effect of Enzyme-resistant Starch on Formation of 1,N<sup>2</sup>-Propanodeoxyguanosine Adducts of *trans*-4-Hydroxy-2-nonenal and Cell Proliferation in the Colonic Mucosa of Healthy Volunteers

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