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Safety evaluation of pectin-derived acidic oligosaccharides (pAOS): Genotoxicity and sub-chronic studies

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ABSTRACT

Pectin-derived acidic oligosaccharides (pAOS) are non-digestible carbohydrates to be used in infant formulae and medical nutrition. To support its safety, the genotoxic potential of pAOS was evaluated. pAOS was not mutagenic in the Ames test. Positive results were obtained in the chromosome aberration test only at highly cytotoxic concentrations. The effects obtained in the mouse lymphoma test were equivocal; pAOS was not mutagenic *in vivo*. A sub-chronic dietary study, preceded by 4-week parental and *in utero* exposure phase, investigated general safety. Administration of pAOS did not affect parental health nor pup characteristics. No effects specific for acidic oligosaccharides were observed in the subsequent sub-chronic study. Slight diffuse hyperplasia of epithelial layer of the urinary bladder was noted to result from concurrently elevated urinary sodium, due to high sodium in pAOS, and elevated urinary pH. This phenomenon was confirmed in a mechanistic (sub-chronic) study. In contrast, in rats fed pAOS in combination with NH_4Cl, an acidifying agent, the induced low urinary pH completely prevented the development of urothelial hyperplasia. Hyperplasia induced by this mechanism in rats is considered not relevant to man. Based on the current knowledge we consider pAOS safe for human consumption under its intended use.

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1. Introduction

Human milk contains a complex mixture of human milk oligosaccharides (HMOs) that play an important role in the establishment of a beneficial microbiota (bifidobacteria and lactic acid bacteria) in the gut of the newborn child. This microbiota can prevent binding of pathogenic bacteria in the gut and stimulates development of a normal gut-barrier function (Salminen et al., 2005). In addition, this beneficial microbiota can stimulate the development of the neonate immune system (Salminen et al., 2005). Naturally available non-digestible oligosaccharide structures, such as short chain (sc) galacto-oligosaccharides (GOS) from cow's milk and long chain (lc) fructo-oligosaccharides (FOS) from chicory, mimic the neutral oligosaccharides of HMOs. In standard infant formulae (IF) these natural oligosaccharide structures have demonstrated to have comparable beneficial effects as HMOs in pre-term and term infants (Boehm and Moro, 2008; Fanaro et al., 2005a,b; Moro et al., 2005). In addition to the neutral oligosaccharides, acidic oligosaccharides, present in human milk at levels of 0.1% (Kunz et al., 2000; Stahl et al., 1994), have also been suggested to exert specific beneficial effects. Coppa and co-workers (2006) demonstrated that the acidic oligosaccharides in human milk can inhibit binding of pathogenic bacteria to epithelial cells. This finding points towards an important role of these acidic oligosaccharides in health promoting effects such as the prevention of diarrhoeal infections (Coppa et al., 2006).

Acidic oligosaccharides of human milk are characterised by their content of sialic acid. Although it is technically possible to synthesise sialic acid containing oligosaccharides, this is not yet feasible for large scale production (Lee and Lee, 1994). Enzymatic cleavage of pectin however is an alternative source of acidic

Abbreviations: HMOs, human milk oligosaccharides; pAOS, pectin-derived acidic oligosaccharides; HAART, highly active antiretroviral therapy; IF, infant formulae; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; sc, short chain; lc, long chain; FOB, Functional Observation Battery; SCFAs, short-chain fatty acids.

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oligosaccharides for use in nutritional products such as infant formulae and medical foods. Pectin is a naturally occurring polymer composed of galacturonic acid and is a commonly found structural component in all higher plants, including fruits and vegetables (Rolin et al., 1998). Pectin degrading enzymes can generate the smaller pectin-derived acidic oligosaccharides (pAOS) through modification of pectin by hydrolysis and β-elimination. Different means of pectin processing (e.g. cooking, juice clarification) actually result in the formation of negatively charged oligosaccharides. Therefore pAOS are considered part of the normal human diet (Doesburg, 1961; Marlett, 1992; Ng and Waldron, 1997). In analogy to lcFOS and scGOS, pAOS is considered to be a non-digestible carbohydrate that resists digestion/hydrolysis in the stomach and small intestine. In the colon, the unabsorbed acidic oligosaccharides are subject to micro biotic fermentation, resulting in the generation of short-chain fatty acids (SCFAs) with the fermentation products subsequently absorbed and utilised in well-characterised biochemical pathways. pAOS that is not fermented in the colon will be excreted in the faeces (Govers et al., 2005; Vaisman et al., 2004).

To alleviate childhood diarrhoea, for more than a century cooked carrot soup has been used of which pAOS was found to be the active ingredient (Guggenbichler et al., 1997; Kastner et al., 2002). Recently, other beneficial effects of pAOS have been demonstrated in animal models including improved Th1-type immune response (Vos et al., 2007a) and reduced disease parameters of allergic asthma in an allergic asthma model in mice (Vos et al., 2007b). Additionally, intake of pAOS by highly active antiretroviral therapy (HAART) -naïve HIV infected individuals resulted in an improvement of gut micro biota composition and immune function (Ben Amor et al., 2008; Gori et al., submitted for publication; Van't Land et al., 2008). These findings demonstrate that, besides the addition of pAOS to infant formulae, pAOS could play a beneficial role in medical foods as well. pAOS is intended for use as an ingredient in IF, cereal products for babies and weaning foods at levels comparable to human milk, i.e., 0.14 g/100 ml formula, which corresponds to approximately 0.2 g/kg bw/day, depending on the infants formula intake and age. In addition, pAOS will be used as ingredient in medical foods. The proposed dose levels of the pAOS preparation in these products will be up to 9 g/day, corresponding to 0.15 g pAOS/kg bw/day, assuming a body weight of 60 kg.

To confirm the safety of commercially produced pAOS in its intended use and to obtain regulatory approval a number of safety studies have been performed. The studies performed are in line with the minimum set of studies requested by regulatory bodies to get approval on novel food ingredients. This minimum set concerns the investigation of the mutagenic potential *in vitro* of the ingredient and depending on the target group of the application, the results of a general toxicity study, in most cases a sub-chronic study in rats. Besides the *in vitro* investigations the mutagenic potential of pAOS was also studied *in vivo* to include breakdown products of pAOS, i.e., SCFAs, that will be formed in the body. The general toxicity of pAOS was investigated in a sub-chronic oral toxicity study in rats. To mimic the intended exposure of infants to pAOS, namely shortly after delivery, the daily administration of pAOS for 13 consecutive weeks was preceded by 4-week parental treatment, an *in utero* exposure phase, and treatment during lactation and weaning. All relevant endpoints which are normally addressed in reprotoxicity and teratogenicity studies were included in the protocol. As reference for dosing high non-digestible oligosaccharides an additional group was incorporated in the study receiving short chain FOS (scFOS). For exploratory purposes, a supplementary sub-chronic study was performed, to elucidate the aetiology of an effect (slight diffuse urothelial hyperplasia) noted in the first oral toxicity study and to examine whether this effect is compound-related.

2. Materials and methods

2.1. Materials

A bulk preparation of pAOS, produced according to an enzymatic method and described in the patent family resulting from WO 02/42482, was used for the present studies. In short, food grade pectin was treated with poly (methoxyl-L-galacturonide) lyase (EC 4.2.2.10) and poly (1,4-alpha-D-galacturonide) glycanhydrolase (EC 3.2.1.15) to generate pAOS. The enzymes were subsequently heat inactivated but not separated from the produced pAOS. Both enzymes used are derived from non-pathogenic and non-toxicogenic strains of *Aspergillus niger* and have a history of use in Europe for food production (primarily in the fruit juice industry). They fulfill the latest JECFA (Joint FAO/WHO Expert Committee on Food Additives) criteria for enzymes used in food processing (WHO, 2007). The final product is a mixture of linear oligomers and small polymers of galacturonic acid. pAOS has predominantly a molecular weight of no more than 3800 Da which corresponds to a maximum degree of polymerisation of 20. The galacturonic acid unit on the non-reduction end of each acidic oligosaccharide (AOS) molecule has a double bond. In Table 1 the compositional analyses of the pAOS (Batch No. 254910) used for the studies described in this paper are presented. The levels of contaminants in pAOS such as heavy metals, pesticide residues, mycotoxins and microbiology are in accordance with the principles of the relevant legislation and Good Agricultural Practice.

scFOS (Beneo^{tm}P95), used as reference substance in the first sub-chronic study, was prepared by partial enzymatic hydrolysis of chicory inulin. The oligofructose content of this batch (PEC-BX7CBX7) was 93.2% on dry matter base and it contained 6.8% glucose, fructose and sucrose. The microbiological analysis reveal total counts of 40/g dry matter and yeast and moulds 0/g dry matter. Ammonium chloride, used as ancillary substance in the second sub-chronic study, was obtained from Merck (Merck product 1.01145).

Table 1
Composition analysis of pAOS of Batch No. 254910.

	Unit	Value
Identity		
Galacturonic acid content	g/100 g	62
Mw below 3800 Da	g/100 g	94
Monosaccharides (GalA)	g/100 g	6.7
Degree of methylation	%	50
Number of double bonds compared to total galacturonic acid content	mol%/mol GalA	23
Physical–chemical characteristics		
Na	mg/100 g	3120
K	mg/100 g	274
Ca	mg/100 g	168
Mg	mg/100 g	18
Cl	mg/100 g	140
Moisture	g/100 g	7.22
Protein	g/100 g	1.44
Contaminants and residues		
Cadmium	μg/kg	1
Lead	μg/kg	21
Mercury	μg/kg	<3
Arsenic	μg/kg	<20
Aluminium	mg/kg	8.18
Pesticides	μg/kg	<5
Nitrate	mg/kg	842
Nitrite	mg/kg	3.4
Microbiological parameters		
Total viable count	g	<99
Moulds	g	<10
Yeast	g	<10

2.2. Genotoxicity tests

All studies were conducted in compliance with the OECD Principles of Good Laboratory Practice at TNO Quality of Life, Zeist, The Netherlands and according to the respective OECD guidelines for the different tests. In all tests, except in the additionally chromosome aberration test, pAOS was dissolved in DMSO (after sonification of 15–30 min at 40 °C), since it was less soluble in aqueous vehicles and to guarantee sterility of the test solution. Negative controls (solvent) and positive controls were run simultaneously with the test substance.

2.2.1. Bacterial reverse mutation assay (Ames test)

The bacterial reverse mutation test was performed in accordance with OECD guideline 471, Bacterial Reverse Mutation Test (OECD, 1997) using the plate-incorporation method with the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and the tryptophan-requiring *Escherichia coli* strain WP2 uvrA, in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix). The test substance pAOS was dissolved in dimethylsulphoxide (DMSO). One test, in triplicate, was performed with all strains in the absence and presence of S9-mix. Five concentrations of the test substance were used including the recommended maximum concentration of 5000 μg/plate, based on AOS content in the test preparation.

2.2.2. Mouse lymphoma assay

The mouse lymphoma assay was performed in accordance with OECD guideline 476 for *In vitro* mammalian cell gene mutation tests (OECD, 1997). The study consisted of two assays, in each assay cells were treated for 24 h in the absence and 4 h in the presence of S9-mix. In both assays five duplicate concentrations of pAOS were evaluated for mutagenicity, for both the absence and presence of S9-mix. The test substance was dissolved in DMSO. The highest feasible concentration tested was 4370 pAOS μg/ml due to limited solubility.

2.2.3. Chromosomal aberration test

The potential of pAOS to induce structural chromosomal aberrations was examined in Chinese Hamster Ovary (CHO) cells, in both the absence and presence of S9-mix. The assay was performed in compliance with OECD guideline 473 for *In vitro* mammalian chromosome aberration tests (OECD, 1997). Two separate tests were conducted. The test substance was dissolved in DMSO. In the first test, the highest dose level (4220 μg/ml) was based on the solubility of the test substance. In the second test, the highest dose level (4220 μg/ml) was based on the results of the mitotic index (as measurement for cytotoxicity) of the first chromosomal aberration test. In all instances duplicate cultures were used. In the first chromosomal aberration test, in both the absence and presence of a S9-mix, the treatment/harvesting times were 4/18 h (pulse treatment). In the second test, in the presence of S9-mix, the treatment/harvesting times were 4/18 h (pulse treatment) and in the absence of S9-mix, the treatment/harvesting times were 18/18 h (continuous treatment). In an additional test both DMSO and culture medium were used as solvents and pH and osmolality were checked before and after incubation. The cells were treated continuously for 18 h in the absence of S9-mix. pAOS was tested up to the recommended maximum dose level of 5000 μg/ml. In all instances duplicate cultures were used and dose level selection for evaluation was based on cytotoxicity.

2.2.4. Genotoxicity parameter in vivo (micronucleus) as part of sub-chronic study

An *in vivo* genotoxicity parameter (micronucleus) was evaluated as part of the sub-chronic oral toxicity study with pAOS in rats, which was preceded by an *in utero* exposure phase. Details of this study are described under paragraph 2.3. The study protocol for the evaluation of the micronucleus parameter was drafted in line with OECD guideline 474 (OECD, 1997). The micronucleus test was conducted in tissues collected at necropsy of the offspring (F_1) of the control group and the high-dose (10% pAOS) group (5 rats/sex/group). In addition, two untreated F_1 reserve male and reserve female rats (kept on control diet during the entire study) were injected 24 h before sacrifice once intraperitoneally with mitomycin C at a dose level of 1.5 mg/kg body weight to serve as positive controls. At necropsy femoral bone marrow cells from the right femur were collected. Two smears of bone marrow cells were prepared per animal. One smear per animal was stained with May-Grünwald-Giemsa solution. Smears were scored for the number of micronucleated polychromatic erythrocytes (MPE) in a total of 2000 polychromatic erythrocytes (PE) and for the number of PE in a total of 200 erythrocytes (E).

2.3. Repeated oral toxicity studies in rats

The studies were conducted in compliance with the OECD Principles of Good Laboratory Practice at TNO Quality of Life, Zeist, The Netherlands.

2.3.1. Study 1: sub-chronic study (13-week) oral toxicity study, preceded by an in utero exposure phase

2.3.1.1. Animals and maintenance. The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Union (Directive 86/609/EEC) as implemented in the Dutch legislation of 1997 on The Experiments on Animals Act.

Parental (F_0) male and female rats Wistar rats (Crl:WI(WU), outbred) were obtained from a colony maintained under SPF-conditions at Charles River Deutschland, Sulzfeld, Germany. The animals were acclimatised to the laboratory conditions for at least 5 days. To form the F_0 groups, rats were allocated to four groups (16 females and 8 males per group) by computer randomization and proportionally to body weight. At the start of the premating period, the parental (F_0) rats were approximately 11 weeks (males) or 10 weeks (females) old. During the mating period, two females were caged with one male from the same group until they were mated. During gestation and lactation, the dams were housed individually. The rats were housed in macrolon cages with wood shavings enriched with shreds of paper.

In the sub-chronic study the F_1 rats were 5 weeks old at the start of the study and housed in groups of five per cage of the same sex. Housing conditions were conventional; room temperature was targeted at 20–24 °C and relative humidity at 40–70%; the number of air changes was about 10 per hour. Lighting was artificial for 12 h continuously. The test- and control diets were provided as powder in stainless steel cans. The animals received a commercial cereal-based rodent diet (RM3 diet, SDS Special Diet Services, Witham, England). Diet and tap water were provided *ad libitum*. The feed in the animal feeders was replaced by fresh portions at least once a week (F_0 rats) or twice a week (F_1 rats in the sub-chronic study).

2.3.1.2. Experimental design. The study comprised two phases: (1) an *in utero* phase, in which parental (F_0) animals were fed the various test- and control diets starting 4 weeks prior to mating, throughout mating, gestation and lactation periods until weaning of the offspring (F_1 rats); and (2) a sub-chronic toxicity study with F_1 rats, in which the administration of the test and control diets was continued for 13 weeks. During and in between the two phases the feeding of the respective test- or control diets to the selected F_1 rats was continued.

There were two control groups, viz. one control group, which received the standard rodent diet supplemented with 10% potato starch, and one control group receiving 10% scFOS in the diet. Two experimental groups received the standard diet supplemented with 5% or 10% pAOS. To keep the total level of added substance (test substance, reference substance and/or starch) equal in each diet, the low-dose diet (5% pAOS) was adjusted with 5% potato starch. All macro- and micronutrients in RM3 breeding diet were well in excess of the minimal requirement of the laboratory rat to allow 10% dilution of the diet, except for vitamin B_{12}. Therefore, all experimental diets (including the control diets) were supplemented with vitamin B_{12} to meet the requirement (50 μg/kg diet) in the finished diet.

2.3.1.3. In utero phase. After a premating period of 4 weeks, during which the rats were fed their respective test diets, two females were caged with one male from the same group until they were mated. The day on which sperm was observed in vaginal smears was considered day 0 of gestation. The morning after birth was considered day 1 *post partum*. On day 4 *post partum*, litters of more than eight pups were adjusted by eliminating surplus pups by random selection, to obtain (as nearly as possible) four males and four females per litter. At day 21 *post partum*, the litters were weaned and F_1 rats were selected for the sub-chronic study. For each group 10 males and 10 females were selected randomly from as many different litters as possible (maximally one animal of each sex was selected from one litter). Parental rats and the remaining pups were sacrificed after selection of the F_1 study groups.

The following observations were made: during premating, mating, gestation and lactation, the general condition and behaviour of all animals were checked daily. Body weight of each animal was recorded, when starting the administration of the test substances and once every week thereafter. Mated females were weighted on days 0, 7, 14 and 21 of gestation and on days 1, 4, 7, 14 and 21 of lactation. The feed consumption of males and females was measured weekly on a cage basis during the premating period. Food consumption of mated females was recorded during pregnancy on days 0–7, 7–14, 14–21 and during lactation on days 1–4, 4–7 and 7–14. For assessment of the fertility and reproductive performance, mating, fertility, fecundity, gestation and live birth were determined for each group. The pups were checked daily on viability. Observations of appearance of pups and recording of the number of live pups per litter were carried out on days 1, 4, 7, 14 and 21 of lactation and viability indices were calculated. The sex ratio was determined on days 1 and 21 of lactation. The weight of the litters as a whole was recorded on days 1, 4 (before and after culling), 7 and 14 *post partum*. The weight and sex of individual pups was recorded on day 21.

2.3.1.4. Sub-chronic study (13-week) with F_1 rats. The sub-chronic study was performed according to the OECD guideline 408 for the testing of chemicals (OECD, 1998) and EC Guideline Annex 5D to Commission Directive 2001/59/EC (2001). The following observations, analysis and measurements were included: intake of the test substance, general daily clinical observations of all rats, neurobehavioral testing (arena testing, FOB and motor activity) in the first week of the study on all rats and at the end of the study in week 13. Ophthalmoscopic observations were made in the first week of the treatment period in all F_1 rats of all groups, and in the last week of the treatment in all rats of the control group, the reference control group (10% scFOS) and the high-dose group (10% pAOS group). Bodyweights were recorded at the start of the 13-week study and once per week thereafter. Feed consumption was measured per cage by weighing the feeders over periods of 3 or 4 days. Water consumption was measured per cage during 5-day periods in week 1, 6 and 11 of the study.

Oestrus cyclicity was investigated by taking daily vaginal smears during the last three weeks prior to sacrifice. Smears were made and stained for all females. Microscopic analysis of the smears was performed for all rats of the control and 10% pAOS group. Sperm analysis was performed on epididymal sperm derived from the left cauda epididymis of all males at necropsy. Sperm motility and, after sonification and DNA-staining, the sperm reserves (sperm count) were measured for all males with a Hamilton Thorne Integrated Visual Optical System (IVOS). Microscopic analysis of stained smears of sperm solution was performed for the control and 10% pAOS group. The testicular parenchyma of the left testis was isolated, weighed, minced and homogenised in saline Triton X-100 solution. After DNA-staining, the homogenisation resistant sperm heads were counted with the IVOS system and the daily sperm production was calculated (number of spermatozoa per gram testicular arenchyma/6.1).

On day 87–88 all F_1 rats were deprived from water for 24 h and of feed during the last 16 h of this period. During the last 16 h of deprivation, the rats were kept individually in metabolism cage and urine was collected. The following determinations were carried out: volume, density, appearance, pH, glucose, occult blood, ketones, protein, bilirubin, urobilinogen, microscopy of the sediment, creatinine, sodium, potassium, calcium and chloride.

After completion of the 13-week treatment period, the animals were killed after overnight fasting under CO_2/O_2 anaesthesia and subjected to a complete gross necropsy. Blood was collected from the abdominal aorta under CO_2/O_2 anaesthesia. Haematology and clinical chemistry was conducted on all F_1 rats. The weights of organs, including caecum (filled and empty), were recorded and related to the final body weight. Histopathology examination was performed on 5 μm, haematoxylin and eosin stained sections of all tissues and organs as listed in the guideline (OECD, 1998) from all animals of the control group and the high-dose (10% pAOS) group. In case no effects were seen in organs or tissues of the highest dose level, no further histopathological examination of the other groups was performed except for the liver, kidneys, urinary bladder and all gross lesions which were examined microscopically in all F_1 rats.

2.3.2. Study 2: supplementary sub-chronic (13-week) oral toxicity study

Information on animals and maintenance and the preparation of the test diets are described under study 1. The study comprised a control group, a high-dose group (10% pAOS in the diet) and an additional high-dose group receiving 10% pAOS in the diet supplemented with NH_4Cl as acidifying substance (1% NH_4Cl until day 11 and 2% NH_4Cl from day 11). To be able to derive a NOAEL level for the species specific effects observed in the first sub-chronic study two additional dose groups were included in which the rats received dietary levels of 1 and 2.5% pAOS. At the start of the treatment period the rats were about 4 weeks old, comparable to the F_1 rats of the previous study (study 1). General clinical observations, determination of body weight, feed and water consumption and intake of the test substance were conducted as described under study 1.

For urinalysis measurements fresh urine samples were collected on day 6, 19, 41 and 83(females)/90(males). In the early morning rats were placed individually in stainless-steel metabolism cages. Urine was collected in glass tubes during the first 2–3 h of the light period. The urinary pH obtained this way is representative for the night period during which the rats had free access to feed and water in their home cages. Urinary pH was determined immediately after sampling by pH meter. Urinary volume, density, appearance, and electrolyte concentration (sodium, potassium, calcium and chloride) were determined on day 6, 41 and 83(females)/90(males). Creatinine was determined for normalisation of the electrolyte excretion values.

At the end of the treatment period, the animals were sacrificed as described under study 1. The caecum (filled and empty) and kidneys (left and right together) were weighed, and the relative organ weights (g/kg body weight) were calculated on the basis of the terminal body weight of the animals. Samples of the kidneys and the urinary bladder were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde. The urinary bladder was embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Histopathology examination (by light microscopy) was performed on the urinary bladder of all animals of all groups.

2.4. Statistical analysis

2.4.1. Genotoxicity studies

Data from the chromosome aberration test were analyzed by Fisher's exact probability test (one-sided) to determine significant differences between treatments and controls. Data from the micronucleus test were subjected to a one-way Anova with factor treatment group. In case of significant effects ($p < 0.05$), pooled error variance *t*-tests or, if variances were not homogeneous, separate variance *t*-tests were performed. These *t*-tests were applied to the negative control group versus 10% pAOS or positive control groups.

2.4.2. Sub-chronic toxicity studies

For data obtained in F_0 rats (study 1) Analysis of variance (Anova) followed by Dunnett's multiple comparison test was used for parental body weight, parental feed intake and pup body weight. Fisher's exact probability test was used for the evaluation of the numbers of mated and pregnant females, females with liveborn pups, females surviving delivery, females with stillborn pups or lost litters, liveborn and stillborn pups, pups lost at various stages and pups surviving for 21 days. Kruskal–Wallis non-parametric Anova followed by the Mann–Whitney *U*-test was used for evaluating precoital time, duration of gestation and litter size.

For data obtained in F_1 rats (study 1) and for data obtained in study 2 one-way Anova was used for body and organ weight, haematology, clinical chemistry and urinalysis data. Homogeneity of variance (Bartlett test) and normality of data distribution (Shapiro–Wilks test) were checked and, if necessary, data were stepwise log or rank transformed before Anova. In case of significant effects ($p < 0.05$), intergroup comparisons were made with the control group by Dunnett's multiple comparison test. One-way Anova followed by Dunnett's multiple comparison test was used for continuous data obtained by FOB, total distance covered in the motor activity test, number of oestrus cycles per animal, sperm counts and continuous sperm motility parameters. Dunnett's multiple comparison test was used for food and water consumption. Kruskal–Wallis non-parametric Anova followed by multiple comparison tests was used for rank order data obtained by FOB. Pearson chi-square analysis was used for categorical data obtained by FOB. Kruskal–Wallis non-parametric Anova followed by the Mann–Whitney *U*-test was used for evaluating oestrus cycle length, motility parameters of sperm (expressed as percentages) and sperm morphology. Fisher's exact probability test was used for analysing the number of acyclic animals, number of animals with prolonged oestrus and indices of histopathological changes.

3. Results

3.1. Genotoxicity studies

In the bacterial reverse mutation assay pAOS was not mutagenic as evidenced by the absence of a dose-related or more than twofold increase in the mean number of revertant colonies in both the absence and presence of S9-mixture. Furthermore, pAOS was not toxic to any of the tested strains as demonstrated by the absence of a decrease in the mean number of revertants or a clearing of the background lawn of bacterial growth. The number of revertants on the DMSO control plates was within acceptable range and the positive controls gave the expected increase (data not shown).

pAOS was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells, both in the absence and presence of a metabolic activation system (S9-mix). In the presence of S9-mix, no cytotoxicity and no in-

Table 2
Effect of pAOS in the mouse lymphoma assay on the mutant frequency (MF, expressed as the trifluorothymidine-resistance mutants per 10^6 cells clonable cells), and the relative total growth (RTG, expressed as per cent of the vehicle control). The results are the mean of two cultures.

Test compound	Dose (μg/ml)	With S9-mix (4 h)		Without S9-mix (24 h)	
		MF	RTG	MF	RTG
Assay 1					
Solvent control (DMSO, 0.1%)	0	90	100	147	100
pAOS	500	94	93	128	121
	990	99	106	130	110
	2020	91	119	108	76
	2890	115	113	175	55
	4130	158	95	173[a]	25
Positive control MCA	10	1807[b]	26		
Positive control MMS	0.1			2100[b]	22
Assay 2					
Solvent control (DMSO, 0.1%)	0	95	100	149	100
pAOS	1030	115	87	106	159
	2050	87	93	126	121
	2920	135	70	301	30
	3590	114	66	251	9
	4370	96	68	478[c]	3
Positive control MCA	10	1666[b]	25		
Positive control MMS	0.1			1784[b]	47

MCA, 3-methyl cholanthrene; MMS, methyl methanesulphonate.
[a] Single culture, duplicate culture had MF of 403 but RTG of 4% and therefore not evaluated.
[b] Single culture.
[c] Not evaluated because RTG was 3%.

crease in mutant frequency (MF) were observed. In the absence of S9-mix, a positive response was observed at the highest concentration tested (i.e., 4130 μg/ml) in the first experiment (Table 2). The mean RTG (relative total growth; a measurement for cytotoxicity) was 15% and the mean MF was 141 above the negative control (DMSO), which is a positive response according to the guidelines. However the variation between duplicate cultures was large; the individual mutant frequencies were 173 and 403 and the RTG values were 25% and 4%, respectively. According to the guidelines cultures with more than 90% cytotoxicity (RTG < 10%) are excluded from evaluation, as increases of the mutant frequency at these levels of cytotoxicity are considered to be an artefact and not indicative of genotoxicity. In the second assay, at the two highest concentrations evaluated a positive (2920 μg/ml) and an equivocal (3590 μg/ml) response was observed (Table 2). Although positive responses were observed, these were again not reproducible (large variation) and there was no clear dose response, therefore under the conditions used in this experiment the results of this test are considered equivocal.

Initially two chromosome aberrations assays with CHO cells and pAOS dissolved in DMSO were performed. In all pulse treatment tests (4 h/18 h) both in the presence and in the absence of S9-mix no increase in the number of aberrant cells was observed and a slightly cytotoxic response at the highest dose level tested (4220 μg/ml) (data not shown). However, in the continuous treatment (18 h/18 h) a dose-related increase in both the number of aberrant cells and cytotoxicity was observed at and above 2530 μg/ml in the absence of a metabolic activation (Table 3). Based on these results pAOS was clastogenic in CHO cells under conditions used in this study. For exploratory reasons the continuously treatment in the absence of S9-mix was repeated. In this experiment pAOS was tested both as solution in DMSO and as suspension in culture medium. With respect to cytotoxity, it was demonstrated that pAOS dissolved in DMSO induced more cytotoxicity compared to suspension of pAOS in culture medium (Table 3). In addition, pAOS in DMSO induced a dose-related significant increase in the number of aberrant cells starting at 3000 μg/ml with simultaneous cytotoxicity (MI of 55% and lower). When pAOS was suspended in culture medium a significant increase of the number of aberrant cells was only observed at the highest recommended dose level tested, i.e., 5000 μg/ml. This was associated with high cytotoxicity (MI of 34%) and the result was, therefore, considered not to be biologically relevant. The increase in number of aberrant cells observed at 4000 μg/ml, although significantly different compared to the controls, was within the historical range of the negative control (TNO data). Based on these results it was concluded that pAOS suspended in the culture medium was cytotoxic but not clastogenic.

In the sub-chronic test exposure to pAOS for 13 weeks was ±7 g/kg bw/day, which is higher compared to the maximum recommended limit dose for the micronucleus tests of 1 g/kg bw/day for treatment longer than 14 days. No increase in the mean number of micronuclei in erythrocytes was observed in rats fed pAOS compared to the negative control group neither in males nor in females (data not shown). The ratio between normochromatic and polychromatic erythrocytes was not changed due to treatment with pAOS, which means that cytotoxicity was not demonstrated. The positive controls gave the expected increase numbers of micronucleated polychromatic erythrocytes.

Histopathological evaluation of the stomach and the small and large intestines of animals exposed to pAOS for 13 weeks revealed no epithelial changes (such as cytotoxicity, irritation or inflammation) that may indirectly lead to (geno)toxic effects (data not shown).

Table 3
Mitotic indices and chromosomal aberrations of cells treated continuously (treatment/harvesting time 18 h/18 h) in the absence of S9-mix with pAOS either dissolved in DMSO (test 2 and 3) or culture medium (test 3).

Dose (μg/ml)	pAOS dissolved in DMSO			pAOS dissolved in Culture medium		
	Mitotic index[a]	Chromosome aberrations[b]	Gaps[c]	Mitotic index	Chromosome aberrations	Gaps
Test 2[#]						
0[d]	100	0	0			
1690	85	1	0			
2530	66	6**	1			
3370	59	16.5**	2			
Positive control[e]	81	52**	0			
Test 3						
0[d]	100	1	6	100	0	6
1000	75	2	4	88	1.5	8
2000	67	4	4	81	2	7
3000	55	8**	6	74	2	8
4000	37	16**	5	59	2.5*	9
5000	ND[f]	ND	ND	34	8.5**	6
Positive control[e]	56	25.5**	0			

[#] Test 1 and test 2, pulse treatment (4 h/18 h): data not shown.
* $p \leqslant 0.05$ (one-sided Fisher's exact probability test) when compared to vehicle control.
** $p \leqslant 0.001$ (one-sided Fisher's exact probability test) when compared to vehicle control.
[a] Percentage of CHO cell in mitosis compared to control.
[b] Percentage of mitotic cells exhibiting chromosome aberrations.
[c] Number of cells exhibiting only gaps.
[d] Either 1% DMSO or culture medium.
[e] Positive control: mitomycin C 0.05 μg/ml.
[f] Not determined, due to limitations in solubility of the test substance.

3.2. Repeated oral toxicity studies

3.2.1. Study 1: sub-chronic (13-week) study, preceded by an in utero exposure phase

The overall intake of the pAOS or scFOS was calculated from the nominal dietary concentration, the feed consumption and the body weight. The calculated intake of pAOS in the F_0 females during the premating and the gestation period was 3.1 and 6.2 g/kg bw/day in the 5% pAOS and 10% pAOS group, respectively. During the lactation period, the overall intake of pAOS in the F_0 females doubled. In the subsequent sub-chronic study the intake of pAOS in the F_1 rats was 3.4 g and 7.1 g/kg bw/day in the 5% and 10% pAOS dietary groups respectively. The overall mean intake of scFOS (10% in the diet) in the reference control group (study 1) was in the same order as the intake of pAOS in the high-dose group.

No clinical signs attributable to pAOS were observed in the F_0 females during premating and gestation period. There were no relevant effects on body weights, growth rate or feed intake of the F_0 rats during the premating, gestation and lactation period. At the end of the lactation period (starting from day 12), most F_0 females of the reference control group (10% scFOS) showed soft faeces and/or diarrhoea, and all F_0 females of this group showed irritation of the anus. Most probably, these effects were caused by the considerable increased consumption of the diet containing 10% scFOS during this period of lactation resulting in a higher exposure to scFOS. These effects on stool consistency were not observed in the pAOS groups. Macroscopic examination of the F_0 male and F_0 female animals of the test groups (5% and 10% pAOS) at sacrifice did not reveal any relevant changes. In addition, no treatment-related effects on reproductive indices were observed. The general condition and macroscopy of pups were not affected by treatment nor were litter size, pup viability or sex ratio. The mean weight of the pups of the high-dose groups was statistically significantly increased on post natal days 1, 4 and 7 as compared to both control groups. This effect was related to the slightly (but not significantly)

Table 4
Urinary pH, calcium and sodium excretion and urinary electrolyte concentration in urinary samples[a] F_1 rats (10/sex/group) after receiving pAOS via the diet for 13 weeks (study 1).

Parameter	Control	Reference control	Test groups	
		10% scFOS	5% pAOS	10% pAOS
Males				
Urinary volume (ml)	4.1 ± 1.2	4.1 ± 1.3	3.5 ± 0.7	4.4 ± 1.4
Urinary density (kg/l)	1.037 ± 0.010	1.039 ± 0.007	1.042 ± 0.010	1.036 ± 0.008
pH	7.6	7.2	8.1[#]	8.2[#]
Calcium excretion (μmol/16 h)	3.8 ± 0.6	8.8 ± 4.4[*]	3.9 ± 1.4[#]	5.8 ± 2.6[*]
Sodium excretion (μmol/16 h)	241.4 ± 83.7	253.8 ± 143.1	414.9 ± 162.5[*,#]	430.9 ± 111.5[*,#]
Calcium concentration (mmol/l)	1.02 ± 0.36	2.13 ± 0.63[*]	1.12 ± 0.31[#]	1.34 ± 0.44[#]
Chloride concentration (mmol/l)	64.4 ± 23.7	71.7 ± 17.5	79.3 ± 29.9	51.7 ± 28.0[#]
Potassium concentration (mmol/l)	226.4 ± 70.9	240.0 ± 44.8	248.8 ± 50.2	236.0 ± 59.4
Sodium concentration (mmol/l)	60.6 ± 19.2	59.3 ± 16.2	121.9 ± 45.0[*,#]	104.7 ± 32.7[*,#]
Females				
Urinary volume (ml)	1.9 ± 0.5	2.1 ± 0.8	2.4 ± 0.8	3.0 ± 1.0[*]
Urinary density (kg/l)	1.052 ± 0.011	1.046 ± 0.008	1.048 ± 0.010	1.042 ± 0.012
pH	5.6	5.6	5.9	6.3[*]
Calcium excretion (μmol/16 h)	3.8 ± 1.0	8.9 ± 8.6[*]	7.5 ± 5.0[*]	10.9 ± 5.0[*]
Sodium excretion (μmol/16 h)	170.3 ± 64.1	164.8 ± 58.5	234.9 ± 85.2	304.2 ± 117.9[*,#]
Calcium concentration (mmol/l)	2.06 ± 0.69	3.89 ± 2.76[*]	3.08 ± 1.25	3.71 ± 1.48[*]
Chloride concentration (mmol/l)	81.5 ± 22.5	79.1 ± 54.5	76.3 ± 35.3	61.4 ± 32.1
Potassium concentration (mmol/l)	215.7 ± 59.3	243.2 ± 53.5	209.1 ± 42.4	189.9 ± 42.2[#]
Sodium concentration (mmol/l)	88.9 ± 26.3	96.9 ± 83.0	104.6 ± 46.5	110.9 ± 49.8

[*] $p < 0.05$; significantly different from controls.
[#] $p < 0.05$; significantly different from reference controls. Values are presented as means ± SD.
[a] Samples were collected in rats deprived of water and feed on day 88.

higher number of pups delivered in the control groups. After culling, this effect gradually disappeared.

In the subsequent sub-chronic study with the F_1 rats no treatment-related clinical signs were observed and none of the rats died during the study. Ophthalmoscopic examination did not reveal any treatment related ocular changes. Neurobehavioural observations and motor activity assessment did not indicate any neurotoxic potential. There were no relevant differences in body weight, growth rate and feed intake. Water consumption was increased in males of all groups and in females of the reference control group and the high-dose group. Since this finding was observed both in the pAOS groups and the reference group it is considered to be a response to the feeding of high dose oligosaccharides rather than a specific effect of pAOS (data not shown).

Haematology measurements did not reveal treatment-related changes in red blood cell- and coagulation parameters, total white blood cell counts and differential white blood cell counts. Significant changes in clinical chemistry parameters were only observed for male rats. Plasma concentrations of cholesterol, phospholipids and triglycerides were significantly decreased and the urea concentration was significantly increased in males of the 10% pAOS group when compared to the control group. Because similar changes were noted in males of the 10% scFOS group, they were considered to reflect physiological responses to the feeding of high fibre diet (data not shown).

Females of the 10% pAOS group showed a statistically significant increase in the urinary volume, but the urinary density was not affected (Table 4). Urinary calcium excretion was significantly increased for groups treated with 10% scFOS (both sexes), 5% pAOS (females), and 10% pAOS (both sexes). The urinary sodium concentration was significantly increased for males treated with 5% and 10% pAOS as a result of the high sodium content of the test substance (Table 4). Urinary sodium excretion was significantly increased for groups treated with 10% pAOS (both sexes) and 5% pAOS (males). The urinary pH was statistically significant increased in females of the 10% pAOS group when compared to the control group, and in males of the 5 and 10% pAOS, when compared to the reference (10% scFOS) control group (Table 4). Semi-quantitative (dipstick) urinary measurements and microscopy of the urinary sediment did not reveal relevant changes (data not shown).

Daily examination of vaginal smears of F_1 females during the last three weeks of the study did not reveal any effect of pAOS on oestrus cycle length and normality. Sperm analysis at the end of the study did not reveal relevant changes in epididymal sperm motility and sperm count, testicular sperm count (including daily sperm production) and sperm morphology.

The absolute (not shown) and relative (Table 5) weights of the full and empty caecum were increased in males and females of the reference (10% scFOS) and the 10% pAOS group as compared to the control group. In the 5% pAOS group, the caecum weights

Table 5
Mean terminal body weight (g) and relevant relative organ weights (g/kg body weight) in F_1 rats (10/sex/group) after receiving pAOS via the diet for 13 weeks (study 1).

Parameter	Control	Reference control	Test groups	
		10% scFOS	5% pAOS	10% pAOS
Males				
Terminal body weight	336 ± 20	317 ± 40	346 ± 28	341 ± 23
Kidneys	5.37 ± 0.34	5.61 ± 0.37	5.69 ± 0.22	6.09 ± 0.40[*,#]
Caecum full	11.0 ± 3.1	19.9 ± 3.0[*]	13.9 ± 1.4[*,#]	17.1 ± 2.2[*,#]
Caecum empty	2.4 ± 0.3	3.6 ± 0.0[*]	2.8 ± 0.5[#]	3.8 ± 0.5[*]
Females				
Terminal body weight	200 ± 11	193 ± 9	198 ± 10	207 ± 13[#]
Kidneys	5.98 ± 0.37	5.80 ± 0.30	6.11 ± 0.46	6.25 ± 0.52
Caecum full	13.6 ± 3.9	19.4 ± 2.5[*]	15.2 ± 2.5[#]	17.4 ± 4.2[*]
Caecum empty	3.2 ± 0.3	4.2 ± 0.2[*]	3.4 ± 0.4[#]	3.6 ± 0.3[*,#]

[*] $p < 0.05$, significantly different from controls.
[#] $p < 0.05$, significantly different from reference controls. Values are presented as means ± SD.

Table 6a
Incidences of changes in urinary bladder of F_1 rats of study 1 after receiving pAOS for 13 weeks.

(a) Study 1								
Treatment	Incidence of lesions (numeric)							
	Males				Females			
	Control	10% scFOS	5% pAOS	10% pAOS	Control	10% scFOS	5% pAOS	10% pAOS
Number of animals examined	10	10	10	10	10	10	10	10
Changes								
Focal hyperplasia[a]	0	0	2	0	0	0	2	0
Diffuse hyperplasia[b]								
Very slight	1	0	1	3	0	0	1	3
Slight	0	0	0	4	0	0	0	1
Score expanded totals	1	0	1	7*	0	0	1	4
Focal mononuclear cell infiltrate	0	0	1	0	0	0	0	0

* $p < 0.05$, significantly different from control.
[a] Focal hyperplasia: thickening of the transitional epithelial layer of only a part of the urinary bladder lining.
[b] Diffuse hyperplasia: thickening of the transitional epithelial layer of the urinary bladder.

Table 6b
Incidences of changes in urinary bladder of rats of the supplementary study after receiving pAOS for 13 weeks (study 2).

(b) Supplementary study (Study 2)										
Treatment	Incidence of lesions (numeric)									
	Males					Females				
	Control	1% pAOS	2.5% pAOS	10% pAOS	10% pAOS + NH_4Cl	Control	1% pAOS	2.5% pAOS	10% pAOS	10% pAOS + NH_4Cl
Number of animals examined	10	10	10	10	10	10	10	10	10	10
Changes										
Diffuse hyperplasia[a]										
Very slight	0	0	0	4	0	0	1	0	2	0
Focal mononuclear cell infiltrate	0	2	1	2	1	0	0	0	0	0

[a] Diffuse hyperplasia: thickening of the transitional epithelial layer of the urinary bladder.

were also increased but to a lesser extent. The absolute and relative weights of the kidneys were statistically significantly increased in males of the 10% pAOS group as compared to both control groups.

Macroscopic examination of the F_1 rats at necropsy did not reveal any adverse effects. Microscopic examination revealed treatment-related histopathological changes in the urinary bladder of animals of the 10% pAOS group (Table 6a). These changes were characterised by thickening of the transitional epithelial layer of the urinary bladder (diffuse simple urothelial hyperplasia). The changes were slightly more prominent in males than in females. One male and one female of the 5% pAOS group, and one male of the control group also showed diffuse hyperplasia, but only in the gradation 'very slight'. In addition, two males and two females of the 5% pAOS group showed simple hyperplasia in only a part of the urinary bladder lining ('focal hyperplasia'). No treatment-related hyperplasia of the transitional epithelium was observed in the kidney. All other histopathology changes observed in organs and tissues were common findings in rats of this age and strain, and their incidences were comparable amongst the examined groups.

3.3. Study 2: supplementary sub-chronic(13-week) study

The overall intake of pAOS, both with and without NH_4Cl, in high-dose rats was comparable to the intake in F_1 rats in the sub-chronic study (study 1), namely 7.2 and 7.1 g/kg body weight/day respectively. The overall intake of pAOS in the 1% and 2.5% dose groups was 0.7 g and 1.7 g/kg body weight/day, respectively. There were no treatment-related clinical signs and none of the rats died during the study. In addition, there were no statistically differences in body weights between the control group and the groups receiving pAOS. Body weights were statistically significantly decreased in males of the additional group (10% pAOS + NH_4Cl) throughout the study. Similar to the first sub-chronic study water consumption was increased with 10% pAOS in both sexes (with and without NH_4Cl; data not shown).

Urinary pH, obtained in fresh urine samples, was consistently high (alkaline) in the 1%, 2.5% and 10% pAOS group as well as in the controls (Table 7). Occasionally, the pH in the pAOS groups was somewhat higher than in the controls. In the additional high-dose group (10% pAOS + NH_4Cl) the urinary pH was consistently lower (neutral or acidic) than in the other groups. Because the acidifying effect was less marked than anticipated, the dietary

Table 7
Urinary pH in freshly obtained (early morning) urine from rats (10/sex/group) receiving pAOS via the diet for 13 weeks (study 2); samples were collected on day 6, 19, 41 and 83 (females)/90 (males) of the study during the first 2–3 h of the light period.

Group	Collection day			
	6	19	41	83(f)/90(m)
Males				
Control	8.2	8.0	8.0	7.7
1% pAOS	8.1	7.9	8.0	7.4
2.5% pAOS	8.3	8.1	8.0	7.8
10% pAOS	8.3**	8.0	8.1	7.7
10% pAOS + NH_4Cl	7.1**	5.6**	5.9**	5.7**
Females				
Control	7.9	7.8	8.3	7.9
1% pAOS	7.8	7.9	8.1*	7.4
2.5% pAOS	7.9	8.1**	8.2	7.9
10% pAOS	8.2*	8.2**	8.2	7.6
10% pAOS + NH_4Cl	7.0**	5.9**	6.2**	6.0**

* $p < 0.05$, significantly different from controls.
** $p < 0.01$, significantly different from controls.

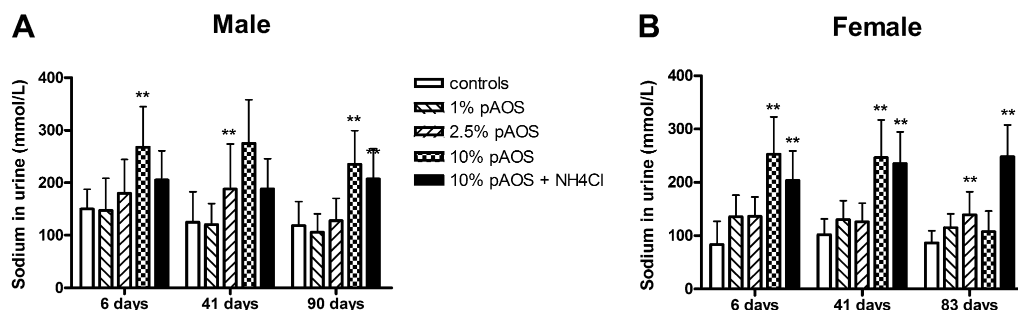

Fig. 1. Sodium concentration in fresh (early morning) samples of male (A) or female (B) rats (10/sex/group) fed pAOS and in combination with NH_4Cl for 13 weeks (study 2). Urine samples were collected on day 6, 19, 41 and 83 (females)/90 (males) of the study during the first 2–3 h of the light period. $^{*}p < 0.05$; $^{**}p < 0.01$, significantly different from controls.

NH_4Cl concentration in this group was raised from 1% to 2% from day 11.

Due to the high sodium content of the test substance (3.1% sodium), urinary sodium excretion (related to creatinine) was increased in rats fed 10% pAOS with and without NH_4Cl (data not shown). As a result, urinary sodium concentration in fresh urine samples was generally higher in males and females fed 10% pAOS (see Fig. 1).

In the additional high-dose group (10% pAOS + NH_4Cl) in females at all stages and in males at day 90 urinary calcium concentration was increased. This finding was ascribed to increased calcium excretion, due to reduced renal tubular re-absorption caused by the (NH_4Cl-induced) acidosis (Lemann et al., 1967; Heusel et al., 1999). In addition to this, it well-known that non-digestible carbohydrates may stimulate the intestinal calcium absorption in rats, which also results in an increased excretion of calcium (Brommage et al., 1993; Hodkinson et al., 1982; Younes et al., 1995). As a result of feeding of NH_4Cl, chloride excretion and concentration values were increased in the additional high-dose group (data not shown).

Similar to study 1, the absolute and relative weights of the full and empty caecum were increased in males and females fed 10% pAOS (with and without NH_4Cl). The relative weights of the kidneys were statistically significantly increased in males fed 10% pAOS (with and without NH_4Cl) as also was seen in study 1 (data not shown).

At necropsy no treatment-related macroscopically changes were observed. Microscopic examination showed diffuse urothelial hyperplasia in four males of the 10% pAOS group. A similar change, albeit less prominent, was observed in two females of this group (Table 6b). Very slight diffuse hyperplasia was also noted in one female of the 1% pAOS group, but this finding, which occurred in one animal only, was not confirmed at the next higher dose-level, and was therefore considered incidental. None of the rats of the additional high-dose group (10% pAOS + NH_4Cl) showed transitional epithelial hyperplasia.

4. Discussion

For the confirmation of the safety of pectin-derived acidic oligosaccharides (pAOS) in its intended use and to obtain regulatory approval, a number of preclinical studies were performed. The genotoxic potential of pAOS was investigated in both *in vitro* and *in vivo* assays. pAOS was not mutagenic in the bacterial reverse mutation assay (Ames test). In the mammalian gene mutation test and the chromosomal aberrations test effects were observed only at concentrations that induced considerable high cytotoxicity in the absence of metabolic activation. Considering the nature and the chemical characteristics of the main product in the test preparation, namely a natural occurring type of non-digestible carbohydrates (oligosaccharides), it was assumed that the AOS itself was not responsible for the apparent positive results. Moreover, a positive response was only observed at highly cytotoxic concentrations and it is known that general cytotoxic effects, like oxidative stress, have been suspected of generating false positive results in *in vitro* genotoxicity tests at high levels of cytotoxicity (Kirkland et al., 2007). Because DMSO has been shown to interfere with the cyto- and genotoxicity of test substances in various *in vitro* genotoxicity assays (Amberg et al., 2008; Eder and Deininger, 2002; Gebel and Koenig, 1999) further investigations were carried out to investigate the effect of the solvent on the mutagenic potential of pAOS. For this purposes a suspension of pAOS in aqueous buffer was included since pAOS when used in commercial applications will be dissolved or suspended in water. With both solvents an increase in number of abberant cells were observed but again only at very high cytotoxic concentrations. However, when pAOS was suspended in culture medium it was clearly less toxic to the cell than when dissolved in DMSO, suggesting an interaction between DMSO and pAOS. This interaction of DMSO with pAOS may also explain the equivocal results obtained in the mammalian gene mutation assay.

The mutagenic potential of pAOS, including the impurities in the preparation and breakdown products of pAOS, was investigated under more relevant exposure conditions, in rats, exposed to pAOS for 13 consecutive weeks, by analysing the bone marrow for chromosomal aberrations. It was demonstrated that pAOS given at 10% in the diet (>7 g/kg bw/day), did not induce chromosomal damage and/or damage to the mitotic apparatus in the bone marrow cells of both male and female rats. Since pAOS is considered non-digestible and therefore not systemically available (Govers et al., 2005; Vaisman et al., 2004) it can be assumed that impurities in the pAOS preparation as provided in Table 1 and breakdown products of pAOS, i.e., SCFAs, that might be well absorbed, did not induce a systemic genotoxic response. Furthermore, there were no histopathological indications that pAOS or its breakdown products might be cytotoxic and as a result indirectly genotoxic at the site of contact.

In the sub-chronic toxicity study, preceded by an *in utero* exposure phase, the administration of pAOS to F_0 rats and dams throughout mating, gestation and lactation did not affect health, growth, fertility or reproduction performance, nor pup characteristics. Also in the subsequent sub-chronic study with the (F_1) offspring, administration of pAOS up to dietary levels of 10% was well-tolerated. Generic effects of dietary fibres at high dose occurred in both reference control rats (10% scFOS) and rats fed pAOS. The weights of the full and empty caecum were increased in these groups, which is regarded as a physiological response to feeding of fermentable carbohydrates (WHO, 1987). It is known that through the fermentation large amounts of short-chain fatty acids are produced which increase the osmotic value of caecal con-

tent and may promote the growth of the mucosal layer (Flamm et al., 2001; Roberfroid, 1993). Both with pAOS and scFOS, the fermentation in the large intestine was associated with increased urinary calcium excretion. Stimulation of the intestinal calcium absorption is a well-known effect of non-digestible but fermentable carbohydrates in rats (Brommage et al., 1993; Hodkinson et al., 1982; Younes et al., 1995). Similarly, the decreases in plasma cholesterol, phospholipids and triglycerides and the increase in urea concentration, which occurred in males of both the 10% scFOS group and the 10% pAOS group, are attributed to the feeding of fermentable carbohydrates rather than to a specific effect of pAOS. This assumption is supported by the literature showing that fermented carbohydrates lower blood cholesterol and triglycerides (Moundras et al., 1994) and can lower blood cholesterol in man (Cummings et al., 2004).

The intake of pAOS (containing approximately 3.1% sodium) resulted in an increased urinary sodium excretion, and the increase in kidney weight in males fed 10% pAOS is probably also due to the higher sodium intake. High salt diets have been shown to increase renal mass of rats (McCormick et al., 1989). Because there were no indications for disturbance of renal function or treatment-related histopathological changes in the kidneys, no toxicological significance was attached to the increased kidney weight. The high sodium content of pAOS may also have contributed to the increased water intake in rats fed pAOS and the slightly higher urinary volume in females fed 10% pAOS. Because of the fact that the products to which pAOS will be added (IF, cereal products for babies, weaning foods and medical foods) have to comply with compositional and nutritional criteria as laid down in the respective legislation (e.g. Directive 2006/141/EC for IF and Directive 1999/21/EC for medical foods) or appropriate guidelines (e.g. Codex standard 72) covering these products, the inclusion of pAOS will not cause adverse effects which are related to high sodium intake since the actual intake of sodium will be well within the acceptable levels. For instance, according to the European legislation for infant formulae these formulae must contain minimal 20 and maximal 60 mg sodium per 100 kcal. In general, around 70% of the minimum sodium requirement, thus 14 mg/100 kcal, is naturally contained in the macro ingredients (protein, carbohydrates and fat) used in the formula. In Europe, it is estimated that the highest formula intake of an infant (5–6 kg) is 1000 ml/day, which corresponds to 600 kcal/day. Assuming that the product contains pAOS at the intended use level of 0.14 g/100 ml, that is 1.4 g/1000 ml, the sodium intake will be 155 mg/day, of which 84 mg is derived from the macro ingredients in the formula and 71 mg from pAOS (maximum sodium content of 5%). This level is well within the legislative limits of 120 up to 360 mg sodium/600 kcal.

Hyperplastic changes of the transitional epithelial layer of the urinary bladder (simple urothelial hyperplasia) were noted in rats fed 10% pAOS and, to a minimal degree, in a few rats of the 5% pAOS group. Because this finding was associated with an increase in both urinary pH and urinary sodium concentration, it was ascribed to the high sodium content of the test substance rather than to the active compound AOS. Urothelial hyperplasia resulting from a concurrent increase in urinary pH and urinary sodium ion concentration is a well-known phenomenon in rats and not frequently observed in other animal species (De Groot et al., 1988; Lina and Woutersen, 1989; Lina et al., 1994). A working mechanism, involving increased sodium ion entry into the cell and increased intracellular pH with an associated increase in DNA synthesis of the bladder epithelial cells has been proposed (Harquindey, 2002; Lina, 2004). To verify the supposed mechanism of action for the findings in the urinary bladder, a supplementary subchronic study with pAOS was performed. In this study it was examined whether the observed urothelial hyperplasia could be prevented by acifidication of the urine through the addition of the acidifying salt NH_4Cl to the 10% pAOS diet. In contrast to the first sub-chronic study, urinary pH was determined in fresh (early morning) urine samples. These pH values are representative for the night period when rats consume most of their feed (De Groot et al., 1988), and, moreover, are not compromised by fasting acidosis as was the case in the first study. The urinary pH obtained in fresh samples was high in both controls and test groups at all time points, and was only occasionally further elevated by the feeding of pAOS. The co-administration of NH_4Cl with pAOS, however, consistently resulted in a marked decrease in urinary pH. Due to the high sodium content of the pAOS material, urinary sodium excretion and sodium concentration were again significantly increased in rats receiving 10% pAOS (with and without NH_4Cl). Similar to the first study, hyperplastic changes of the transitional epithelial layer of the urinary bladder were noted in rats fed 10% pAOS. The incidence and severity of urothelial hyperplasia in this study was lower than in the first study. Nevertheless, the findings confirmed those obtained with the 10% pAOS group of the previous study. In rats fed pAOS in combination with NH_4Cl, the low urinary pH completely prevented the development of urothelial hyperplasia. This finding shows that the hyperplasia was not due to AOS *per se*, but to the concurrent increase in urinary sodium concentration and pH; a condition known to predispose to hyperplasia of the urothelium in rats (De Groot et al., 1988; Lina and Woutersen, 1989; Lina et al., 1994). A persistent combination of elevated urinary pH and high sodium levels, however, is unlikely to occur in humans since human urinary pH generally tends to be acidic and the sodium concentration is generally lower. Therefore, and because of the species specificity of the bladder response urothelial hyperplasia induced by this mechanism in rats is considered not to be of relevance to man (Lina, 2004).

The assumption that the addition of pAOS to food would not affect the urinary pH was demonstrated in a recent study with IF containing pAOS above the anticipated dose level of pAOS. As part of this prospective, placebo-controlled, double blind study, the possible effect of dietary prebiotics on urine pH was examined in 22 pre-term infants. All infants were similar fed with breast milk and/or standard pre-term formula (Nenatal Start). Starting with postnatal age of three days they were randomised to be supplemented either with a prebiotic mixture (GOS/long chain FOS in a ratio 9:1 plus 20% pectin-derived AOS) or with the placebo (maltodextrine). The prebiotics and the placebo were provided as sachets in a dosage of 1.5 g/kg bw/day, corresponding to 0.3 g pAOS/kg bw/day. There was no difference in the baseline characteristics between the groups (prebiotic group: birth weight 1251 ± 317 g, gestational age 28.9 ± 2.2 weeks, $n = 12$; control group: birth weight 1102 ± 165 g, gestational age 28.1 ± 2.2 weeks, $n = 10$). The urine pH after 7 days of supplementation was 5.58 ± 0.70 in the prebiotic group and 5.20 ± 0.42 in the control group. The difference was not significant (two sided Students *t*-test: $p = 0.13$). [Personal communication provided by Professor Harrie Lafeber and Dr. Ruurd van Elburg, Department of Paediatrics, Division of Neonatology, University Medical Center Amsterdam]. In addition, recently it has been shown that administration of twice the anticipated level pAOS (18 g/day) for 12 weeks to HAART-naïve HIV infected adults was well-tolerated, with no unexpected treatment related adverse effects and did not result in changes in both liver and renal function (Gori et al., submitted for publication). On the basis of these results it is, therefore, not to be expected that urinary pH will increase due to the intake of products containing pAOS.

5. Conclusion

Taken into account the results obtained in the various genotoxicity studies it is concluded that pAOS is not mutagenic under the

circumstances of use. Furthermore, there was no indication that pAOS or its breakdown products might be cytotoxic and as a result indirectly genotoxic at the site of contact by the absence of any histopathological changes in the gastro-intestinal tract. In the subchronic studies the administration of pAOS at dietary levels up to 10% (equivalent to 7.1 g/kg bw/day) did not reveal any effects specific for its main constituent acidic oligosaccharides. The only effects observed in the studies were related to a relative high sodium intake via the pAOS preparation. These concern the increased kidney weight (without corroborating changes in kidney function or pathological effects) in rats fed 10% pAOS and an increased sodium concentration in the urine. In addition, slight hyperplasia of epithelial cells of the urinary bladder noted in the rats fed 10% pAOS and in a few rats fed 5% pAOS was the result of a concurrently elevated urinary sodium concentration and urinary pH, a condition known to predispose to hyperplasia of the urothelium in rats. This was confirmed in a mechanistic study. Urothelial hyperplasia was not observed in rats fed 2.5% pAOS, the NOAEL for rats was, therefore, allocated at this level (equivalent to 1.7 g/kg body weight/day). This is 8.5 and 11 times the anticipated intake in infants and patients respectively. Since the final applications (IF, cereal products for babies, weaning foods and medical foods) to which pAOS will be added have to comply with compositional and nutritional criteria laid down in legislation and guidelines, these effects related to a high sodium intake will have no implications for the end user. The addition of pAOS to the specific products will not result in a sodium intake above safe levels. We conclude, therefore, that pAOS is safe under the conditions of its intended usage.

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