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Sucralose: Lack of Effects on Sperm Glycolysis and Reproduction in the Rat

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Abstract—Certain chlorine-substituted sugars with chemical similarities to sucralose have been demonstrated previously to diminish or inhibit sperm glycolysis and fertility in the rat (Ford and Waites, 1978a). In order to investigate this potential for sucralose, epididymal spermatozoa were recovered from rats exposed *in vivo* to oral doses of one of three of these substituted sugars: 6-chloroglucose (6-CG, 24 mg/kg/day, positive control), sucralose (500 mg/kg/day, over 300 times the expected human daily intake), or a 6′-substituted isomer of sucralose, trichloro de-oxy sucrose (TCDS, 100 mg/kg/day, a potential trace impurity in commercial sucralose); distilled water served as the negative control. After incubation of the spermatozoa with D-[U-^{14}C] glucose, measurements of $^{14}CO_2$ and of ATP content showed no impairment of the glycolytic ability of spermatozoa in any of the groups except for a marked inhibition for those exposed to 6-CG, the positive control. In order to determine whether other parameters of reproduction and fertility could be affected, reproductive endpoints were examined following oral exposure of male and female rats to sucralose. Sucralose was fed in the diet at concentrations of 0, 0.3, 1.0 and 3.0% (approx. 100, 365 and 1150 times the EDI) to groups of 30 male and 30 female rats for 10 weeks prior to mating, and continued through two subsequent generations until weaning of the F_2 pups. Two litters were produced per generation. Food consumption and weight gain in the F_0 and F_1 generations were depressed in all sucralose groups before mating and in all four litters prior to weaning. The decrease in initial average weight for newborn pups probably reflects the increased litter sizes noted for sucralose-treated groups and the reduced food consumption of the dams during gestation and lactation. The latter is a result primarily of the unpalatability of sucralose to rats (McNeil, 1987). Caecal enlargement (a common animal response to large doses of indigestible material) occurred in both the F_0 and F_1 parents. Increased kidney weights, possibly associated with increased water intake, were observed primarily among animals receiving 3% sucralose (no renal histopathology has been detected). Decreased thymus weights occurred in F_1 males and in both F_1 and F_2 females at the 3% level. Subsequent studies specifically designed to investigate the potential for adverse immune system effects of sucralose (McNeil, 1987) showed no adverse effects. These findings are consistent with investigations by others showing that decreases in thymus weights occur in young rats in response to stressful conditions associated with reductions in weight gain. All reproductive indices (oestrous cycles, mating behaviour, fertility, gestation, maternal and foetal viability, foetal development, parturition, pup maturation and lactation) were comparable between the control and sucralose-treated groups. We conclude from these results that sucralose has no effect on sperm glycolysis or on male or female reproductive performance in the rat. © *2000 Elsevier Science Ltd. All rights reserved*

Keywords: sucralose; rat reproduction; sperm glycolysis; monosaccharides; disaccharides; artificial sweeteners; 6-chlorosugars.

Abbreviations: 6-CG = 6-chloro-6-deoxy-D-glucose; EDI = expected daily intake; PBS = phosphate buffered saline; TCDS = 1,6-dichloro-1; 6-dideoxy- (β-D-fructofuranosyl-6-chloro-6-deoxy-(α-D-glucopyranoside; TLC = thin-layer chromatography.

INTRODUCTION

This paper describes two series of studies that were undertaken to investigate the possible effects of sucralose on reproduction and fertility. One series concerned the assessment of possible biochemical effects on epididymal spermatozoa, and the other involved a reproductive toxicological investigation covering two sequential generations of rats, each of

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two litters. The rat was considered to be the preferred species for such studies on sucralose because the rat metabolic profile for sucralose is very similar to that of man. Also, certain 6-chloro-6-deoxysugars have been reported to have biochemical effects on rat sperm glycolysis that were associated with reversible alterations in fertility (Ford and Waites, 1978). Owing to the metabolic similarities between man and rat regarding sucralose, and the apparent chemical similarities between sucralose and 6-chloro sugars, it was important to determine whether exposure to sucralose could result in adverse reproductive consequences.

Certain chlorinated monosaccharides such as 6-chloro-6-deoxy-D-hexoses and 6-chloro-6-deoxy-D-hexitols, or disaccharides which can be hydrolysed *in vivo* to such compounds, exert a reversible antifertility effect in male rats and marmoset monkeys (but not in hamsters, mice, guinea pigs or rabbits) without any effect on the numbers of spermatozoa or on the androgen status of the animals (Ford *et al.*, 1981). One hypothesis for this antifertility effect is that these compounds, in common with α-chlorohydrin which also causes male infertility, inhibit the utilisation of glucose by epididymal sperm resulting in a reduction in the concentration of ATP and in sperm motility (Ford, 1982; Ford and Waites, 1978). This effect on glycolysis has been attributed to inhibition of the enzyme glyceraldehyde 3-phosphate dehydrogenase by a metabolite of the 6-chloro-6-deoxy-D-hexoses or -hexitols, which, by analogy with α-chlorohydrin, is presumed to be either 3-chloroglyceraldehyde or a derivative thereof. As the mechanism of action is focused on mature sperm, it is not necessary for these agents to be present throughout spermatogenesis to exert their activity. Although there was no evidence for any effect of sucralose on fertility in male rats (Ford, 1982; Ford and Waites, 1978). Ford and Waites (1978) originally reported effects of sucralose and a related compound (TCDS) on rat sperm glycolysis similar to those observed with the 6-chloro-6-deoxy monosaccharides. In these experiments they found that spermatozoa obtained from male rats treated for 14 days with sucralose (96 mg/kg/day) or with 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-6-chloro-6-deoxy-α-D-glucopyranoside (TCDS, a potential trace impurity in commercial sucralose; 95 mg/kg/day), oxidized less glucose to CO_2 than spermatozoa from control rats, and contained a lower concentration of ATP after a 2-hr incubation. As these sperm metabolism findings were inconsistent with the *in vivo* fertility observations, further investigations into the potential effects of both sucralose and TCDS on rat sperm glycolysis were considered prudent. The rat spermatozoal metabolism experiments reported in the present paper were conducted in Dr Ford's laboratory as a "single-blind" trial in rats using 6-chloro-6-deoxy-D-glucose (6-CG) as a positive control.

In addition, a detailed *in vivo* analysis of sucralose was conducted to investigate thoroughly the potential for sucralose to affect the reproductive performance and fertility of male and female rats from gametogenesis through two successive generations of offspring.

MATERIALS AND METHODS

Materials

As shown below, three batches of sucralose were used, with purities determined by HPLC ranging from 97.7 to 99.4%. The purities of TCDS and of 6-CG were determined by gas–liquid chromatography.

Sperm-glycolysis study. Sucralose (batch 163003), purity 99.4%; TCDS batch 53/84/39(b), purity >98%); 6-CG (batch SD-86/01, purity >98%).

Two-generation study. Sucralose (batch 167001, purity 97.9%; batch 167002, purity 97.7%; batch 163003, purity 99.4%).

Chemical analysis at the commencement and at the completion of each study, or at the depletion of each batch, showed the test materials to be stable for the duration of the studies.

Animals

Male and female rats of the CD strain (Sprague–Dawley origin) were obtained from Charles River (UK) Ltd (Margate, Kent, UK).

Methods

Sperm glycolysis study. Adult sexually naive male rats, weighing 225–268 g on arrival, were allowed from 5 to 12 days' acclimatization before being distributed randomly to four groups of 10 animals. Each rat was individually identified by an ear notch corresponding to its identity number. The animals were housed singly in high-density polypropylene cages with stainless-steel lids and grid floors. The room temperature was maintained at 20–25°C and the relative humidity at 58–69%. A 12-hr light/dark cycle operated throughout. Tap water and a commercially available rodent diet (Labsure Laboratory Animal Diet No. 1, K & K Greeff Chemicals Ltd, Croydon, Surrey, UK), were available *ad lib.* throughout the study.

The four treatment groups consisted of a negative control group, and three experimental groups which received 6-CG (24 mg/kg/day, positive control), sucralose (500 mg/kg/day) and TCDS (100 mg/kg/day), respectively. The daily dose was administered orally by gavage in aqueous solution, or in the case of the negative control group, distilled water alone was administered, all at a volume-dosage of 4 ml/kg. Treatment continued for 28 days. All animals were weighed on days 1, 8, 15, 22 and 28 of treatment and were observed for any clinical signs and reaction to treatment. On completion of treatment,

rats were killed by cervical dislocation and the spermatozoa were flushed out of the cauda epididymides with phosphate buffered saline (PBS). The contents of both epididymides were combined and made up to a volume of about 2.0 ml with PBS buffer and transferred to a 5.0 ml conical flask. The sperm suspension was allowed to stand at room temperature for about 5 min to allow the dense mass of spermatozoa and epididymal plasma to disperse. The concentration of the spermatozoa was measured with a haemocytometer, each suspension being counted at least twice. Two 0.8-ml portions of the sperm suspensions from each animal were taken and transferred to 10 ml conical flasks (one sample per flask) containing 0.2 ml 10 mM D-[U-^{14}C]glucose (0.5 Ci/mol) dissolved in PBS buffer. The flasks were sealed with Suba-seal caps and 0.5 ml 1.0 M perchloric acid was added to one immediately. The acidified flask was placed on ice. The other was transferred to a shaking water-bath at 34°C and incubated for 2 hr. The reaction was stopped by the addition of 0.5 ml 1.0 M perchloric acid and the flask was placed on ice. $^{14}CO_2$ was collected by piercing the Suba-seal cap of each flask with two sharpened tubes, and drawing a stream of air through the flask via the tubes and then through two scintillation vials lined with glass-fibre paper soaked with 1.0 ml 0.2 M hyamine hydroxide dissolved in toluene. Air was drawn through to the scintillation vials for a period of 1 min. Scintillation fluid (5 ml) was added to the scintillation vials and the ^{14}C content determined by liquid scintillation counting; the external channels ratio procedure was used to correct for counting efficiency. The conversion of D-[U-^{14}C]glucose to $^{14}CO_2$ was calculated in terms of nm glucose oxidized/10^8 spermatozoa. The concentration of ATP in perchloric acid extracts of spermatozoa was measured by a fluorimetric technique (Williamson and Corkey, 1969). The concentration of ATP was expressed as nm/10^8 spermatozoa.

Two-generation reproductive study in rats. Weanling litter-mate rats, in the weight range 162–210 g for males and 120–165 g for females, were randomly distributed into four groups each consisting of 30 males and 30 females. After a 16-day acclimatization period all animals were identified by unique ear notching. Except during mating, littering and lactation, animals were housed in groups of five, by sex, using the same type of cage described for the sperm glycolysis experiments. During littering and early lactation, each female and her litter were housed in cages with solid polypropylene bottoms and were provided with autoclaved wood shavings as bedding; they were transferred to cages with grid floors during the latter stages of lactation. Other housing conditions were as previously described, except that the rodent diet employed was slightly different (Labsure Laboratory Animal Diet No. 2, K & K (Greeff Chemicals Ltd). Tap water and this diet, or this same diet supplemented with sucralose, were available *ad lib.* throughout the study.

The four treatment groups consisted of an untreated control group and three treated groups which received sucralose at 0.3, 1.0 or 3.0% of the diet. Diets were made up freshly each week. The diet formulations were satisfactorily homogenous and sucralose was stable for at least 15 days in diets stored at 23 ± 2°C. Analysis of diets throughout the treatment period showed that all diets contained sucralose at concentrations within 10% of the target levels.

Both sexes of the F_0 and F_1 generations were fed sucralose-treated diets beginning at least 10 wk (males) or 2 wk (females) prior to the initial mating. Exposure to control or sucralose-containing diets was continuous for 26 or 28 wk (males and females, respectively) at which time the parental F_0 and F_1 generations were terminated.

First generation (F_0) parental animals were paired twice during the study. The first pairing produced the F_{1A} litters that were discarded at weaning following examination for macroscopic abnormalities. After the second pairing, delivery and observation of the F_{1B} pups, 30 male and 30 female weanling offspring were selected for continuation of the study as the F_1 parental generation. Where possible, one male and one female were randomly selected from each litter, each selected pup being identified by a unique ear notch. These F_1 animals were treated comparably to their F_0 parents, that is for 10 wk (males) or 2 wk (females) before being paired twice in succession, to produce F_{2A} and F_{2B} offspring that were discarded after observation and weaning. As described above, the animals were continuously exposed to either control or sucralose-containing diets until termination of the parents.

During the 10-wk premating period, F_0 and F_1 animals were monitored for clinical signs of adverse reaction to treatment and individual weekly records of body weight gain (males weekly until termination; females weekly until confirmation of mating, then at specified intervals during gestation and lactation), food and water consumption, and food conversion efficiency (weight gain/food consumed × 100) were maintained. For 10 days before pairing for the first (F_A) mating of each generation, daily vaginal smears were taken from all females to establish the normality of the oestrous cycle. This was continued after pairing with the male until evidence of mating was observed. Females were paired on a one-to-one basis with males from the same treatment group, but avoiding the pairings of siblings. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs. Mating was further confirmed by preparing a vaginal smear from each female and examining it for the presence of spermatozoa. The day on which evidence of mating was found was

designated day 1 of gestation. Once mating had occurred, the males and females were separated and vaginal smearing discontinued. Females who failed to mate within 21 days of pairing were removed and placed with a proven male from the same treatment group for a period of up to 7 days. The time elapsing between initial pairing and detection of mating was noted. The uteri of "non-pregnant" females were examined for the presence of implantation sites by the staining method of Salewski (1964). F_0 parental animals were rested for a minimum of 10 days before the second pairing (F_B), and the procedure repeated. Different pairing combinations were used for the second mating of each generation, again avoiding mating between siblings. The same procedure was adopted for the F_1 parental animals after a minimum of 10 wk of treatment.

Females were weighed on days 1, 7, 14 and 21 *post coitum* and on days 1, 4, 7, 14, 21 and 25 *post partum*. From day 21 *post coitum*, females were inspected at least twice daily for the onset and completion of parturition, and the time elapsing between the detection of mating and commencement of parturition was noted. Food consumption during gestation and lactation was recorded during the F_0-F_{1A} littering alone. All litters were examined at approximately 24 hr after birth (day 1) and the following recorded for each litter: number born (alive and dead); litter weight; individual sexes; observations on individual offspring. Live birth indices (number alive on day 1/number offspring at day 1×100), viability indices (number alive on day 4/number alive on day 1×100) and lactation indices (number alive on day 25/number in standardized litters on day 4×100) were calculated for each litter. All offspring were examined daily and any visible signs of reaction to treatment were recorded. Any offspring found dead were examined externally and internally. On day 4 *post partum*, all litters containing more than eight offspring were randomly culled to four males and four females where possible. Offspring were weighed as litters on days 1, 4, 7, 14, 21 and 25 *post partum* and were sexed on days 1, 4, 14 and 25 *post partum*. The rate of physical development of offspring was assessed on a total litter basis by recording the days on which onset and completion of the following parameters occurred: pinna unfolding; hair growth; tooth eruption; and eye opening. Auditory and visual responses were assessed in offspring of B litters at weaning by using the startle response to a sudden sharp noise and by examination of the pupil closure response to a bright source of light and assessment of the visual placing response. All rats, except the discarded offspring, were subjected to a necropsy. Tissues from all the major systems were removed, weighed and retained in buffered formal saline (4%, v/v, formaldehyde in PBS) but were not examined microscopically.

Statistical analysis

In the sperm-glycolysis study, the significance of inter-group differences in the oxidation of D-[U-^{14}C]glucose to $^{14}CO_2$ and the concentration of ATP was assessed by analysis of variance followed by Tukey's studentized range test.

In the two-generation study, body weights, body weight change and litter size were evaluated by Student's *t*-test, absolute and body weight-related organ weights by Dunnett's *t*-test, pre-coital interval and gestation length by Mann–Whitney U-test, mating performance, conception rate, fertility index, gestation index, live birth index, viability index, lactation index and sex ratio by chi-square test, Fisher's exact probability test or Mann–Whitney U-test.

RESULTS

Sperm glycolysis study

Clinical condition and growth rate. The general condition of males in all treated groups was similar to that of the negative control animals receiving distilled water. There were no deaths. Body weight gains of treated animals were similar to, or slightly greater than, those of the negative control group.

Concentrations of spermatozoa. The concentrations of spermatozoa during incubation are shown in Table 1. It was observed that the epididymides of a rat from group 4 (TCDS; animal no. 27) were much smaller than normal for a rat of its body weight, and the tubules appeared small and pink in colour rather than packed with white masses of spermatozoa. Few spermatozoa were recovered and they had a very low ATP concentration at zero time. The reproductive system of all other rats appeared normal and there were no significant inter-group differences in the mean concentrations of spermatozoa recovered in the flushings from the epididymides. As animal 27, group 4, was the only animal presenting with this condition, it was concluded that the lesion was not related to treatment. Nevertheless, the data were evaluated both including and excluding the values for this animal in group 4. While group 4 means differed somewhat between the two methods, as expected, the overall conclusions were not affected by excluding animal 27.

$^{14}CO_2$ production. There was no difference in the production of $^{14}CO_2$ from [U-^{14}C]glucose (nm/10^8 spermatozoa; mean $\pm$ SD, n = 10) between spermatozoa from rats treated with sucralose (group 3: 79.4 ± 56.17) or with TCDS (group 4: 83.6 ± 42.06, excluding animal no. 27) and those from the negative control (group 1; 73.5 ± 19.28) (Table 2). Spermatozoa from rats treated with 6-CG produced considerably less $^{14}CO_2$ (group 2; 2.3 ± 0.78). This was highly significantly different from the values for

Table 1. Concentration ($\times 10^8$spermatozoa/ml) of sperm suspensions during incubation

Group	Treatment	Mean	SD	Minimum value	Maximum value
1	Negative control (water)	0.79	0.19	0.54	1.10
2	Positive control (6-CG 24 mg/kg/day × 28)	0.73	0.23	0.48	1.10
3	Sucralose (500 mg/kg/day × 28)	0.75	0.18	0.53	1.15
4	TCDS (100 mg/kg/day × 28)	0.62	0.24	0.22	0.97
4	TCDS Excluding rat no. 27	0.66	0.20	0.32	0.97

all other groups ($P < 0.001$) by analysis of variance followed by Tukey's studentized range test.

The amount of $^{14}CO_2$ produced varied greatly within each group, but, if animal 27 is excluded, the maximum CO_2 production in group 2 (6-CG; 4.2 nm/10^8 spermatozoa) is less than the minimum in all the other groups (group 3; 16.7 nm/10^8 spermatozoa).

ATP concentrations. The concentration of ATP at zero time (Table 3) varied between 40 and 175 nm/10^8 sperm (excluding animal 27). The mean concentration ($\pm$SD, n = 10) was least in sperm from rats treated with 6-CG (Group 2; 92 ± 35.2), which was slightly less than that from TCDS, when the complete data set was analysed (negative control, 120 ± 28.1; sucralose, 119 ± 32.6; TCDS, 133 ± 31.7). The mean concentration of ATP (nm/10^8 sperm) after the incubation (Table 4) was very similar in the negative control group (group 1; 28.8 ± 14.79), the sucralose-treated group (group 3; 29.9 ± 21.91) and the TCDS-treated group (group 4; 39.9 ± 18.62). However, the mean concentration of ATP after 2 hr of incubation was significantly less in the positive control group receiving 6-CG (Group 2; 4.2 ± 4.24). This difference was statistically highly significant ($P < 0.001$) by one-way analysis of variance).

Two-generation study: F_0 and F_1 parental generations

Clinical condition and mortality. No treatment-related effects on clinical condition were observed. Two F_0 animals (one male receiving 0.3% of sucralose and one female receiving 1.0% sucralose) and one F_1 animal (a female receiving 1.0% sucralose) died *in extremis* but the cause of ill-health was not attributed to treatment.

Growth, food and water consumption. Body weight gain of F_0 and F_1 males and females during the maturation phases was reduced significantly at all treatment levels except for male F_1 animals receiving 1.0% sucralose (Table 5). Food intake of F_0 and F_1 parents was reduced in all treatment groups throughout the maturation period before the first pairings, the effect being less marked in F_1 animals than F_0 animals. During the F_{1A} gestation and lactation phases, food intake by treated females was essentially similar to that of controls. Likewise, body weights of dams at day 1 *post coitum* and day 1 *post partum* were significantly reduced in all sucralose-treated groups compared to controls.

Food conversion efficiency for F_0 males and for F_1 males and females during the maturation period was similar in the treated and control groups. Food conversion efficiency of F_0 females, however, was slightly reduced in all treated groups during the maturation period, particularly in the first 5 wk of treatment.

Mean cumulative water intake of sucralose-treated F_0 males during maturation was similar to the controls, but water intake of F_0 females showed a slight, dose-related increase that was statistically significant at the 1.0% and 3.0% dietary levels. Cumulative water intake during maturation was significantly increased for F_1 males at 1.0% and 3.0% sucralose, and for F_1 females at all levels.

Mating performance and fertility. Oestrous cycles, pre-coital interval, mating performance and fertility indices were unaffected by dietary concentrations of sucralose up to 3.0% throughout two generations, with two litters per generation (Table 6). Most matings occurred during the first oestrous after pairing. Conception rates and fertility indices for treated groups in the F_1 generation were slightly higher than those of the control group at both matings.

Gestation length and gestation index. Gestation length was similar in all groups and for all pairings. Body weight gain during gestation was reduced significantly at 0.3% in the F_{1A} pairing and at all treatment levels in the F_{1B} pairing (Table 6).

Table 2. The oxidation of D-[U-^{14}C]glucose to $^{14}CO_2$ (ηmol glucose converted/10^8 spermatozoa) during incubation for 2 hr at 34°C

Group	Treatment	Mean	SD	Minimum value	Maximum value
1	Negative control (water)	73.5	19.28	42.0	105.3
2	Positive control (6-CG 24 mg/kg/day × 28)	2.3***	0.78	1.5	4.2
3	Sucralose (500 mg/kg/day × 28)	79.4	56.17	16.7	181.1
4	TCDS (100 mg/kg/day × 28)	75.6	47.11	3.2	147.6
4	TCDS Excluding rat no. 27	83.6	42.06	26.3	147.5

***Significantly less than all other groups, $P < 0.001$ (analysis of variance followed by Tukey's studentized range test).

Table 3. Concentration (ηmol/10^8 spermatozoa) of ATP at the start of the incubation

Group	Treatment	Mean	SD	Minimum value	Maximum value
1	Negative control (water)	120	28.1	73	173
2	Positive control (6-CG 24 mg/kg/day × 28)	92	35.2	40	154
3	Sucralose (500 mg/kg/day × 28)	119	32.6	68	164
4	TCDS (100 mg/kg/day × 28)	121	50.0	6.7	175
4	TCDS Excluding rat no. 27	133*	31.7	89	175

*Significantly different from group 2, $P < 0.05$ (analysis of variance followed by: Tukey's studentized range test).

Two-generation study: F_{1A}, F_{1B}, F_{2A} and F_{2B} offspring

Litter responses. The general condition and appearance of offspring before weaning were similar in all groups (Table 6).

Live litter size in both the 1.0% and 3.0% groups was significantly greater than that of the control group for the F_{2A} litters ($P < 0.05$). Initial litter sizes of F_{1A}, F_{1B} and F_{2B} litters followed the same trend toward greater numbers of foetuses in the sucralose-treated groups, but without statistical significance. Offspring viability was unaffected by treatment with sucralose. Sex ratio, offspring development and auditory and visual sensory functions were also unaffected by treatment.

Body weight of offspring. Body weight of F_{1A}, F_{1B}, F_{2A} and F_{2B} offspring at day 1 *post partum* showed a slight reduction in all sucralose-treated groups, but statistical significance was only achieved by F_{1B} and F_{2A} offspring receiving 3.0% sucralose. Subsequent body weight gain up to day 25 *post partum* was significantly reduced for offspring in all sucralose-treated groups except for F_{1B} pups at the 0.3% level (Table 6).

Pathology. Examination of the few offspring that died before weaning revealed the absence of milk in the stomach, indicating failure to suckle, as the only consistent finding. Although offspring killed after weaning presented a number of common minor abnormalities, there was no evidence for any adverse response to treatment with sucralose. Analysis of absolute organ weights and of organ weights relative to body weight of both the F_0 and F_1 adults showed a number of statistically significant inter-group differences which were, however, considered to be influenced by the marked depression in body weight in all treated groups. Consequently, organ-weight data were evaluated by analysis of covariance using terminal body weight as the covariate. The results are shown in Table 7 (males) and Table 8 (females). Caecal enlargement was apparent in both F_0 and F_1 animals. Increased kidney weight occurred with statistical significance in F_0 males and in F_1 animals of both sexes fed 3% sucralose. There was a statistically significant reduction in thymus weight for F_0 females and F_1 males fed 3% sucralose, and a non-dose-related reduction in thymus weight in F_1 females at all treatment levels. Some occasional inter-group variations in the weights of other organs were noted, but were considered to be unrelated to treatment.

DISCUSSION

Sucralose, a disaccharide chlorinated at three strategic carbon atoms to enhance sweetness intensity and stability, was studied to determine whether consuming it orally would pose any potential adverse consequences to the reproductive process. One particular possibility for infertility, that of inhibition of epididymal sperm glycolysis, was investigated specifically. Other potential points of interference with the ability to procreate were examined comprehensively by exposure of animals to sucralose continually from gametogenesis through mating and throughout the subsequent F_1 and F_2 generations. Consumption of sucralose through the F_2 generation was conducted to determine the ability of exposed germ cells to give rise to normal fertile offspring.

In earlier experiments designed to identify a biochemical means of contraception in males, Ford and Waites (1978a,b) evaluated a series of chlorinated sugars for their effects on fertility. They treated male rats with several sugars chlorinated at various carefully selected carbon positions (hydroxyl substitution) and found that those mono- or disaccharides chlorinated at the 6 or 6′ position produced reversible antifertility effects. The most likely mechanism was inhibition of glycolysis with a

Table 4. Concentration (ηmol/10^8 spermatozoa) of ATP after 2 hr of incubation at 34°C

Group	Treatment	Mean	SD	Minimum value	Maximum value
1	Negative control (water)	28.8	14.79	6.4	53.2
2	Positive control (6-CG 24 mg/kg/day × 28)	4.2***	4.24	1.5	15.5
3	Sucralose (500 mg/kg/day × 28)	29.9	21.91	3.4	66.1
4	TCDS (100 mg/kg/day × 28)	36.2	21.04	3.2	68.2
4	TCDS Excluding rat no. 27	39.9	18.62	7.3	68.2

***Significantly less than all other groups, $P < 0.001$ (analysis of variance followed by Tukey's studentized range test).

Table 5. Two-generation reproduction and fertility study in rats: parental generation parameters

		F_0 Generation				F_1 Generation			
Group		1	2	3	4	1	2	3	4
Level of sucralose (%)		0	0.3	1.0	3.0	0	0.3	1.0	3.0
					Males				
Body weight (g):	Wk 0	185	185	185	184	105	100	96***	93***
	Wk 10	518	482***	483***	458***	507	475	491	466
	Wk 26	687	633***	635***	595***	673	626*	647	614**
Body weight gain (g):	Wk 0–10	333	297***	298***	274***	402	375*	395	373**
	Wk 0–26	502	448***	400***	411***	568	526*	551	521**
Food intake (% of control):	Wk 1–10	100	94	93	91	100	96	98	96
Achieved doses (mg/kg/day):	Wk 10	0	155	514	1580	0	154	524	1680
Water intake (% of control):	Wk 1–10	100	98	102	105	100	107	115++	124++
					Females				
Body weight (g):	Wk 0	142	144	144	141	94	89*	88*	86**
	Wk 10	280	265***	259***	244***	285	263***	259***	255***
	Day 1 pc[1] F_A	288	272*	262***	248***	283	266**	263**	260***
	Day 1 pp[2] F_A	319	300***	293***	276***	331	298***	295***	294***
	Day 1 pc F_B	283	266**	263**	260***	332	312*	303***	304***
	Day 1 pp F_B	324	305***	305***	283***	368	346*	333**	337**
Body weight gain (g):	Wk 0–10[3]	138	121***	115***	103***	191	174**	171***	169***
Food intake (% of control):	Wk 1–10	100	95	94	91	100	95	96	97
Achieved doses (mg/kg/day):	Wk 10	0	194	656	2090	0	192	642	2100
Water intake (% of control):	Wk 1–10	100	107	116+	123*	100	120+	112	136++

*Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.01$. ***Significantly different from control, $P < 0.001$. +Significantly different from control, $P < 0.05$ (Mann–Whitney U-test). ++ Significantly different from control, $P < 0.01$ (Mann–Whitney U-test). [1]pc = post coitum. [2]pp = post partum. [3]Adult females were paired with males at wk 10. Body weight gain during gestation for these females is presented in Table 6.

Table 6. Two-generation reproduction and fertility study in rats: mating and litter responses

	F_0–F_1 Generation				F_1–F_2 Generation			
Group	1	2	3	4	1	2	3	4
Level of sucralose (%)	0	0.3	1.0	3.0	0	0.3	1.0	3.0
				Matings F_A				
Males cohabited	30	30	30	30	30	29	30	30
Females cohabited	30	30	30	30	30	30	30	30
Females oestrus cycle 4-5 days	29	28	28	29	29	27	27	29
Females mated ⩽ 4 days	29	30	30	29	29	27	28	29
Females littered	29	28	28	30	24	28	28	30
Mean gestation length (days)	22.6	22.6	22.4	22.5	22.6	22.6	22.5	22.6
Body weight gain (g) during gestation	120	110*	123	124	129	122	128	129
Mean live litter size (day 1)	12.7	12.1	13.2	13.1	11.8	12.6	13.5*	13.4*
Live birth index (%)	100	100	100	100	99	93	100	100
Viability index–day 4 (%)	99	98	98	99	93	87	92	95
Lactation index–day 25 (%)	98	100	100	99	99	91	98	97
Mean offspring weight (g) day 1	6.6	6.5	6.3	6.2	6.5	6.2	6.2	6.1*
Mean offspring weight (g) day 14	32.4	31.3	31.7	30.4	31.1	30.0	30.6	30.2
Mean offspring weight (g) day 25	75.1	70.0	69.7	68.1	71.4	67.1	67.3	66.3
Offspring body weight gain (g) day 1–25	68.5	63.5**	63.4***	61.9***	64.9	60.9*	61.1*	60.2*
				Matings F_B				
Males cohabited	29	30	30	30	29	30	30	30
Females cohabited	29	30	30	30	29	30	30	30
Females mated ⩽ 4 days	29	30	30	30	28	28	29	29
Females littered	28	28	28	30	21	26	27	29
Mean gestation length (days)	22.8	22.9	22.7	22.6	22.6	22.6	22.6	22.6
Body weight gain (g) during gestation	136	121*	119**	124*	130	125	126	132
Mean live litter size (day 1)	13.1	13.0	13.3	14.5	13.1	12.6	13.2	14.0
Live birth index (%)	99	99	100	100	99	96	97	100
Viability index–day 4 (%)	99	89	96	93	99	98	92	97
Lactation index–day 25 (%)	100	97	100	96	97	99	95	99
Mean offspring weight (g) day 1	6.7	6.3	6.3	6.1**	6.5	6.4	6.4	6.1
Mean offspring weight (g) day 14	34	32.2	32.1	30.9	32.6	32.1	31.9	30.9
Mean offspring weight (g) day 25	77.9	73.6	70.6	68.7	76.6	72.6	72.6	69.8
Offspring body weight gain (g) day 1–25	71.2	67.3	64.3***	62.6***	70.1	66.2*	66.2*	63.7***

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 7. Effect of dietary sucralose (0–3%) on F_0 and F_1 male organ weights[1]

	Males (F_0)			
Dietary level (%)	0	0.3	1.0	3.0
Body weight (g)	680.9	627.7	626.9	593.5
Adrenals	0.0476	0.0462	0.0484	0.0476
Caecum (empty)	1.28	1.28	1.24	1.63***
Caecum (full)	5.26	5.38	6.16	9.65***
Epididymides	1.40	1.35	1.37	1.42
Kidneys	4.00	3.97	4.14	4.33
Liver	21.9	21.4	21.3	22.3
Lungs	2.14	2.04	2.09	2.05
Pituitary	0.0114	0.0118	0.0131*	0.0111
Prostate	0.622	0.598	0.626	0.627
Seminal vesicles	2.47	2.48	2.57	2.64
Spleen	0.811	0.813	0.837	0.830
Testes	3.58	3.46	3.56	3.57
Thymus	0.505	0.518	0.468	0.457
	Males (F_1)			
Dietary level (%)	0	0.3	1.0	3.0
Body weight (g)	671.3	622.5**	645.4	608.9**
Adrenals	0.0473	0.0420**	0.0453	0.0472
Caecum (empty)	1.19	1.28	1.36**	1.60***
Caecum (full)	4.96	5.65*	5.81**	8.63***
Epididymides	1.25	1.27	1.32	1.32
Kidneys	3.90	3.95	3.89	4.15**
Liver	21.1	21.2	20.5	20.8
Lungs	2.02	2.01	2.06	1.99
Pituitary	0.0110	0.0104	0.0101	0.0105
Prostate	0.672	0.631	0.612	0.702
Seminal vesicles	2.64	2.46	2.61	2.83
Spleen	0.834	0.824	0.825	0.818
Testes	3.65	3.59	3.72	3.54
Thymus	0.417	0.376	0.367	0.349*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [1]Males were killed after 26 wk of treatment. Organ weight differences were statistically analysed using body weight as the covariant.

corresponding decrease in ATP levels in the exposed sperm. From the results of these and further studies (Ford, 1980, 1982), it was determined that: (1) removal of the 6 or 6′ hydroxyl group alone was not sufficient to produce infertility; (2) chlorination at multiple sites in addition to 6 or 6′ not only did not increase the antifertility effect, but actually eliminated it; (3) stable substitutions (CH_3) in addition to chlorine at position 4 also eliminated the effect; and (4) that the antifertility effect is reduced when an additional chlorine atom is introduced at the 1′ position. All 6-chloro sugars that inhibited fertility were also found to inhibit sperm glycolysis and reduce ATP production. The investigators were thus able to categorize those 6-chlorinated sugars that resulted in infertility from those that did not, and, at least partially, explain the molecular and biochemical basis for that distinction. To their stated surprise, two compounds they designated as 4,1′,6′-trichloro-4,1′,6′-trideoxygalactosucrose and 6,1′,6′-trichloro-6,1′,6′-trideoxysucrose (which are identical, respectively, to sucralose and 6,1′,6′ TCDS, a potential trace impurity, Fig. 1) did not have antifertility activity but did seem to inhibit sperm glycolysis and reduce sperm ATP production. According to their hypothesis, sugars chlorinated at multiple sites in addition to the 6 or 6′ position were expected to be neither active antifertility agents nor inhibitors of sperm glycolysis. This apparent discrepancy went unresolved and appeared to be an exception to the hypothetical rule they had established. These findings raised the prospect, however, that sucralose, while not targeted as having antifertility effects, might produce some untoward influence on sperm metabolism.

The sperm glycolysis studies reported here serve to resolve that apparent contradiction. The chlorosugar, 6-chloroglucose (6-CG), known to produce both male infertility and inhibition of sperm glycolysis in rats, was used as the positive control and compared against sucralose and TCDS as to their ability to affect glycolysis in sperm recovered from the cauda epididymides of treated male rats. Both the sucralose and TCDS preparations in the present studies were highly purified, whereas in the earlier studies only cruder, perhaps degraded, samples were available. In addition, the technical skill level in maintaining the physical integrity of the sperm preparations was considerably more experienced at the time of the present work. The doses of sucralose and TCDS used presently exceeded the amounts dosed in the earlier studies. Even so, when the present sperm samples were analysed (blinded) for glycolytic activity and ATP production, only the

Table 8. Effect of dietary sucralose (0–3%) on F_0 and F_1 female organ weights[1]

	Females (F_0)			
Dietary level (%)	0	0.3	1.0	3.0
Body weight (g)	3.589	334.3**	332.6**	305.0**
Adrenals	0.0642	0.0631	0.0649	0.0644
Caecum (empty)	0.96	1.02	1.03	1.26***
Caecum (full)	4.03	4.71*	5.70***	8.11***
Kidneys	2.38	2.46	2.43	2.47
Liver	12.8	12.7	12.2	12.7
Lungs	1.45	1.47	1.48	1.45
Ovaries	0.0836	0.0895	0.0892	0.0939*
Pituitary	0.0137	0.0144	0.0129	0.0128
Spleen	0.528	0.533	0.547	0.539
Thymus	0.335	0.303	0.299	0.270*
Uterus	0.692	0.629	0.647	0.598
	Females (F_1)			
Dietary level (%)	0	0.3	1.0	3.0
Body weight (g)	358.3	341.4	329.4**	321.9
Adrenals	0.0622	0.0669	0.0643	0.0621
Caecum (empty)	1.14	1.20	1.29	1.74**
Caecum (full)	4.06	5.05*	6.14***	7.56***
Kidneys	2.44	2.47	2.52	2.56*
Liver	12.4	12.4	12.6	12.6
Lungs	1.47	1.51	1.55	1.51
Ovaries	0.0882	0.1004*	0.0963*	0.0939
Pituitary	0.0161	0.0145	0.0140	0.0147
Spleen	0.529	0.552	0.551	0.560
Thymus	0.283	0.238**	0.248*	0.246*
Uterus	0.722	0.657	0.682*	0.698

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [1]Females were killed after 28 wk of treatment. Organ weight differences were statistically analysed using body weight as the covariant.

6-chloroglucose produced any decrease in glycolysis or ATP, as would have been originally predicted. Neither sucralose nor TCDS had any effect.

From the earlier work of Ford *et al.* (1978a,b), one would have anticipated that hydrolysis of the disaccharide, 6,1′,6′-TCDS to its respective monosaccharides, 6-chlorodeoxyglucose (6-CG, the present positive control) and 1,6-dichlorodideoxyfructose (1,6-DCF), would have resulted in some inhibition of sperm glycolysis and at least a partial re-

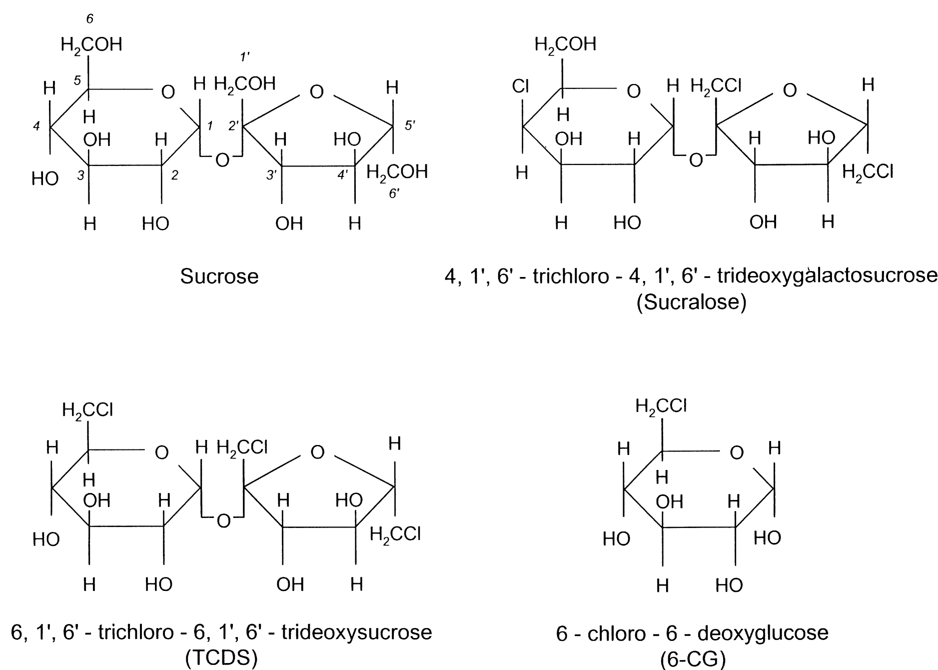

Fig. 1. Sucrose and selected 6-chlorosugars (sucralose, TCDS, 6-CG).

duction in fertility since half the hydrolysis products would be represented by the monosaccharide 6-CG with a single chlorine at the 6 position. The 1,6-DCF would have been expected to be negative in these respects due to the additional chlorine atom at the 1 position. Further investigations into the hydrolytic susceptibility of sucralose, however, show that the presence of the chlorine atom at the 1′ position renders the sucralose disaccharide highly resistant to hydrolysis (McNeil, 1987) to its component monosaccharides. Presumably, this hydrolytic stability is conferred on the disaccharide, 6,1′,6′-TCDS, as well and 6-CG is not produced, at least not in sufficient quantity to affect sperm metabolism or fertility.

With regard to human exposure levels, the 500 mg/kg dose of sucralose used in the present studies is over 300 times the expected daily intake (EDI) for humans. TCDS, at 100 mg/kg, is several orders of magnitude greater than the trace amount that may be produced in the commercial sucralose preparation as an impurity.

In order to confirm and expand the original observation that there was no effect of sucralose on fertility, a comprehensive toxicological evaluation of reproductive parameters was conducted. These included investigation of a range of potential effects on gametes, oestrous cycles, mating behaviour, fertility, maternal toxicity, gestation, foetal development, parturition, lactation, newborn physical and sensory development, and the potential that foetal exposure to sucralose could impair fertility of the succeeding generation (germ cell function). Rats were exposed to sucralose at 0.3, 1.0 or 3.0% in the diet which, on the basis of the calculated achieved doses (Table 5), corresponds to over 100, 365 and 1150 times the expected daily human intake of sucralose (*Federal Register*, 1998). At these levels of sucralose consumption no adverse effects on any parameters of reproductive performance were observed throughout two generations, with two litters per generation.

A few non-reproductive changes were observed in this two-generation study that bear comment. Reduced food intake, corresponding reduced growth rate, increased water intake and changes in the weight of the caecum, kidneys and thymus were observed in both generations in male and female parental animals fed sucralose for 26 and 28 wk, respectively. Reduced food intake has been a common finding in studies of sucralose using the rat animal model. Independent studies (Grice and Goldsmith, 2000); (McNeil, 1987) have shown the reductions in food intake and growth rate are primarily consequences of the unpalatability of the sucralose-containing diet in rats. At very low concentrations, rats prefer sucralose-containing diets or water to those without sucralose. At higher concentrations (starting below 0.3%), however, rats have an aversion to diet containing sucralose. The unpalatability of the food has ramifications extending to reduced food intake, and decreased body weight and body weight gain, all of which were observed in these studies. As sucralose is poorly absorbed (only about 10% in the rat), it is not surprising that caecal enlargement resulting in increased caecal weight was observed. This is a common finding, particularly in rats, following ingestion of osmotically active, poorly absorbed compounds (Newberne *et al*., 1988), and is considered a physiological, rather than a toxicological, response (IPCS, 1987). Increased kidney weights may simply be a reflection of the significantly increased water intake at the higher sucralose doses Since no pathologic findings, either macroscopic or microscopic (independent studies, same doses, (McNeil, 1987), have been found to suggest renal toxicity. The increased foetal thymus weights are thought to be associated with the sensitivity of this organ to the stress of under-nutrition at this stage of its development (McAnulty and Dickerson, 1973; Pfeiffer, 1968). Further independent studies failed to detect any adverse effect of sucralose on immune system anatomy or function (Grice and Goldsmith, 2000). The only finding related to reproduction was an increase in litter size at the higher doses of sucralose. As larger litters are commonly known to contain foetuses with reduced average body weights compared to foetuses from small litters, this, in addition to the reduced food intake of the sucralose-fed dams during gestation, probably contributed to the decreased average body weight of newborn pups. The slightly reduced growth of young pups may be related to the reduced caloric intake of the dams during lactation resulting from lower food consumption.

These results demonstrate that ingestion of sucralose up to over 1150 times the expected daily human intake level produces no effect on sperm glycolysis or on male or female reproductive performance in the rat.

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