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Toxicological Evaluation of Neosugar: Genotoxicity, Carcinogenicity, and Chronic Toxicity

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ABSTRACT

Neosugar, a fructooligosaccharide mixture, was tested for genotoxicity in three assays: (1) microbial reverse mutation assays in *Salmonella typhimurium* (Ames assay) and *Escherichia coli* WP2 *uvr*A, (2) the L5178Y mouse lymphoma TK^{+} mammalian cell mutation assay, and (3) an assay for the induction of unscheduled DNA synthesis (UDS) in human epithelioid cells (HeLa S3). Each assay was conducted at a wide range of dose levels, both with and without metabolic activation. Test results gave no indication that neosugar possessed any genotoxic potential. The carcinogenicity and chronic toxicity of neosugar were examined in Fischer 344 rats. Rats were fed diets containing 0, 8000, 20,000, or 50,000 ppm neosugar for 104 weeks. No dose-related effects on survival, growth, hematology, blood chemistry, organ weights, or nonneoplastic lesions were observed. The incidence of rare and spontaneous tumors was comparable between control and neosugar treatment groups, with the exception of pituitary adenomas in male rats. In light of the background incidence of this tumor and an equivocal dose–response trend, it is unlikely that neosugar treatment is related to the incidence of pituitary adenomas in male rats. The results of this study indicate that neosugar is nonmutagenic and that rats are not adversely affected by chronic neosugar exposure.

INTRODUCTION

NEOSUGAR IS A FRUCTOOLIGOSACCHARIDE MIXTURE of 1^{F}-(1-β-fructofuranosyl)$_{n-1}$-sucrose oligomers in which n may vary from 2 to 4. That is, it consists of sucrose molecules (glucose–fructose disaccharides) to which one, two, or three additional fructose units have been added by $\beta(2-1)$ linkage to the fructose unit of sucrose. These components are abbreviated as GF_2, GF_3, and GF_4, and their chemical structures are shown in Figure 1. Similar or identical fructooligosaccharides are found in a variety of plants, including Jerusalem artichoke,[1] asparagus root,[2]

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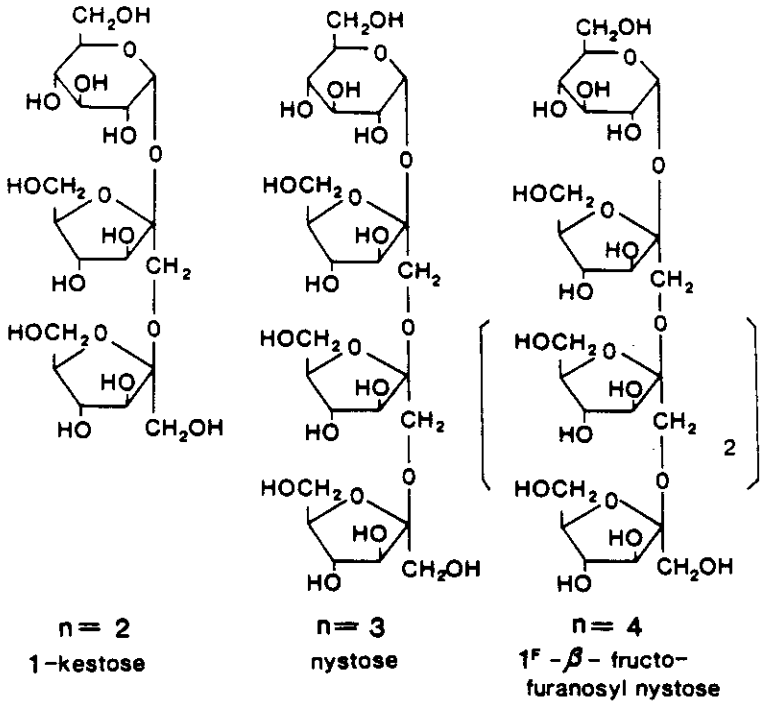

FIG. 1. Structure of major components of neosugar.

onion,[3] wheat,[4,5] rye,[5] and triticale.[5,6] These oligomers are resistant to hydrolysis by mammalian α-amylase, sucrase, and maltase.[7,8] They are sweet-tasting, however, and, therefore, have potential as low-calorie sweeteners.[9] When fed to weanling pigs, neosugar is reported to accelerate weight gain,[10] and it is used as a feed additive in poultry and livestock in Japan.

The present genotoxicity and chronic rat studies were conducted as part of an investigation to examine the safety of neosugar. The genotoxicity studies were conducted by Huntingdon Research Center, Huntingdon England, and the chronic rat study was conducted by the Biosafety Research Center, Foods, Drugs and Pesticides (An-Pyo Center), Shizuoka-ken (437-12), Japan.

MATERIALS AND METHODS

Mutagenicity study

Test material. Neosugar, a clear viscous liquid, was obtained from Meiji Seiki Kaisha Ltd., Pharmaceuticals Division, Tokyo 104, Japan. Neosugar was dissolved in distilled water for testing.

Microbial gene mutation assay. Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100 were obtained from Dr. Bruce Ames, University of California, Berkeley, CA, and *Escherichia coli* WP2 *uvr*A was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. The bacteria were stored at −80°C and grown in Oxoid nutrient broth at 37°C for 18 hr before use in mutation assays.

Neosugar was tested in a standard plate incorporation assay at 0, 50, 150, 500, 1500, and 5000

μg/plate in each tester strain, with and without metabolic activation, using procedures complying with OECD and Japanese Ministry of Health and Welfare guidelines. Three test plates per strain per treatment condition were used. Appropriate negative and positive controls were used with each strain. The positive controls used in the absence of metabolic activation were *N*-ethyl-*N'*-*nitro*-*N*-nitrosoguanidine with TA 1535, TA 100, and *E. coli* WP2 *uvr*A, 2-nitrofluorene with TA 98 and TA 1538, and 9-aminoacridine with TA 1537. With metabolic activation, 2-aminoanthracene was used with all strains.

Mammalian cell mutation assay. Mouse lymphoma L5178Y cells (3.7.2c) were obtained from Dr. J. Cole, Sussex University. The cells, which are heterozygous at the thymidine kinase locus ($TK^{\pm}$) were grown routinely as suspensions in roller culture in sodium bicarbonate-buffered RPMI 1640 medium supplemented with sodium pyruvate (110 μg/ml), pluronic F68 (1 mg/ml), gentamicin (50 μg/ml), and 10% heat-inactivated horse serum, in an atmosphere of 5% CO_2 in air. For cloning, the serum content of the medium was doubled to 20%, the pluronic content reduced to 0.2 mg/ml, and 0.3% Noble agar was incorporated. Chemical treatment of the cells took place in hepes-buffered RPMI 1640 medium without added serum, pluronic, or sodium pyruvate.

Preliminary toxicity tests were conducted by treating cells in suspension with neosugar at 50, 100, 250, 1000, 2500, and 5000 μg/ml at 37°C for 3 hr. The highest dose used represented the maximum dose used routinely in this assay to avoid confounding physical effects. The cells were then washed and resuspended in normal growth medium and cultured at 37°C. Cell population growth was monitored at 24 and 48 hr using a Coulter counter.

Mutagenicity was tested by treating populations of 12×10^6 cells for 3 hr at 37°C with neosugar at 2000, 3000, 4000, and 5000 μg/ml in the presence and absence of an Aroclor-induced rat liver microsomal metabolic activation system. After treatment in serum-free medium, the cells were washed and grown for 48 hr in normal growth medium to allow expression of any induced mutations, then cloned in soft agar to test for viability (600 cells/plate) and mutations (10^6 cells/plate, plus trifluorothymidine at 4 μg/ml). The procedures used are based on those of Clive and Spector[11] and Amacher et al.[12–14]

Unscheduled DNA synthesis (UDS) assay. UDS was examined using the procedures of Martin et al.[15,16] and complying with OECD guidelines. Briefly, HeLa S3 epithelioid cells, originally derived from a human cervical carcinoma, were obtained from Flow Laboratories, Ltd., and cultured in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and gentamicin at 50 μg/ml, plus 15% fetal calf serum. For the UDS assay, cells were grown to confluence on 22 mm coverslips in multiwell tissue culture dishes in normal EMEM. The medium was then replaced with arginine-deficient medium containing 2.5% dialyzed fetal calf serum and kept at 37°C for an additional 72 hr to suppress normal scheduled DNA replication. ^{3}H-Thymidine (20 Ci/mmole) was then added to a final concentration of 5 μCi/ml, together with 100 μl of an appropriate concentration of the test chemical to give a final concentration of 25, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, 25,600, or 51,200 μg/ml. Each dose level was tested with and without metabolic activation. Appropriate negative (water and dimethylsulfoxide) and positive (4-nitroquinoline-1-oxide and 2-aminoanthracene) controls also were tested.[17] After treatment for 3 hr, the coverslips and cells were removed, washed, fixed, stained in orcein, mounted on slides, and processed for autoradiography using Kodak AR-10 stripping film. After application of the photographic emulsion to the coverslips, they were kept in the dark for 13 days to expose the emulsion. The autoradiographs were then developed, fixed, rinsed, and air dried.

UDS was quantitated by counting silver grains overlying 100 non-S-phase cells and over equal areas of cytoplasm (background counts).

Metabolic activation system. For all three tests, a metabolic activation system was used, consisting of liver microsomal fraction (S-9) from Charles River CD rats, 6–8 weeks old, given a single intraperitoneal injection of Aroclor 1254 at 500 mg/kg body weight 5 days before killing. The livers were removed and homogenized in three times their weight of ice-cold 0.15 *M* KCl. The homoge-

nate was centrifuged at 900 *g* for 10 min, and the supernatant (S-9 fraction) was collected and stored at −70°C. The S-9 fraction was mixed with appropriate cofactors immediately before use. In all cases, the functioning of the S-9 mix was tested by including positive control materials that required metabolic activation to express a genotoxic effect. For the microbial tests, the S-9 mix contained 10% vol/vol S-9 fraction, 8 m*M* $MgCl_2$, 33 m*M* KCl, 100 m*M* sodium phosphate buffer (pH 7.4), 5 m*M* glucose-6-phosphate, 4 m*M* NADPH, and 4 m*M* NADH; 0.5 ml of this mixture was added to 2 ml of top agar with 0.1 ml bacterial suspension and 0.1 ml test solution.

For the mouse lymphoma assay, the S-9 fraction was added to a threefold larger volume of ice-cold RPMI 1640 medium containing isocitric acid at 15 mg/ml and NADP at 8 mg/ml immediately before use, and 8 ml of that mixture was added to 12 ml of cell suspension with 0.2 ml of test solution.

For the UDS assay, S-9 mix was prepared by mixing 4 ml of S-9 fraction with 2 ml of 0.15 *M* KCl, 0.36 g glucose-6-phosphate, 0.05 g NADP, and 4 ml distilled water. Aliquots of 0.1 ml of that mixture were added to each 2 ml of medium in the tissue culture dishes containing cells.

Chronic animal study

Animals and diet. Male and female specific pathogen-free Fischer 344 (F344) rats (4 weeks of age) were obtained from Charles River Japan, Inc. The animals were housed singly in wire mesh cages. A 12-hr light–dark cycle was provided. The temperature was maintained at 22–24°C, and relative humidity was 50–60%. Test animals received a modified NIH open formula rat and mouse diet (Oriental Yeast Co., Tokyo, Japan) containing neosugar. Control animals received the same diet without neosugar. Food and water were provided ad libitum. Neosugar powder containing greater than 95% fructooligosaccharides was obtained from Mie Kariyo Co., Mie, Japan. The oligosaccharide ratio (GF_2:GF_3:GF_4) of the test material was 37:51:12.

Experimental design. After 1 week of acclimation, groups of 50 rats of each sex began receiving neosugar at concentrations of 0, 8000, 20,000, or 50,000 ppm in their diet. Because of the low toxicity of neosugar, the selection of the highest exposure concentration was based on the maximum volume of neosugar that could be incorporated as a food additive. The experiment was terminated after 104 weeks of treatment. Animals were observed at least twice daily. Body weights were determined weekly for the first 26 weeks and biweekly thereafter. Food consumption was determined weekly for all animals throughout the study. Food efficiency and neosugar intake were calculated from body weight and food consumption data.

At the termination of the study, surviving animals were anesthetized with ether, and blood samples were collected from the abdominal aorta. Hematology measurements were performed on all samples using a Coulter Counter Model SP (Coulter Electronics, USA). Blood smears fixed with methanol and stained with Wright-Giemsa were used for differential leukocyte counts and morphological evaluation. Blood chemistry measurements using serum were performed using a Centrifichem System 400 (Union Carbide Co., USA) except for Na, K, and Cl, which were measured with an automatic electrolysis analyzer (Toa Electronics, Tokyo, Japan). Brain, adrenals, heart, spleen, lungs, testes, liver, ovaries, and kidney weights were recorded for all animals surviving the length of the experiment. All animals, including those dying spontaneously or killed in a moribund condition, were necropsied. Tissues were fixed in 10% neutral buffer formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical evaluation. A two-tailed Yates corrected Chi-square analysis was used to determine the significance of differences in survival and differences in the incidence of nonneoplastic lesions. When pairwise comparisons of nonneoplastic lesions revealed a significant difference between treatment and control groups, logistic regression analysis was performed to test for dose-related trends.[18] One-way analysis of variance (ANOVA) was used to assess the significance of differences in body weight, food consumption, food efficiency, organ weight, hematology, and blood

chemistry. Where significant effects were found, the Student-Newman-Keuls (SNK) multiple range test for multiple comparisons among means[19] was performed to determine where differences among the group means were located.

A one-tailed Yates corrected Chi-square test was used to determine the significance of differences in the incidence of tumors.[20] Logistic regression and Cochran-Armitage Chi-square[20] tests for trend were used to determine significant dose-related trends. A significance level of $P \leq 0.05$ was used in all analyses.

RESULTS

Mutagenicity study

Microbial gene mutation assay. Two independent trials were conducted, both with and without metabolic activation. No signs of toxicity were seen at any of the doses of neosugar tested, and there was no increase in mutants per plate in any bacterial strain either with or without metabolic activation. The positive control chemicals produced the expected clear increases in mutation frequency. The results of the first trial are shown in Table 1. The second trial gave very similar results.

Mammalian cell mutation assay. Two independent trials were conducted both in the presence and in the absence of metabolic activation (Table 2). In both trials, no significant toxicity at any dose level was detected, as measured by cell population growth after treatment or colony-forming ability. Also, no increase in mutation frequency was seen in either trial, either with or without metabolic activation. As with the microbial assays, the positive control chemicals produced the expected clear increases in mutation frequency.

UDS assay. Two independent trials were conducted, both with and without metabolic activation (Table 3). In the first trial, a statistically significant ($P < 0.05$, ANOVA) increase in net nuclear grains occurred at a concentration of neosugar of 1600 μg/ml in the absence of metabolic activation. There was no indication of a dose-related trend in grain counts, however, and the increase was not reproduced in the second trial. In neither trial with metabolic activation was there any significant effect on net nuclear grain counts.

Chronic animal study

Survival. Survival curves for male and female rats are shown in Figure 2. At the termination of the experiment, survival of male rats administered 0, 8000, 20,000 and 50,000 ppm neosugar was 88%, 72%, 68%, and 84%, respectively. The survival rates of the male 8000 and 50,000 ppm groups were comparable to the control group, but mortality in the 20,000 ppm group was significantly higher than in controls. The survival of female rats administered 0, 8000, 20,000, and 50,000 ppm neosugar was 82%, 74%, 74%, and 88%, respectively. All female neosugar-treated groups had survival rates comparable to controls.

Growth, food efficiency, and chemical intake. Growth curves for male and female rats are shown in Figure 3. Mean body weights of all male and female neosugar treatment groups were comparable to their respective controls. Overall food consumption by neosugar-treated male rats was comparable to the control group. When overall food consumption data for females were analyzed by ANOVA, significant variation among groups was found; however, subsequent pariwise group comparisons by the SNK test procedure showed no significant intergroup differences involving the control group. Overall food efficiencies by neosugar-treated male and female groups were comparable to their control groups.

Neosugar intake by the 8000, 20,000, and 50,000 ppm groups was calculated to be approxi-

TABLE 1. RESULTS OF MICROBIAL GENE MUTATION ASSAY WITH NEOSUGAR (TRIAL 1)

Material	Test concentration (μg/plate)	With or without S-9	Reverse mutation (No. of colonies/plate)[a]					
			Base pair exchange type			Frame shift type		
			TA 100	TA 1535	WP2 uvrA	TA 98	TA 1537	TA 1538
Solvent control		–	119	20	46	27	14	11
Neosugar	5000	–	130	23	57	18	9	6
	1500	–	118	19	47	23	16	9
	500	–	106	23	45	24	11	12
	150	–	119	21	48	21	11	12
	50	–	121	20	42	21	12	10
Solvent control		+	118	23	53	23	17	16
Neosugar	5000	+	118	20	53	21	13	9
	1500	+	115	19	43	22	17	17
	500	+	99	19	49	22	12	11
	150	+	105	16	52	21	15	12
	50	+	128	16	51	23	12	12
Positive controls								
ENNG[b]	2	–	–	–	1257	–	–	–
	3	–	399	–	–	–	–	–
	5	–	–	737	–	–	–	–
NF	1	–	–	–	–	52	–	–
	2	–	–	–	–	–	–	38
9 AC	80	–	–	–	–	–	1231	–
AA	0.5	+	245	–	–	115	–	71
	2	+	–	89	–	–	83	–
	80	+	–	–	265	–	–	–

[a]Values are the mean of 3 plates.
[b]ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; 9 AC, 9-aminoacridine; AA, 2-aminoanthracene; NF, 2-nitrofluorene.

TABLE 2. SUMMARY OF RESULTS OF MOUSE LYMPHOMA TK$^{\pm}$ MUTATION ASSAY WITH NEOSUGAR

Concentration of neosugar (μg/ml)	Without metabolic activation				With metabolic activation			
	% Survival		Mutant frequency (× 10^{-6})		% Survival		Mutant frequency (× 10^{-6})	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
0	100	100	48	72	100	100	85	57
2000	69	134	49	58	157	97	71	63
3000	77	114	53	76	143	73	76	90
4000	64	124	44	65	111	98	89	54
5000	64	112	63	67	120	87	73	78
Positive control[a]	35	53	489	478	18	28	228	239

[a]Ethyl methanesulfonate (500 μg/ml) without metabolic activation; cyclophosphamide (5 μg/ml) with activation.

mately 341, 854, and 2170 mg/kg/day, respectively, for male rats and 419, 1045, and 2664 mg/kg/day, respectively, for female rats.

Hematology and blood chemistry. Hematology results are presented in Table 4. Neosugar exposure had no significant effect on any of these parameters. In addition, neosugar treatment had no effect on differential leukocyte counts (data not shown).

Blood chemistry results are presented in Table 5. Male rats fed neosugar showed a slight but significant elevation of Na and Cl. Male rats fed 20,000 ppm neosugar had slightly elevated levels of blood glucose and creatinine. The male 50,000 ppm group had slightly decreased creatinine levels. All other parameters for male neosugar-treated rats were similar to control values. In females, all blood chemistry parameters were similar to controls except for a slight elevation of uric acid in the 8000 and 20,000 ppm groups.

Organ weights. Organ weights are shown in Table 6. Neosugar treatment had no effect on organ weights. Organ/body weight ratios also were similar among groups except for adrenal weight ratios of females. However, pairwise comparisons showed no significant differences involving the control group.

Nonneoplastic lesions. As might be expected in aging rats, many types of nonneoplastic lesions were observed in all groups, including controls. Numerous lesions were present in liver, kidneys, adrenals, and lymph nodes (Table 7). Other age-related lesions that occurred in the majority of animals were hyaline bodies in brain tissue, pigment deposits in the spleen, and atrophy of the thymus. Stomach fibrosis, seminal vesicle atrophy, and decreased spermatogenesis were common in males, and pituitary and adrenal angiectasis were extremely common in females.

Nonneoplastic lesions were classified as slight, moderate, or marked. The majority of lesions were slight. Exceptions were chronic nephropathy in males, where 20–40% of these lesions were moderate in severity. In females, renal calcium deposits and protein casts and pigment deposits in the spleen were of moderate or marked severity in 15–40% of animals with these lesions.

Neosugar treatment did not affect the severity of these lesions except for renal protein casts in male rats. Renal casts of moderate severity were found in 0, 4, 7, and 6 rats in the male control,

TABLE 3. SUMMARY OF RESULTS OF UDS ASSAY WITH NEOSUGAR

Concentration of neosugar (μg/ml)	Without metabolic activation				With metabolic activation			
	Net grains/100 nuclei		% Nuclei with >3 net grains		Net grains/100 nuclei		% Nuclei with >3 net grains	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
0 (water)	−2	12	0.6	0	4	30	0.9	0.6
0 (DMSO)	5	18	0.6	0.4	13	8	0.5	1
25	14	16	0	0	18	−17	2	1
50	15	23	0	0.5	26	24	1.5	1
100	5	27	0	0.5	10	25	0.5	0
200	−2	5	0.5	0.5	7	0	1	0
400	−2	17	0.5	2	14	8	2.5	0
800	−16	17	0	3	17	44	1	1.5
1600	24[a]	8	1	0	12	5	1.5	0
3200	6	27	1	1	12	1	1	0
6400	7	42	0	1.5	14	25	0	1
12,800	10	33	0.5	1	2	7	0	0
25,600	−3	25	0	0.5	12	22	0.5	0
51,200	−9	21	0	0.5	−19	15	0.5	0
Positive control[b]								
x	321	529	41	67.5	32	31	1	0
2x	1051	845	75.5	82	40	26	3	1.5
4x	1185	853	92	81.5	53	58	2.5	5.5
8x	1646	1237	95.5	95	50	88	1.5	8
16x	1732	1351	97.5	99	40	71	3	7.5

[a]Significantly greater than control ($P < 0.05$, one-way analysis of variance).
[b]4-Nitroquinoline-1-oxide at 0.02, 0.04, 0.08, 0.16, and 0.32 μg/ml without metabolic activation; 2-aminoanthracene at 2.5, 5, 10, 20, and 40 μg/ml with activation.

8000, 20,000, and 50,000 ppm groups, respectively. One male in the 20,000 ppm group had renal casts of marked severity.

The incidence of the majority of nonneoplastic lesions was similar in all groups whether fed control diet or neosugar. However, pairwise comparisons revealed that some lesions occurred in greater or lesser incidence in neosugar groups than in controls. These lesions are shown in Table 8. It is apparent from the incidence data that simple pairwise comparisons alone were inadequate for determining a cause–effect relationship between neosugar treatment and the occurrence of a particular lesion. Therefore, additional intergroup comparisons were made, which included a quantitative dose–response trend analysis, comparison of the severity of lesions, and consideration of the background incidence of these lesions in historical controls.

Logistic regression is a parametric statistical test that analyzes for dose-related trends. Table 8 presents the results of logistic regression analysis of the nonneoplastic lesions data. Positive and

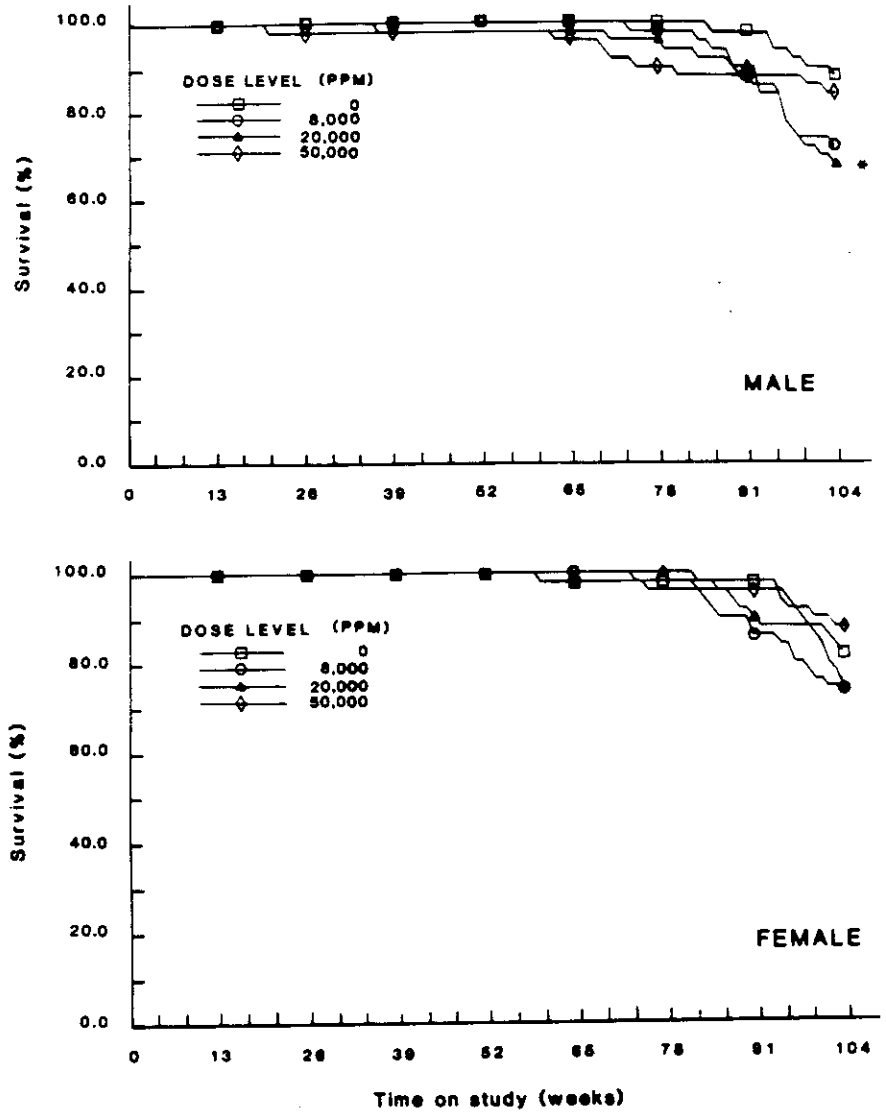

FIG. 2. Survival curves for male and female rats administered neosugar in the diet. *Significantly different from control, $P \leq 0.05$.

negative trends are denoted by the sign of the dose coefficient and Student's t value. Of the 17 lesions with significant findings by pairwise comparisons, only 3 were common to both males and females, and for 2 of the 3 (lymph node granulation and stomach fibrosis), opposite trends were observed. However, as indicated by the P values, no significant positive or negative dose-related trends were detected by logistic regression for any of the lesions in either sex.

A borderline positive trend ($P = 0.07$) was noted for lymph node granulation and prostatic atrophy in males. In females, only two lesions, adrenal angiectasis and adrenal hyperplasia, showed a positive (although nonsignificant) dose-related trend. The severity of these particular lesions in males and females was not affected by neosugar treatment. Furthermore, their incidence in neo-

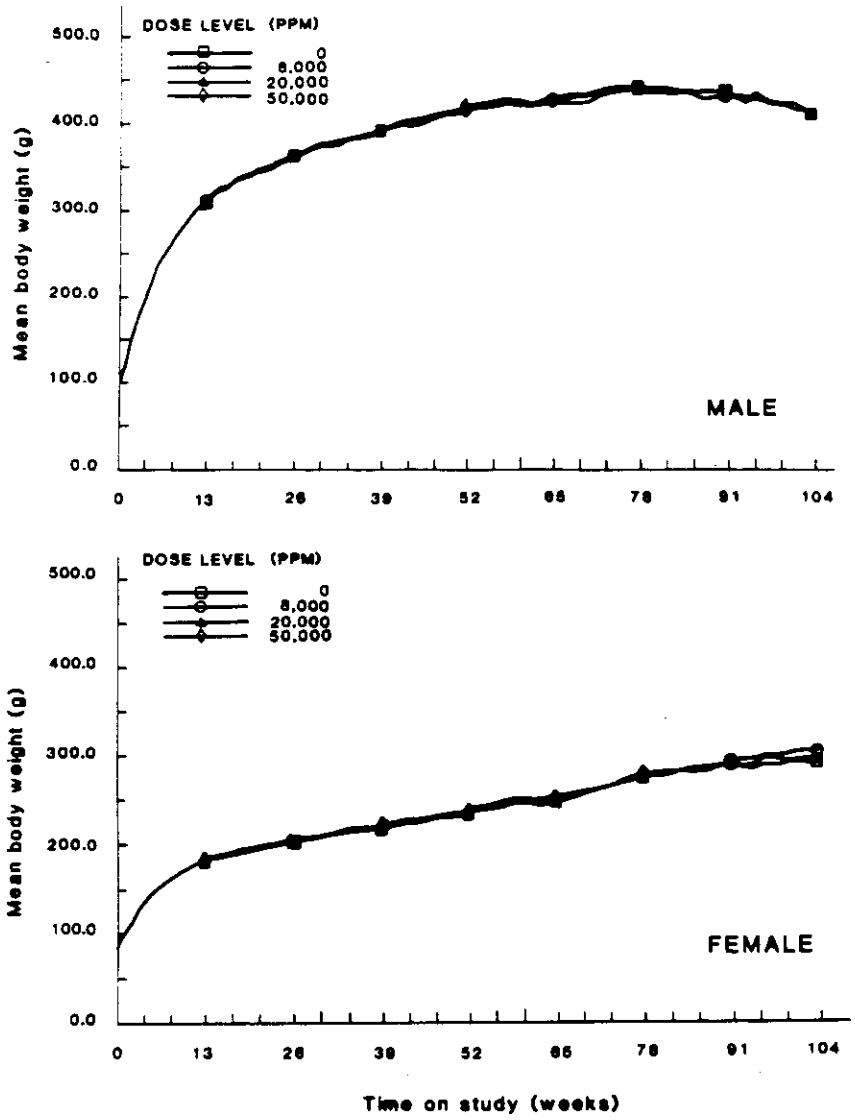

FIG. 3. Growth curves for male and female rats administered neosugar in the diet.

sugar groups was within the historical control range (see Discussion). With regard to lymph node granulation, opposite dose-related trends were noted in males and females.

Table 8 shows the incidence of the nonneoplastic lesions in historical control F-344 rats from this laboratory. Although most of these lesions were quite common, there were also wide variations in occurrence in historical controls. Despite the wide historical range, the incidence of some lesions in the neosugar study controls was outside the historical control range. The incidence of prostatic atrophy in concurrent controls was 10%, whereas the historical mean was 58%, and the range was 15–100%. Similarly, the incidence of dilated gastric glands and lymph node granulation in concurrent male controls were at the lower extreme of the historical ranges. Consequently, lesions that were significantly increased in neosugar groups as compared by pairwise comparison to concurrent

TABLE 4. HEMATOLOGY RESULTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

Dietary level (ppm)	*HCT*[b] *(%)*	*HGB (g/dl)*	*RBC (× 10⁶/mm³)*	*MCV (μm³)*	*MCH (pg)*	*MCHC (%)*	*PLT (× 10³/mm³)*	*WBC (× 10³/mm³)*
Males								
0	46.0 ± 9.1	16.5 ± 3.2	8.95 ± 1.99	51.9 ± 4.8	18.7 ± 1.9	35.9 ± 0.8	811 ± 176	5.6 ± 2.3
8000	49.2 ± 6.3	17.6 ± 2.1	9.62 ± 1.39	51.2 ± 2.3	18.4 ± 1.0	35.8 ± 0.6	770 ± 154	5.2 ± 1.5
20,000	46.2 ± 7.5	16.5 ± 2.6	8.94 ± 1.79	52.5 ± 6.9	18.8 ± 2.6	35.8 ± 0.6	807 ± 176	5.4 ± 1.7
50,000	47.8 ± 6.6	17.2 ± 2.3	9.41 ± 1.41	50.8 ± 1.1	18.3 ± 0.5	36.0 ± 0.5	835 ± 150	5.1 ± 2.8
Females								
0	45.5 ± 7.8	16.0 ± 2.6	8.05 ± 1.83	57.9 ± 8.0	20.5 ± 3.1	35.3 ± 0.7	742 ± 308	4.6 ± 4.9
8000	47.3 ± 7.0	16.5 ± 2.3	8.51 ± 1.40	55.9 ± 3.0	19.6 ± 1.2	35.1 ± 0.8	738 ± 95	3.5 ± 2.3
20,000	44.1 ± 6.3	15.5 ± 2.1	7.94 ± 1.40	56.0 ± 3.7	19.7 ± 1.4	35.1 ± 0.6	832 ± 264	3.7 ± 1.8
50,000	44.9 ± 5.6	15.9 ± 1.9	8.14 ± 1.25	56.0 ± 7.1	19.9 ± 2.7	35.4 ± 0.4	735 ± 135	4.9 ± 8.1

[a]Values represent mean ± SD, n = 34–44.
[b]HCT, hematocrit; HGB, hemoglobin; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; WBC, white blood cell count.

TABLE 5. BLOOD CHEMISTRY RESULTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

	Dietary level (ppm)							
	Males				*Females*			
	0	*8000*	*20,000*	*50,000*	*0*	*8000*	*20,000*	*50,000*
Na (mEq/L)	146 ± 1	147 ± 1	148 ± 2[c]	149 ± 2[c]	148 ± 1	148 ± 2	148 ± 1	148 ± 2
K (mEq/L)	5.1 ± 0.3	5.1 ± 0.4	5.1 ± 0.3	5.5 ± 0.5	4.9 ± 0.4	5.1 ± 0.4	5.0 ± 0.6	4.6 ± 0.5
Cl (mEq/L)	106 ± 1	107 ± 1[c]	109 ± 2[c]	108 ± 1[c]	107 ± 2	107 ± 2	106 ± 2	107 ± 2
Ca (mg/dl)	9.8 ± 0.6	9.8 ± 0.5	9.5 ± 0.9	9.8 ± 0.3	10.0 ± 0.6	10.4 ± 0.9	10.4 ± 0.9	10.8 ± 0.8
IP[b] (mg/dl)	5.0 ± 0.8	4.1 ± 0.4	4.4 ± 0.8	4.5 ± 0.8	4.8 ± 0.9	4.0 ± 0.5	4.8 ± 1.1	4.6 ± 0.6
Glu (mg/dl)	84 ± 25	86 ± 24	107 ± 10[c]	76 ± 22	112 ± 17	111 ± 10	101 ± 34	121 ± 16
BUN (mg/dl)	26 ± 6	26 ± 6	22 ± 7	23 ± 5	15 ± 2	14 ± 1	13 ± 2	18 ± 4
UA (mg/dl)	1.4 ± 0.4	1.4 ± 0.4	1.1 ± 0.3	1.7 ± 0.4	0.8 ± 0.3	1.1 ± 0.2[c]	1.3 ± 0.5[c]	0.7 ± 0.2

Creat (mg/dl)	0.59 ± 0.05	0.65 ± 0.04	0.73 ± 0.12[c]	0.49 ± 0.11[c]	0.53 ± 0.05	0.52 ± 0.04	0.49 ± 0.02	0.51 ± 0.06
T-Chol (mg/dl)	131 ± 39	135 ± 53	138 ± 75	129 ± 40	121 ± 29	110 ± 19	114 ± 38	95 ± 18
Alb (g/dl)	2.7 ± 0.3	2.8 ± 0.2	2.6 ± 0.3	2.9 ± 0.2	3.2 ± 0.2	3.3 ± 0.3	3.3 ± 0.2	3.3 ± 0.3
T-Bili (mg/dl)	0.45 ± 0.06	0.47 ± 0.04	0.45 ± 0.12	0.44 ± 0.09	0.48 ± 0.11	0.40 ± 0.04	0.47 ± 0.07	0.43 ± 0.06
AL-P (IU/L)	121 ± 49	118 ± 20	140 ± 73	90 ± 23	91 ± 31	90 ± 40	92 ± 32	90 ± 18
SGOT (IU/L)	104 ± 16	109 ± 47	103 ± 78	134 ± 18	68 ± 24	95 ± 43	95 ± 36	86 ± 24
SGPT (IU/L)	43 ± 15	41 ± 9	40 ± 16	47 ± 17	42 ± 16	50 ± 19	45 ± 16	40 ± 18
LDH (IU/L)	122 ± 32	195 ± 128	212 ± 140	186 ± 44	88 ± 17	104 ± 53	128 ± 63	206 ± 182

[a]Values represent the mean ± SD, $n = 10$.

[b]IP, inorganic phosphorus; Glu, glucose; BUN, blood urea nitrogen; UA, uric acid; Creat, creatinine; T-Chol, total cholesterol; Alb, albumin; T-Bili, total bilirubin; AL-P, alkaline phosphatase; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; LDH, lactate hydrogenase.

[c]Significantly different from control, $P \leq 0.05$.

TABLE 6. ORGAN WEIGHTS OF MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS[a]

Dietary level (ppm)	*Brain*	*Heart*	*Lungs*	*Liver*	*Kidneys*	*Spleen*	*Adrenals*	*Testes*	*Ovaries*
Males									
0	2.20 ± 0.05	1.18 ± 0.11	1.44 ± 0.17	11.64 ± 2.84	3.14 ± 0.46	1.42 ± 1.63	0.082 ± 0.053	5.51 ± 2.78	
8000	2.20 ± 0.05	1.18 ± 0.08	1.43 ± 0.14	10.46 ± 1.16	2.98 ± 0.22	1.20 ± 0.96	0.075 ± 0.024	5.49 ± 1.93	
20,000	2.19 ± 0.06	1.20 ± 0.07	1.47 ± 0.28	11.66 ± 3.39	3.06 ± 0.43	1.23 ± 0.81	0.113 ± 0.205	4.95 ± 1.56	
50,000	2.19 ± 0.06	1.16 ± 0.07	1.42 ± 0.13	10.89 ± 1.00	3.13 ± 0.33	1.10 ± 0.75	0.080 ± 0.020	5.11 ± 2.12	
Females									
0	1.98 ± 0.05	0.94 ± 0.11	1.11 ± 0.39	7.74 ± 1.75	2.21 ± 0.40	1.57 ± 3.46	0.071 ± 0.013		0.064 ± 0.016
8000	1.99 ± 0.04	0.93 ± 0.09	1.03 ± 0.13	7.40 ± 1.04	2.16 ± 0.19	0.79 ± 1.45	0.069 ± 0.005		0.069 ± 0.012
20,000	1.99 ± 0.05	0.93 ± 0.08	1.07 ± 0.24	7.57 ± 1.49	2.13 ± 0.25	1.39 ± 2.59	0.077 ± 0.030		0.069 ± 0.014
50,000	2.00 ± 0.05	0.93 ± 0.10	1.10 ± 0.25	7.49 ± 1.39	2.14 ± 0.16	1.22 ± 2.69	0.076 ± 0.010		0.068 ± 0.015

[a]Values represent the mean ± SD, n = 34–44; weights are expressed in grams.

TABLE 7. INCIDENCE OF COMMONLY OCCURRING NONNEOPLASTIC LESIONS IN MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS

	Male (50 rats/group)				*Female (50 rats/group)*			
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*0 (ppm)*	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*
Liver								
Fatty change	30 (60%)	27 (54%)	19 (38%)	35 (70%)	44 (88%)	40 (80%)	37 (74%)	40 (80%)
Granulation	15 (30%)	21 (42%)	26 (52%)	27 (54%)	29 (58%)	27 (54%)	24 (48%)	31 (62%)
Fibrosis	22 (44%)	20 (40%)	20 (40%)	26 (52%)	0 (0%)	0 (0%)	2 (4%)	0 (0%)
Hyperplasia of bile ducts	47 (94%)	48 (96%)	47 (94%)	45 (90%)	10 (20%)	12 (24%)	11 (22%)	12 (24%)
Necrosis	0 (0%)	1 (2%)	1 (2%)	2 (4%)	12 (24%)	11 (22%)	9 (18%)	10 (20%)
Kidney								
Basophilic change	47 (94%)	43 (86%)	43 (86%)	46 (92%)	23 (46%)	26 (52%)	24 (48%)	21 (42%)
Deposit of pigment	48 (96%)	43 (86%)	45 (90%)	47 (94%)	44 (88%)	45 (90%)	42 (84%)	42 (84%)
Lymphocytic infiltration	42 (84%)	29 (58%)	39 (78%)	31 (62%)	5 (10%)	4 (8%)	4 (8%)	0 (0%)
Protein cast	49 (98%)	48 (96%)	49 (98%)	48 (96%)	44 (88%)	43 (86%)	37 (74%)	37 (74%)
Chronic nephropathy	33 (66%)	27 (54%)	39 (78%)	33 (66%)	16 (32%)	9 (18%)	5 (10%)	4 (8%)
Glomerulosclerosis	39 (78%)	35 (70%)	44 (88%)	40 (80%)	26 (52%)	21 (42%)	17 (34%)	9 (18%)
Hyaline droplet	1 (2%)	0 (0%)	3 (6%)	0 (0%)	21 (42%)	21 (42%)	14 (28%)	14 (28%)
Lymph nodes								
Granulation	1 (2%)	8 (16%)	12 (24%)	16 (32%)	10 (20%)	5 (10%)	5 (10%)	3 (6%)
Plasma cell increase	21 (42%)	27 (54%)	33 (66%)	29 (58%)	22 (44%)	15 (30%)	19 (38%)	19 (38%)
Deposit of pigment	2 (4%)	0 (0%)	0 (0%)	1 (2%)	16 (32%)	16 (32%)	19 (38%)	17 (34%)
Sinus histiocytosis	1 (2%)	0 (0%)	0 (0%)	0 (0%)	19 (38%)	9 (18%)	10 (20%)	9 (18%)
Adrenal								
Angiectasis	1 (2%)	2 (4%)	2 (4%)	8 (16%)	20 (40%)	26 (52%)	25 (50%)	32 (64%)
Vacuolic change	17 (34%)	17 (34%)	16 (32%)	15 (30%)	34 (68%)	42 (84%)	37 (74%)	37 (74%)
Hyperplasia	13 (26%)	14 (28%)	26 (52%)	22 (44%)	0 (0%)	1 (2%)	7 (14%)	4 (8%)

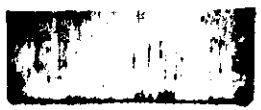

TABLE 8. INCIDENCE OF SIGNIFICANT NONNEOPLASTIC LESIONS IN HISTORICAL CONTROL AND NEOSUGAR STUDY RATS AND LOGISTIC REGRESSION ANALYSIS OF NEOSUGAR STUDY DATA

	Neosugar study incidence (%)[a]				*Logistic regression analysis*			*Historical control incidence (%)*[b]	
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*Dose coefficient (× 10^{-5})*	*Student's t*	*P value*	*Mean*	*Range*
Males									
Lymph node granulation	2	16[d]	24[d]	32[d]	3.3017	3.54	0.07	16	0–40
Lymph node plasma cell increase	42	54	66[d]	58	1.0270	1.35	0.31	35	2–53
Liver granulation	30	42	52[d]	54[d]	1.7053	2.25	0.15	15	0–38
Liver fatty changes	60	54	38[d]	70	1.0329	1.36	0.31	61	29–82
Prostatic atrophy	10	42[d]	48[d]	52[d]	2.7417	3.50	0.07	58	15–100
Adrenal hyperplasia[c]	26	28	52[d]	44	1.5754	2.06	0.18	3	0–19
Dilated gastric glands	4	14	20[d]	30[d]	2.6775	2.87	0.10	31	0–79
Stomach fibrosis	70	74	90[d]	82	1.4815	1.50	0.27	23	0–74
Kidney infarct	0	6	16[d]	10	2.1169	1.64	0.24	3	0–17
Kidney lymphocytic infiltration	84	58[d]	78	62[d]	−1.2196	−1.52	0.27	37	0–86
Brain hyaline bodies	84	80	68	62[d]	−2.1676	−2.63	0.12	69	31–90
Heart fibrosis	42	10[d]	12[d]	22	−1.1098	−1.16	0.36	37	4–79
Females									
Lymph node sinus histiocytosis	38	18[d]	20	18[d]	−1.6147	−1.70	0.23	11	0–56
Lymph node granulation	20	10	10	4[d]	−3.4264	−2.14	0.17	16	0–68
Adrenal angiectasis	40	52	50	64[d]	1.6960	2.21	0.16	37	0–79
Adrenal hyperplasia[c]	0	2	14[d]	8	2.5522	1.73	0.23	6	0–17
Pituitary angiectasis	76	60	72	46[d]	−2.1673	−2.80	0.11	51	18–78
Stomach fibrosis	12	40[d]	52[d]	16	−0.6052	−0.73	0.54	18	0–80
Chronic nephropathy	32	18	10[d]	8[d]	−3.6171	−2.76	0.11	8	0–30
Glomerulosclerosis	52	42	34	18[d]	−3.1184	−3.49	0.07	39	18–58

[a] $n = 50$ for each group.
[b] $n = 330$ males, $n = 334$ females.
[c] Medullary cell hyperplasia.
[d] Significantly different from control, $P \leq 0.05$, by pairwise comparison.

control groups and that had borderline dose-response trends (lymph node granulation and prostatic atrophy in males) were within the historical control range.

Similarly, the incidence of adrenal angiectasis and adrenal hyperplasia in historical female controls was highly variable, and the incidence of these lesions in neosugar-treated females was within the historical control range.

If neosugar treatment were responsible for the occurrence of a particular lesion, the severity of the lesion would be expected to increase with increasing dose. Most of the lesions listed in Table 8 were slight in severity in control as well as neosugar groups. Neosugar treatment had no effect on severity of any of the lesions where significant differences in incidence were observed by pairwise comparisons, which further supports the conclusion that the observed lesions were not related to neosugar treatment.

Tumor incidence. Tumors that occurred in greater than 5% incidence in any group are shown in Table 9. Pituitary adenomas, pheochromocytomas, thyroid C-cell adenomas, Langerhans' islet adenomas, and leukemias occurred commonly in control groups of both sexes. Interstitial cell tumors of the testis were extremely common in control male rats, with an incidence greater than 80%. In the female control group, mammary gland fibroadenomas and endometrial stromal polyps were common. All of these tumors are considered to be spontaneous in F-344 rats.[21,22]

Neosugar treatment did not increase the incidence of rare tumors in male or female rats. The incidence of spontaneous tumors in neosugar-treated animals was comparable to their incidence in concurrent controls, with the exception of pituitary adenomas. In male rats, the incidence of pituitary adenomas for the 0, 8000, 20,000, and 50,000 ppm dose groups was 20%, 26%, 38%, and 44%, respectively. The average background rate of pituitary adenomas in control male F-344 rats in this laboratory is 31%, with a range of 17–49%. Thus, the incidence of this tumor in male rats used in this experiment was within the historical range. However, pairwise comparisons between neosugar treatment groups and the concurrent control group showed the 20,000 and 50,000 ppm dose groups to have a significantly higher incidence of pituitary adenomas. To test for dose-related trends, two widely accepted trend tests were performed on these data. The Cochran-Armitage Chi-square test indicated a dose-response trend ($P = 0.007$), whereas logistic regression analysis showed no such trend ($P = 0.51$).

In female rats, the incidence of pituitary adenomas for the 0, 8000, 20,000, and 50,000 ppm dose groups was 48%, 39%, 38%, and 28%, respectively. The incidence of pituitary adenomas in females showed an apparent negative trend (opposite to that seen in males), but the difference between the high-dose group and control was not significant using a two-tailed Chi-square test ($P = 0.06$). The background rate in control female F-344 rats ranges from 24% to 49%, with a mean incidence of 40%.

DISCUSSION

The results of the mutagenicity study demonstrate that, over a wide range of dose levels, neosugar does not cause gene mutations in bacteria or mammalian cells in culture and does not induce UDS in mammalian cells in culture either in the absence or in the presence of a functional, Aroclor-induced, rat liver microsomal metabolic activation system. Because neosugar was not appreciably toxic to the cells, it was necessary to impose an arbitrary maximum testing dose in these experiments rather than to test at doses up to and including toxic levels as is normal practice in mutagenicity testing.

In the chronic rat study, survival of both sexes was unaffected by neosugar treatment. The only significant finding was a decreased rate of survival in the male 20,000 ppm dose group. This is not believed to be treatment related, since it was an isolated occurrence and no dose-response relationship was evident. Body weight gain, food intake, hematology, and organ weights of both sexes were likewise unaffected by neosugar treatment.

TABLE 9. INCIDENCE OF NEOPLASMS IN MALE AND FEMALE RATS ADMINISTERED NEOSUGAR IN THE DIET FOR 2 YEARS

	Male (50 rats/group)				*Female (50 rats/group)*			
	0 (ppm)	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*	*0 (ppm)*	*8000 (ppm)*	*20,000 (ppm)*	*50,000 (ppm)*
Skin								
Keratoacanthoma	3 (6%)	2 (4%)	2 (4%)	3 (6%)	2 (4%)	0 (0%)	0 (0%)	2 (4%)
Subcutaneous tissue								
Fibroma	0 (0%)	3 (6%)	1 (2%)	2 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pituitary								
Adenoma	10 (20%)	13 (26%)	19 (38%)[a]	22 (44%)[b]	24 (48%)	19 (38%)	19 (38%)	14 (28%)
Adrenal								
Pheochromocytoma	6 (12%)	5 (10%)	7 (14%)	6 (12%)	1 (2%)	1 (2%)	4 (8%)	2 (4%)
Thyroid								
C-cell adenoma	3 (6%)	6 (12%)	5 (10%)	3 (6%)	3 (6%)	0 (0%)	3 (6%)	6 (12%)
Pancreatic islets								
Langerhans' islet adenoma	6 (12%)	5 (10%)	9 (18%)	6 (12%)	1 (2%)	0 (0%)	4 (8%)	0 (0%)
Testis								
Interstitial cell tumor	41 (82%)	41 (82%)	37 (74%)	40 (80%)	–	–	–	–
Mammary gland								
Fibroadenoma	0 (0%)	0 (0%)	1 (2%)	0 (0%)	7 (14%)	4 (8%)	3 (6%)	5 (10%)
Uterus								
Endometrial stromal polyp	–	–	–	–	8 (16%)	10 (20%)	11 (22%)	10 (20%)
Spleen								
Leukemia	2 (4%)	4 (8%)	6 (12%)	4 (8%)	4 (8%)	7 (14%)	12 (24%)	7 (14%)

[a]Significantly different from control, $P = 0.04$.
[b]Significantly different from control, $P = 0.01$.

Nonneoplastic lesions were extremely common in all rats. Sporadic increases and decreases in the incidence of some lesions were found by pairwise comparisons between control and neosugar treatment groups. If neosugar treatment were responsible for these significant findings, a dose-response relationship with regard to incidence and severity would be expected. Logistic regression analysis of the incidence data showed no significant positive or negative dose-related trends. In addition, the severity of the lesions with significant findings was not increased by neosugar treatment. Any decrease in severity would be undetectable, since almost all lesions were very mild. It should be noted that of the 17 lesions with significant findings, only 3 were common to both males and females, and for 2 of the 3 (lymph node granulation and stomach fibrosis), opposite trends were observed. No biological basis for a sex difference was apparent. A survey of the historical control incidence of these lesions showed that these lesions are common and highly variable in aging F-344 rats. Consideration of all of these factors leads to the conclusion that neosugar treatment did not affect the incidence of nonneoplastic lesions.

A slight but significant increase in blood Na and Cl was observed in male neosugar-treated rats, which is suggestive of mild renal impairment. However, other indicators of renal function, including K, Ca, phosphorus, BUN, uric acid, creatinine, and albumin, were not affected by neosugar treatment.

Neosugar did not increase the incidence of rare tumors in male or female rats, and pituitary adenoma was the only spontaneous tumor that was significantly increased in a neosugar treatment group. The increased incidence of pituitary adenomas occurred only in male rats. Pituitary adenoma is one of the most frequently occurring spontaneous tumors in male and female F-344 rats based on the historical incidence in this laboratory as well as National Cancer Institute and National Toxicology Program databases.[21,22] The background incidence also is highly variable, ranging from 20% to 50% in this laboratory. Although the incidence of this tumor in this experiment was well within the historical range for all male rats, the incidence in the two highest dose groups was significantly greater than the incidence in concurrent controls. However, the significance of a dose-related trend was equivocal in that one trend test showed a significant trend, whereas another test did not. If males are compared to females, a similar but opposite dose-response trend is noted. This dichotomy has no apparent biological basis. If male and female pituitary adenoma incidences are combined, no significant across-dose group differences are found. All of these observations point toward the conclusion that the higher incidence of pituitary adenomas in neosugar-treated male rats is a chance artifact. Such chance artifacts can arise when large numbers of statistical comparisons are made. In this study, 54 comparisons were made, and one to three significant results would be expected by chance alone at the significance levels of 0.01 and 0.05, respectively. It is unlikely, therefore, that neosugar was responsible for the occurrence of pituitary adenomas in male rats.

The results of this study indicate that neosugar is not mutagenic and does not produce chronic toxicity in rats. This lack of toxic activity is expected because, as described in the Introduction, neosugar is a mixture of simple oligosaccharides comprised of glucose and fructose. There is no reason to believe that such oligosaccharides or their metabolites would have genotoxic, carcinogenic, or chronic toxic potential.

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