A Comparison of the Chronic Toxicities of Synthetic Sweetening Agents*

By O. GARTH FITZHUGH, ARTHUR A. NELSON, and JOHN P. FRAWLEY

Data are given on the chronic oral toxicities of the synthetic sweetening agents, saccharin, sodium cyclohexyl sulfamate, dulcin, and P-4000. The results indicate that these four substances may be divided into a toxic group and a relatively nontoxic group. Dulcin and P-4000 were toxic to rats at dosage levels of 0.1 per cent and above, whereas saccharin and sodium cyclohexyl sulfamate had only slight effects at a dosage level of 5 per cent.

THE DISCOVERY and proposed use of two new synthetic noncaloric sweetening agents, sodium cyclohexyl sulfamate (hereinafter called cyclamate sodium) in this country (1), and P-4000 (1-*n*-propoxy-2-amino-4-nitrobenzene) in the Netherlands (2), led us to compare the relative chronic toxicities of these substances with the older sugar substitutes. The following experiments were designed to evaluate the relative safety of cyclamate sodium, P-4000, saccharin, and dulcin (4-ethoxy-phenylurea).

METHOD

Four parallel experiments were conducted in which groups of weanling albino rats (twenty-one days), from our colony of Osborne-Mendel strain and equally divided between the sexes, were started on diets containing one of the above-named sweetening agents. A total of 528 animals was used.

In the individual experiments, each of which included the dosage levels of a single substance and a control group, the number of rats per group was 20 except for the experiment with P-4000, which had 24. All animals were randomized into groups according to litter mates. The rats were started on dosage levels of 1, 0.5, 0.1, and 0.01% for each substance. Additional dosage levels of 5% saccharin and cyclamate sodium, 0.25% dulcin, and 0.25 and 0.001% P-4000 were used. Ground commercial rat biscuits with 1% added cod-liver oil served as a basic diet. The sweetening agents were mixed with the basic diet by means of a rotary batch mixer. All animals were kept in individual cages in a room with the temperature and humidity controlled for the duration of the experiment and were given free access to their respective diets and water. The weights of individual animals and their food consumption were determined at weekly intervals. Most animals were allowed to live out their natural life span; however, those living at the end of two years on the experiment were sacrificed.

RESULTS

Since the four experiments were started at the same time and were part of the same study, they will be discussed together.

* Received June 28, 1951, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

The Effect on Growth Rate and Food Consumption—An inspection of Table I shows that dosage levels of 0.5 and 1% dulcin and P-4000 retarded the growth rate significantly. Likewise the animals on 5% saccharin and cyclamate sodium were smaller than the controls. These differences were not significant ($p < 0.05$) for the groups of males on cyclamate sodium and females on saccharin. All other dosage levels of each substance showed no retardation of growth. Individual growth curves from the same litter and the composite growth curves of the several groups showed little variation during the fast-growing period and during the plateau period. Analyses, therefore, for the gain in weight for the first twelve weeks and for the first twenty-six weeks of the experiment showed the same statistical differences as those for fifty-two weeks (Table I).

When the data for the weekly food consumption of each group during the first twenty-six weeks and during the second twenty-six weeks of the experiment were analyzed, the differences between the control and experimental animals were not statistically significant. A slight increase in food intake occurred with groups of animals on 5% cyclamate sodium and saccharin, and this can be accounted for by the amount of bulk noncaloric material added to the diet.

The Effect on Mortality.—Statistical tests cannot be applied to the mean survival time since all surviving animals were sacrificed at the conclusion of two years on the experiment. Most of the deaths during the first year, except for a group on dulcin, were distributed throughout all groups and in most cases represented animals killed because of middle ear infection. The single exception was the group of animals on 1% dulcin, which produced a 25% mortality during the first year. When the number of animals surviving at eighteen months and at two years were selected as estimates of relative toxicity, the dosage levels of 0.5% dulcin and 1% P-4000 also increased the mortality rate. Other dosage levels had no significant effect on mortality. The mortality rates for control animals at one year and two years were 14% and 68%, respectively.

The Effect on Organ Weights.—As is shown in Table II, dulcin and P-4000 increased the relative size of livers, kidneys, and spleens of the animals on the higher dosage levels. This was most evident in the case of the spleens in animals receiving 1% dulcin, where the spleens weighed up to 4.6 Gm. compared with an average control weight of 0.9 Gm. Spleen, liver, and kidney weights were significantly

TABLE I.—MEAN GAIN IN WEIGHT OF RATS FED DIETS CONTAINING SYNTHETIC SWEETENING AGENTS FOR ONE YEAR

Substance	Dosage, %	No. of Animals	Sex	Mean Gain in Wt., Gm.
Saccharin	Control	7	M	475.4 ± 27.25
		9	F	245.4 ± 8.93
	1.0	10	M	467.0 ± 22.24
		10	F	270.1 ± 12.93
	5.0	9	M	409.5 ± 11.23[a]
		9	F	226.6 ± 10.16
Cyclamate sodium	Control	9	M	439.7 ± 24.25
		11	F	286.4 ± 11.14
	1.0	6	M	468.8 ± 24.96
		9	F	241.7 ± 12.22
	5.0	9	M	408.5 ± 8.08
		7	F	209.3 ± 6.94[b]
Dulcin	Control	7	M	492.1 ± 29.80
		7	F	289.2 ± 15.01
	0.25	8	M	462.8 ± 22.86
		7	F	251.8 ± 24.87
	0.1	9	M	499.7 ± 24.63
		9	F	277.2 ± 6.80
	0.5	10	M	418.0 ± 15.09[a]
		7	F	204.4 ± 7.90[a]
	1.0	7	M	337.0 ± 18.73[b]
		7	F	174.0 ± 8.15[b]
P-4000	Control	10	M	478.9 ± 19.53
		9	F	256.7 ± 15.77
	0.25	10	M	489.8 ± 23.09
		10	F	246.8 ± 7.73
	0.1	10	M	514.7 ± 23.80
		9	F	254.2 ± 13.46
	0.5	11	M	403.5 ± 14.13[a]
		12	F	235.3 ± 9.61[a]
	1.0	9	M	397.3 ± 18.58[b]
		12	F	208.1 ± 5.72[b]

[a] $p < 0.05$.; [b] $p < 0.01$.

different from the controls at 0.5% dulcin, but not at 0.25% or less. At dosage levels of 1.0 and 0.5%, P-4000 significantly increased the relative weights of liver and kidney, while increase in splenic weight was not significant. Certain correlations and explanations of the above-mentioned weight increases will be found with the description of the microscopic pathology. Saccharin and cyclamate sodium had no effect on organ weights.

Pathology.—Of the 528 treated and control animals started on the experiment, all but a few had a gross examination of their tissues at autopsy, and 407 were examined microscopically. Detailed examination was done in 185 instances and routinely included hematoxylin-eosin stained paraffin sections of lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal, thyroid, testis (or uterus and ovary), leg muscles, leg bones, and bone marrow; the remaining 222 were usually limited to liver, kidney, and (in males) testis. Various special stains were done in a limited number of instances.

Dulcin.—At the higher dosage levels, two effects of dulcin were frequent, and distinctly noticeable grossly. Liver tumors began appearing in rats autopsied after fifty-nine weeks, and eventually involved 10 of the 1% rats, 2 at 0.5%, and 1 at 0.1%. These tumors were up to 2.5 cm. in diameter, although usually about 8 mm. in diameter, and were sometimes multiple. They accounted for most, if not all, of the increased average organ weight. Enlargement and a purplish darkening of the spleen were noted as early as thirteen weeks on the experiment. The incidence and degree of this lesion, present at levels as low as 0.1%, diminished more gradually with decreasing dosage than did the liver tumors.

On microscopic examination the liver tumors were seen to be hepatic cell adenomas, composed of irregular cords and masses of cells slightly to moderately larger than the surrounding hepatic cells, and markedly but by no means completely similar to them in shape and staining quality. Some of the larger tumors were histologically malignant. Apart from the tumors, the livers showed no other direct effect of the dulcin. There were certain indirect effects, namely hepatic cell atrophy, Kupffer cell hemosiderosis and bile duct proliferation, all in relatively slight degree. The hemosiderosis was the most significant of these; it was very slight at 0.25%, slight at 0.5%, and slight to moderate at 1.0%.

The enlargement and darkening noted grossly in the spleen were associated microscopically with chronic congestion and to a lesser extent with hyperplasia of the pulp and an increased amount of hemosiderin. At 0.01% these changes were absent; at 0.1% they were present in a slight degree at seventy-nine weeks and thereafter, whereas at 0.25% they were definite in the first animal autopsied at twenty-seven weeks, and as time and dosage increased there was a parallel increase in the microscopic changes related to the enlargment and darkening. At the two highest dosage levels these generalized changes were sometimes overshadowed by localized changes. The local lesions appeared in the animals on 0.25% dulcin at fifty-eight weeks and thereafter, and consisted of rounded areas about

TABLE II.—LIVER, KIDNEY, AND SPLEEN WEIGHTS OF RATS FED THE HIGHER DOSAGE LEVELS OF SWEETENING AGENTS FOR ONE YEAR OR MORE

Substance		Mean Weight in Gm./Kg. of Body Weight		
		Liver	Kidney	Spleen
Control		30.4 ± 1.5	7.2 ± 0.4	2.0 ± 0.3
Saccharin	5%	29.4 + 1.9	8.5 ± 0.4	2.1 ± 0.2
	1%	38.4 ± 1.9	8.5 ± 0.5	2.1 ± 0.2
Cyclamate sodium	5%	27.8 ± 1.9	7.6 ± 0.5	2.1 ± 0.2
	1%	26.7 ± 1.9	7.5 ± 0.5	1.9 ± 0.2
P-4000	1%	46.5 ± 3.3[a]	12.0 ± 1.1[a]	3.0 ± 0.3
	0.5%	42.3 ± 1.9[a]	12.1 ± 0.8[a]	2.7 ± 0.4
	0.25%	34.4 ± 2.7	9.3 ± 0.4	1.6 ± 0.4
	0.1%	34.3 ± 1.5	10.5 ± 0.8	1.9 ± 0.2
Dulcin	1%	58.3 ± 7.7[b]	11.7 ± 1.0[a]	9.3 ± 1.6[b]
	0.5%	39.9 ± 2.8[a]	9.3 ± 0.7[a]	5.0 ± 0.7[a]
	0.25%	33.7 ± 3.2	7.2 ± 0.6	4.8 ± 1.6
	0.1%	31.4 ± 1.5	8.7 ± 0.5	3.0 ± 0.6

[a] $p < 0.05$.
[b] $p < 0.01$.

1 mm. in diameter, poor in lymphoid cells and congested. The sinuses were not distended and contained moderate numbers of hemosiderin-laden macrophages. These local changes increased in severity with dosage and at the 1.0% dosage level several spleens had a markedly distorted structure because of the irregular distribution of hyperplasia, atrophy, and fibrosis.

In the kidney, pigmentation of the proximal convoluted tubular epithelium by a brown, chiefly nonferrous pigment which paralleled in degree and relation to dosage the hemosiderosis in the liver, was the only change attributable to dulcin. The relatively increased kidney weight (Table II) was not accompanied by an increased incidence of focal nephritis, fibrosis, etc., as will be mentioned in connection with P-4000. It should be remembered that the weight difference was smaller with dulcin, and that the dulcin animals showed greater growth retardation.

P-4000.—The chief gross and microscopic change caused by P-4000 was a pigmentation of the thyroid gland, seen at 0.25% and more in the diet. At 1.0% the thyroids were uniformly a dark reddish black in the fresh condition and almost coal black after formalin fixation. Slight enlargement and epithelial hyperplasia were also present. The pigment, in brown granules of about 1 μ diameter, filled the epithelial cells and to a lesser extent the follicular lumens. The appearance of the pigment, together with the results of various special staining reactions, eliminated P-4000 itself, hemofuscin, hemosiderin, ceroid, or carotenoid as the pigment, and narrowed the possibilities to either a wear-and-tear pigment or, perhaps more likely, a melanin. We have not seen such thyroid pigmentation before in any of our numerous chronic toxicity experiments. Our normal older rat thyroids may contain a very small amount of pigment, differing somewhat in appearance from that seen with P-4000; the quantitative difference between the two is of the order of 100:1 at the 1.0% level.

At 1.0%, some of the urinary bladders contained a soft yellowish gelatinous precipitate which dried into a light powder. Analysis showed the principal constituent of this powder to be protein, together with some uric acid and ammonium urate crystals. The yellow color came from P-4000, which likewise stained the urine, gastrointestinal tract, and fur of the animals.

The slight enlargements of the liver and spleen at the two highest dosage levels (Table II) were reflected microscopically in slight hepatic cell enlargement and, in the spleen, slight pulp hyperplasia and congestion, in some of the animals. Similarly, the increased kidney weights (and, grossly, increased pitting and fibrosis) correlated with an increased incidence of focal nephritis (nephrosis) at 0.1% or more of P-4000; the histological features of the nephritis were not changed. In tabulating the incidence of nephritis, the break came between 0.01% and 0.1%. Combining the two lowest dosage groups and the similarly appearing controls into one group gave 21% with marked nephritis and 36% with moderate or marked nephritis. Similarly combining the rats on 0.1% or more gave percentages of 31 and 59, respectively.

Saccharin.—No pathological effect whatever could be attributed to saccharin at levels of 1.0% or less. At 5% only one effect was noted, in the latter part of the experiment, namely an increased incidence of the ordinarily uncommon condition of abdominal lymphosarcoma. In the 5% group, there were seven animals with lymphosarcomas; this number is not out of line with the incidence in comparable groups of rats, but the fact that in four of the seven rats abdominal as well as thoracic lymphosarcomas were present is unusual, since ordinarily the ratio is about 1 to 15–20. Three of these four combinations occurred in animals on experiment one hundred and two or more weeks.

Cyclamate Sodium.—As with saccharin, the groups on dietary levels of 1% or less showed no changes which could be attributed to the cyclamate sodium. At the 5% level the only gross and microscopic effects were those of mild inanition. The animals at this dosage level had a moderate amount of diarrhea throughout the experimental period.

Hematology.—Blood studies were made once during the first year and twice during the second year of the experiment on 10 rats from each group of the higher dosage levels of each substance. The group on 1% dulcin showed a significant reduction in the erythrocyte count from an average of about 8 million in the controls to about 5 million in the experimentals and numerous normoblasts in the blood stream. The groups on 0.5% dulcin had a few normoblasts and a slightly, but not significantly, reduced average erythrocyte count. All other groups were normal.

DISCUSSION

The results of the above experiments show that the four synthetic sweetening agents may be divided into a relatively nontoxic group and a toxic group. Saccharin and cyclamate sodium belong to the former, and dulcin and P-4000 to the latter. The nontoxicity of saccharin has been well known both from extensive animal experimentation (3, 4) and from a long use. The present experiment on cyclamate sodium supports our earlier report (5) and the recent data of Richards, *et al.* (6). Richards, *et al.* (6), likewise found that the only toxic symptom of cyclamate sodium to animals was diarrhea at high levels. This also was observed clinically if persons took several grams of cyclamate sodium at one time. Although the sweetness equivalent of cyclamate sodium is about eight times less than saccharin, it appears to be safe for use as a saccharin substitute. There were no distinct toxic effects at levels as high as 5% in the diet of rats, and diarrhea seems to be the warning signal for the maximum tolerated dose both in animals and in man. It has been known that large doses of dulcin would produce toxic effects (7, 8); however, no previous experiment has shown the extent of such damage from small repeated dosages. From experiments in dogs lasting only twenty days, Kling, *et al.* (9), suggested the possibility of an accumulative effect of dulcin. Because of the extensive damage observed in our experiment, dulcin cannot be considered safe for food or drug use, even in small quantities. Lehmstedt (10), although mentioning the possibility of toxicity from compounds of the nature of P-4000, emphasized its high sweetening power. Since about a tenth as much P-4000 is required to give the sweetness equivalent to saccharin, this should be considered in a final analysis of its usefulness; however, in our experiments the ratio of toxicity is less than this ratio of effectiveness. P-4000 also has an undesirable anesthetic property. Thus, P-4000 cannot be considered to be a safe or desirable sweetening agent.

SUMMARY

1. In two-year feeding studies on rats, saccharin and cyclamate sodium were found to be without effect at levels of 1.0 per cent or less in the diet, and to cause only slight toxic effects at 5.0 per cent.

2. Dulcin (4-ethoxy-phenylurea) was toxic to rats at dosage levels of 0.1 per cent and upward. Adenomas of the liver up to 2.5 cm. in diameter occurred in half of the animals on 1.0 per cent, and in a few at lower levels down to 0.1 per cent of the diet. Splenic enlargement and darkening, associated with chronic congestion, pulp hyperplasia, and increased hemosiderin content, began at the 0.1 per cent level and increased parallel with dosage. Anemia and decreased growth rate were present at 1.0 and 0.5 per cent, as were increases in mortality rate, and in the number of circulating normoblasts.

3. P-4000 (1-*n*-propoxy-2-amino-4-nitrobenzene) was toxic to rats at dosage levels of 0.1 per cent and upward. An unusual pathological change was the presence of large amounts of a melanin-like pigment in the thyroid; at the 1.0 per cent level the thyroids were slightly enlarged and almost black. Pigmentation was pronounced at 0.5 per cent and slight but distinct at 0.25 per cent. A somewhat increased incidence of focal nephritis was present at 0.1 per cent and upward. Dosage levels of 1.0 and 0.5 per cent decreased growth rate and increased the relative size of the liver and kidney. The 1.0 per cent level increased mortality rate.

REFERENCES

(1) Audrieth, L. F., and Sveda, M., *J. Org. Chem.*, **9**, 89 (1944).
(2) Verkade, P. E., van Dijk, C. P., and Meerburg, W., *Proc. Koninkl. Nederland. Akad. Wetenschap.*, **45**, 630(1942).
(3) Fantus, B., and Hektoen, L., THIS JOURNAL, **12**, 318(1923).
(4) Carlson, A. J., Eldridge, C. J., Martin, H. P., and Foran, F. L., *J. Metabolic Research*, **3**, 451(1923).
(5) Fitzhugh, O. G., and Nelson, A. A., *Federation Proc.*, **9**, 272(1950).
(6) Richards, R. K., Taylor, J. D., O'Brien, J. L., and Duescher, H. O., THIS JOURNAL, **40**, 1(1951).
(7) Rost, E., and Braun, A., *Arb. Reichsgesundh.*, **57**, 212(1926).
(8) Pesman, J., and Luten, J., *Nederland. Tijdschr. Geneesk.*, **90**, 261(1946).
(9) Kling, A., Bovet, D., and Ruiz, J., *Bull. acad. méd.*, **125**, 69(1941).
(10) Lehmstedt, Kurt, *Seifen-Öle-Fette-Wachse*, **75**, 81 (1949).