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Influence of Diet on Chronic Oral Toxicity of Safrole and Butter Yellow in Rats.* (30388)

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Chronic oral toxicity studies are generally conducted in dogs and rats according to methods described by Fitzhugh(1). Such methodology, while noting that dietary effects on the toxicity of chemicals represent a problem, does not prescribe the use of specific standardized diets. "Proved commercial diets" are usually employed and the composition of such diets varies considerably.

In the case of a relatively weak hepatic carcinogen such as Safrole(2,3,4,5), dietary composition may have significant bearing on the outcome of experiments, and a study was therefore made to determine hepatotoxic effects of Safrole as well as those of a more potent hepatic carcinogen, butter yellow, in rats placed on diets containing 5, 10 and 30% protein. Fat content was varied to 5 and 15% in the case of the 30% diet. One group of rats was also fed a riboflavin-free 10% protein, 5% fat diet to compare the butter yellow-carcinogenesis-enhancing effect of this deficiency with its effect on Safrole carcinogenesis.

Material, methods and experimental design. The animals used were Osborne-Mendel male rats obtained from Camm Research Institute. Animals received Achromycin†† (0.001%) in drinking water for three days on arrival. Starting weight averaged 114 g (range, 92-135 g) for each group of 10 rats. Food consumption measurements were made during the first year of the experiment. The animals were given 0.5% Safrole† or 0.06% butter yellow mixed in their diet. The evaporation rate of Safrole was found by chemical analysis to be 11.3% per 3-day period(3). The diets were prepared in 2-kg lots as needed, kept refrigerated in sealed jars, and fed freshly every third day. All animals were kept individually in hanging cages. Animal room temperature was 24°C (± 2°C), and tap water was given *ad libitum*. All rats were killed when they appeared to be moribund.

Autopsies were conducted on all animals and included measurement of organ weights and histological study of lungs and livers and of any organ which appeared upon inspection to be diseased. The experimental design and diets are shown in Table I.

Results. The results are illustrated in Fig. 1 to 5. Significant differences, where noted, indicate a probability level of 5% or less by Student's t-test.

Weight curves (Fig. 1) reveal that during the first year, the controls showed the anticipated growth variations dependent on the protein and fat content of the diet with only

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†† Achromycin was generously supplied by Lederle Laboratories Division of American Cyanamid Company, Pearl River, New York.

† Safrole was obtained from Matheson, Coleman & Bell, Rutherford, N. J., and p-dimethylaminoazobenzene came from K & K Laboratories, Inc., Plainview, N. J.

TABLE I. Dietary Variables and Experimental Design for Chronic Oral Toxicity Study Using Male Osborne-Mendel Rats.

Test substance		Diet composition, %			
		Protein*	Fat†	Carbohydrate‡	Salts§‖
Control		5	5	85	5
.5% Safrole		5	5	85	5
.06% Butter yellow		5	5	85	5
Control		10	5	80	5
.5% Safrole		10	5	80	5
.06% Butter yellow		10	5	80	5
Control		30	5	60	5
.5% Safrole		30	5	60	5
.06% Butter yellow		30	5	60	5
Control		30	15¶	50	5
.5% Safrole		30	15¶	50	5
.06% Butter yellow		30	15¶	50	5
Control	*No riboflavin*	10	5	80	5
.5% Safrole	*No riboflavin*	10	5	80	5
.06% Butter yellow	*No riboflavin*	10	5	80	5

* Vitamin-free casein, Nutritional Biochemicals Co.
† Mazola corn oil, Corn Products Corp.
‡ Amijel, partially hydrolyzed corn starch, Corn Products Corp.
§ H. M. W. Salt Mixture, Nutritional Biochemicals Co.
‖ Purified vitamins added to supply per 100 g of diet, except riboflavin where noted: thiamine, 1 mg; pyridoxine, 3 mg; riboflavin, 3 mg; pantothenate, 10 mg; choline, 300 mg; biotin, 0.1 mg; folic acid, 0.8 mg; α-tocopherol, 45 mg; Vit. A, 180 I.U.; Vit. D, 200 I.U.
¶ 10% Mazola corn oil, 5% Crisco, Proctor & Gamble Co.

the riboflavin-deficient and the low-protein, low-fat diets (relative to the 15% fat diet) falling into the range of significantly inhibited growth. The addition of Safrole depressed growth rate of the animals fed at the 30% protein level and at the 10% protein level, but only after more than 10 weeks of feeding, and it did not inhibit growth markedly at the 5% protein concentration.

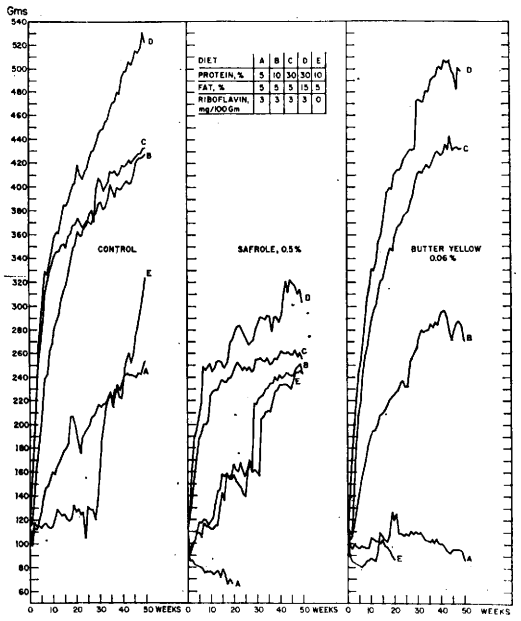

FIG. 1. 50-week body weight curves for various diets with added Safrole or butter yellow.

The removal of riboflavin had little effect on the toxic action of Safrole upon these animals' growth. In contrast, the toxic effect of butter yellow as expected(6), is accentuated in the riboflavin-deficient diet, but otherwise appears to be less growth-depressing than Safrole at the other protein levels studied.

Weights of butter yellow-fed rats at the time of autopsy (Fig. 2) were significantly higher than those of Safrole-fed animals on all diets except the riboflavin-deficient and the low-protein, low-fat groups.

The weights of the livers (Fig. 3) are high in the butter yellow-treated animals, mostly because of the high density and large size of the tumors which occurred. In Safrole-fed rats, the livers of the animals receiving the 30% protein diet appear normal, while on the other diets, the livers of the Safrole-treated animals are smaller than those of the controls. The relative liver weight of the ani-

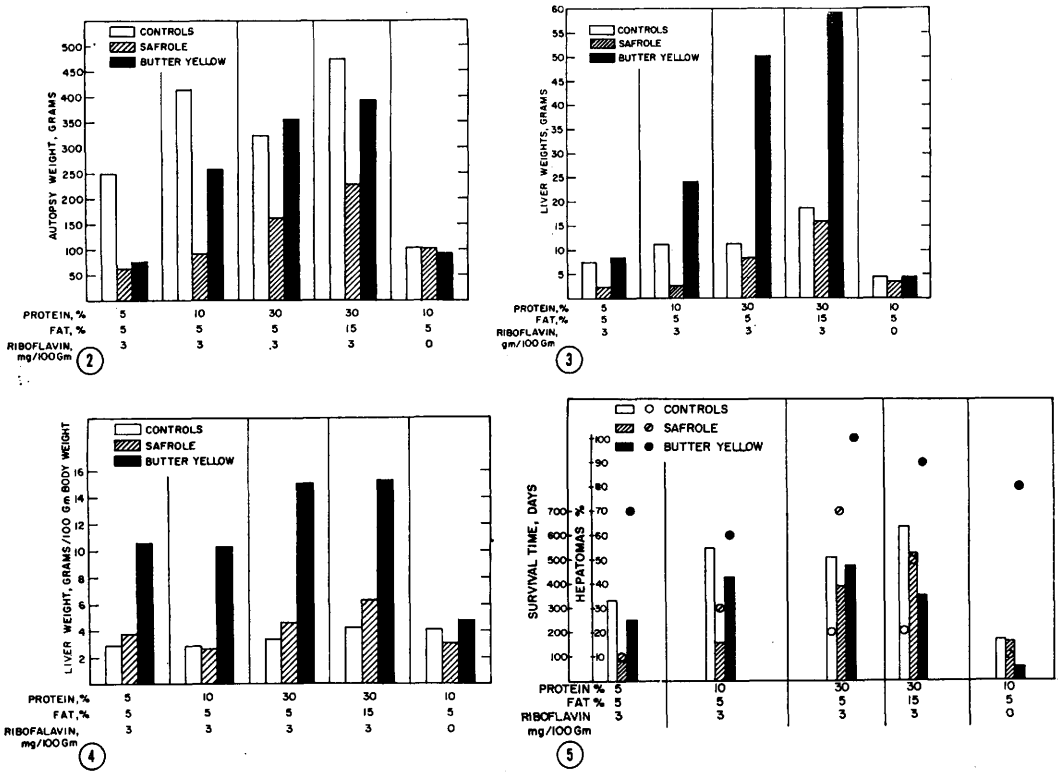

FIG. 2. Toxicity and hepato-carcinogenicity of Safrole and butter yellow as influenced by diets of various composition.

FIG. 3. Toxicity and hepato-carcinogenicity of Safrole and butter yellow as influenced by diets of various composition.

FIG. 4. Toxicity and hepato-carcinogenicity of Safrole and butter yellow as influenced by diets of various composition.

FIG. 5. Toxicity and hepato-carcinogenicity of Safrole and butter yellow as influenced by diets of various composition.

mals fed a 30% protein and Safrole diet was slightly heavier than was relative liver weight of animals on all other diets (Fig. 4).

Histological observations (Table II) showed no fat in the livers of any of the animals fed Safrole or butter yellow, whereas fat was seen in the livers of about one-half of the corresponding control animals.‡ The pathological examination of organs other than the liver was negative, except for some cases of murine pneumonia.

Organ weights listed in Table III showed no significant changes except those already discussed.

Studies of hepatoma incidence and longevity (Fig. 5) reveal the following: 1) Safrole-treated animals live longer at higher protein levels, than at lower ones, especially on 30% protein and 15% fat, whereas butter yellow-treated animals benefit from an increase of protein from 5% to 10%, but gain no further lifetime from further increases in proteins. 2) At the doses used (0.5% Safrole and 0.06% butter yellow), the hepatoma-producing effect of Safrole was greater with the high-protein diets, and minimal with 5 and 10% protein, whereas with butter yellow, over half of the animals had tumors on the low-protein diets also. 3) In the presence of riboflavin-deficiency, butter yellow was of maximal toxicity and high carcinogenicity, whereas Safrole was no more toxic than the corresponding control diet and only one tumor occurred.

‡ It is possible that a relative niacin deficiency was instrumental in causing the fatty livers of some of the controls, but we do not consider that this bears on the principal conclusions of our paper.

TABLE II. Liver Pathology of Osborne-Mendel Rats Fed Semi-Synthetic Diets Supplemented with Safrole (0.5%) or Butter Yellow (0.06%).

Treatment*	Days on diet	Normal	Adeno-matosis	Fatty	Bile duct prolif.	Cirrho-sis	Malignancy, hepatoma-carcinoma	Others†
5% Prot.—5% fat	335	5	—	4	—	—	0	1
+ .5% Safrole	85	7	—	—	1	—	1	1
+ .06% butter yellow	250	1	1	—	—	1	7	—
10% Prot.—5% fat	546	7	—	3	—	—	0	—
+ .5% Safrole	158	5	—	—	1	—	3	1
+ .06% butter yellow	422	3	—	—	—	—	6	1
30% Prot.—5% fat	508	3	—	4	—	—	2	1
+ .5% Safrole	393	1	—	—	1	—	7	1
+ .06% butter yellow	479	—	—	—	—	—	10	—
30% Prot.—15% fat	634	2	—	4	—	—	2	2
+ .5% Safrole	524	3	1	—	—	—	5	1
+ .06% butter yellow	354	1	—	—	—	—	9	—
No riboflavin								
10% Prot.—5% fat	168	5	—	3	—	—	0	2
+ .5% Safrole	158	6	1	—	—	—	1	2
+ .06% butter yellow	55	—	1	—	—	1	8	—

* Ten ♂ O-M rats per diet. † Abscess, hyperemia, cysts, etc.

TABLE III. Actual Organ Weights, Means ± Standard Deviation (Values in Parentheses Are mg/100 g Body Weight).

Treatment*	Autopsy wt, g	Kidney, g	Adrenal, mg	Gonad, g	Heart, g	Liver, g
5% Prot.— 5% fat	250 ± 45	1.51 ± .21 (623 ± 138)	30.1 ± 9.1 (12.5 ± 4.8)	2.55 ± .41 (1033 ± 154)	.82 ± .20 (337 ± 104)	7.29 ± 1.27 (2991 ± 670)
+ .5% Safrole	62 ± 12	.89 ± .17 (1431 ± 154)	30.4 ± 6.6 (49.5 ± 10.3)	.33 ± .24 (509 ± 286)	.38 ± .08 (602 ± 78)	2.45 ± 1.04 (3805 ± 875)
+ .06% butter yellow	76 ± 12	.95 ± .19 (1258 ± 170)	25.1 ± 6.2 (34.0 ± 8.3)	.59 ± .37 (808 ± 528)	.47 ± .15 (616 ± 168)	8.52 ± 7.29 (10640 ± 8607)
10% Prot.— 5% fat	416 ±141	2.63 ± .44 (714 ± 300)	37.3 ± 7.4 (10.5 ± 4.5)	3.06 ± .72 (802 ± 269)	1.29 ± .27 (367 ± 238)	11.22 ± 3.80 (2836 ± 766)
+ .5% Safrole	92 ± 35	1.07 ± .59 (1133 ± 142)	34.5 ± 6.6 (40.2 ± 8.7)	1.03 ± .56 (1124 ± 565)	.43 ± .23 (458 ± 74)	2.75 ± 2.75 (2639 ± 1043)
+ .06% butter yellow	258 ± 61	2.10 ± .45 (796 ± 315)	47.8 ± 5.1 (19.7 ± 5.6)	2.40 ± .67 (871 ± 298)	.88 ± .29 (322 ± 130)	24.27 ± 17.22 (10290 ± 8236)
30% Prot.— 5% fat	326 ± 96	3.00 ± 1.08 (992 ± 505)	57.6 ± 19.0 (20.5 ± 11.1)	2.37 ± .82 (755 ± 274)	1.08 ± .25 (343 ± 58)	11.20 ± 3.70 (3444 ± 1033)
+ .5% Safrole	161 ± 39	1.95 ± .54 (1217 ± 235)	50.7 ± 11.3 (33.4 ± 10.5)	2.00 ± .37 (1348 ± 515)	.63 ± .18 (391 ± 58)	8.13 ± 7.67 (4659 ± 3852)
+ .06% butter yellow	356 ± 90	2.71 ± .51 (787 ± 167)	52.6 ± 14.9 (16.3 ± 6.5)	2.70 ± .72 (781 ± 228)	1.36 ± .31 (401 ± 116)	50.41 ± 21.25 (15070 ± 7157)
30% Prot.— 15% fat	474 ±138	3.80 ± .66 (869 ± 311)	89.2 ± 21.8 (20.6 ± 8.3)	2.51 ± .38 (569 ± 168)	1.78 ± .29 (404 ± 115)	18.68 ± 6.29 (4249 ± 1870)
+ .5% Safrole	229 ± 75	2.30 ± .70 (1021 ± 134)	21.7 ± 12.8 (24.0 ± 7.4)	2.17 ± .81 (973 ± 269)	.88 ± .37 (386 ± 122)	15.95 ± 12.79 (6235 ± 4772)
+ .06% butter yellow	393 ±115	2.84 ± .42 (753 ± 124)	56.6 ± 14.6 (16.0 ± 9.0)	3.16 ± .36 (855 ± 209)	1.19 ± .25 (314 ± 62)	59.47 ± 31.03 (15290 ± 7430)
No riboflavin						
10% Prot.— 5% fat	105 ± 35	1.31 ± .54 (1230 ± 193)	29.4 ± 6.2 (31.7 ± 6.7)	.88 ± .54 (786 ± 363)	.50 ± .15 (486 ± 77)	4.65 ± 3.42 (4079 ± 1715)
+ .5% Safrole	102 ± 58	1.13 ± .38 (1177 ± 171)	34.4 ± 6.5 (38.2 ± 10.9)	.96 ± .78 (907 ± 446)	.46 ± .19 (472 ± 64)	3.44 ± 3.35 (3020 ± 779)
+ .06% butter yellow	93 ± 8	1.36 ± .12 (1462 ± 108)	37.5 ± 5.3 (40.1 ± 5.4)	.77 ± .43 (822 ± 461)	.47 ± .04 (503 ± 38)	4.52 ± 4.67 (4794 ± 482)

* Semi-synthetic diets fed to Osborne-Mendel male rats.

Discussion. The incidence of liver tumors produced by a potent carcinogen such as butter yellow is but little changed by variations of dietary protein levels. In contrast, with a weak hepatic carcinogen such as Safrole, only one tumor occurred among 10 animals receiving a low-protein diet. When 30% of protein was fed, 7 out of 10 animals had liver tumors. Thus, a conclusive demonstration of carcinogenicity can be made with animals fed high-protein diets because of their extended lifespan, even though some hepatomas occurred in the 30% protein controls. However, an inconclusive result is obtained when using a low-protein diet.

From a practical point of view, the safety testing of intentionally low-caloric diet foods on diets covering a wide range of protein content is especially important because the increasing use of sweeteners(7)§ in place of sugar results in low-carbohydrate, relatively high-protein diets, since persons accustomed to consuming large amounts of carbohydrates today are switching in large numbers to the low-caloric products.

The varying response to different modifications of the diet in rats receiving Safrole or butter yellow suggests that these substances may achieve similar end results such as shortening life and increasing tumor incidence by different metabolic mechanisms. The clarification of this question, however, requires additional experiments having more exact control of food intake than that required by conventional chronic oral toxicity feeding techniques.

§ For example, cyclamate consumption alone was reported as being 7 million pounds in 1964, up from 0.5 million pounds in 1958.

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Glucose Uptake Related to Proliferation of Animal Cells *in vitro*. (30389)

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The literature on glucose consumption by animal cells *in vitro* is voluminous. The most consistent conclusion has been the lack of any correlation among glucose uptake, cell numbers, and rates of proliferation (*e.g.*, 1-8). This report presents data which clarifies this apparent anomaly by demonstrating that these factors are directly related when animal cells are provided a suitable environment *in vitro*; also, the magnitude of uptake may be fundamentally different in cells of fibroblast- *vs* epithelial-like morphology. To our knowledge, neither of these aspects has been demonstrated previously in animal cell culture systems.†

Methods. Animal cells of diverse origins and morphology were employed. Experiments were conducted in a perfusion system for replicate T-60 flask cell cultures(9), which has several advantages compared with conventional culture systems. These include provisions for pH control, maintenance of nutrient levels, elimination of "waste" accumulations, and production of unusually dense cell populations.

Jensen rat sarcoma and Walker rat car-

† A relationship between glucose utilization and proliferation of WI-38 cells has recently been reported by Cristofalo and Kritchevsky (Proc. Soc. Exp. Biol. and Med., 1965, v118, 1109).