The Genesis and Growth of Tumors

V. Effects of Varying the Level of B Vitamins in the Diet*

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As parts of a broad, integrated program on nutrition in relation to cancer, the influence of underfeeding, caloric restriction, and the proportions of fat and protein in the diet of mice have been investigated (9–12). The work reported in this publication deals with the possible modification of tumor formation consequent to varying certain B vitamins from dietary levels considered to represent minimal requirements for mice to amounts several-fold higher. Two experiments are concerned with the spontaneous mammary carcinoma and two with skin tumors deliberately induced with chemical carcinogens.

Vitamins of the B complex were chosen for these investigations on the assumption that large dietary differences in these essentials may exert a more significant influence on metabolism—and, therefore, possibly on carcinogenesis—than might other vitamins. Particularly, the consequences of stepwise gradation of six vitamins were studied: thiamine, riboflavin, pyridoxine, pantothenate, niacin, and choline. The minimal dietary requirements for the first four are known with a fair degree of accuracy. The needs for niacin and choline and the remaining known members of the B complex have not been defined for mice; apparently, they were adequately supplied by the small amounts in the "vitamin-free" casein and yeast extract employed in the present experiments, and by the considerable contribution of intestinal synthesis. It should be noted that the mice were housed under relatively good conditions, and not exposed to toxic agents, or to antibiotics that might hinder synthesis of vitamins by the intestinal flora. In all four experiments the vitamins were varied as a group, it being our purpose to ascertain over-all effects before considering the possible influence of altering the consumption of a particular vitamin.

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METHODS

The mice utilized in the investigations were of inbred strains, raised in our laboratory. At weaning they were divided into three groups, littermate distribution being employed so far as possible. They were housed five to a cage and were fed Purina Laboratory Chow checkers until institution of the experimental diets. During the study the animals were kept in cages with wire-meshed bottoms in order to minimize coprophagy.

In all experiments the mice received a semipurified diet composed of:

Casein	21 per cent
Gelatin	2 per cent
Partially hydrogenated cottonseed oil	5 per cent
Salt mixture (14)	4 per cent
Cornstarch	68 per cent

The casein was Borden's Labco Vitamin-Free brand, for which a typical vitamin assay was kindly supplied by the manufacturer: in micrograms per gram it contained thiamine, 0.03; riboflavin, 0.35; pyridoxine, 0.03; pantothenate, 0.9; niacin, 0.35; biotin, 0.03; folic acid, 0.13; vitamin B_{12} is present in undetermined amount. Ten U.S.P. units of Vitamin A, 1 U.S.P. unit of Vitamin D, and 0.4 mg. of Vitamin E in 0.005 ml. of cottonseed oil were incorporated into the daily ration. The B vitamins were supplied in yeast extract and as crystalline compounds, described below. The diets were prepared each week and stored in a refrigerator at 4° C. The materials and methods employed in preparing the diets have been described previously (12).

Small supplements of yeast extract were utilized to supply a limited amount of B vitamins—and also unknown factors. The vitamin content of the Yeast Extract No. 3 (Anheuser-Busch Co.) is given in Table 1. The actual designed levels of vitamins were achieved by the addition of crystalline synthetic compounds.

Each experiment included three groups of mice which were fed diets differing only in their content of B vitamins. The diet with the lowest levels of B vitamins was designed to contain thiamine, riboflavin, pyridoxine, and pantothenate in amounts approximately minimal for maintenance or growth—an attempt to provide enough to avoid vitamin deficiency and consequent voluntary food restriction. The actual quantities were based on information in the literature (6), modified by the results of pilot experiments with our colonies of mice. The ration with 3 times these levels was considered to have reasonably adequate amounts of B vitamins (control or "normal" levels), whereas the one with 5 or 9 times the minimal levels contained super-abundant amounts. For simplicity, these three diets and the groups of mice consuming the rations are designated as low, moderate, and high.

In the first studies with the spontaneous mammary carcinoma and the induced skin tumor, the same amounts of yeast extract were used in the preparation of the several rations: 0.007 ml/gm of diet in Experiment 1, and 0.005 ml. in Experiment 3. Synthetic thiamine, riboflavin, pyridoxine, Ca pantothenate, niacin, and choline were added in such quantities that (except for choline) they were present in the ratio of approximately 1:3:5 in the low, moderate, and high vitamin diets, respectively.

A second set of experiments, differing slightly from the preceding design, was performed with the spontaneous mammary carcinoma (Experiment 2) and the induced skin tumor (Experiment 4). The low vitamin rations contained only 0.002 ml of yeast extract/gram of food plus supplements of synthetic thiamine, riboflavin, pyridoxine, pantothenate, and choline. The moderate and high vitamin rations contained, respectively, 3 and 9 times the amounts of both yeast extract and synthetic B vitamins, so that the experimental diets differed in the ratio of approximately 1:3:9.

The calculated amounts of vitamins/gram of respective rations given to the mice of the four experiments are shown in Table 1. The calculations include the estimated amounts contributed by the yeast extract and the alcohol-extracted casein plus the actual amounts of added synthetic vitamins. The latter account for the major proportion of the totals in the moderate and high vitamin diets of Experiments 1 and 3 and of all diets of Experiments 2 and 4.

It is known that the level of caloric intake—and perhaps other factors related to body weight—influence the rate of formation of tumors, and that differences in caloric intake and body weight between groups may render interpretation of the effects of an experimental procedure difficult. It was not expected that both of these aspects could be kept constant for the three experimental groups consuming diverse vitamin-containing rations. This resulted in the decision to feed equicaloric amounts, at slightly below ad libitum levels, in three of the experiments, and ad libitum in the fourth. Actually, the degree of scattering of food by the mice of the three groups of an experiment was not uniform, resulting in slightly unequal food intakes.

The mice were allowed drinking water ad libitum. The routine execution of the experiments—determining food consumption and body weights, inspection for physical condition and gross recognition of tumors, the removal and histologic study of lesions—are described in previous publications (7, 12).

EXPERIMENTS AND RESULTS

Genesis of Spontaneous Mammary Carcinoma

Experiment 1.—Three groups, each consisting of 47 DBA female mice ranging from 21 to 26 weeks of age, were placed on semipurified diets that differed only in their B vitamin content (graded in the ratio of 1:3:5, as listed in Table 1). The daily ration per mouse was 2.7 gm. During the course of the experiment the mice of the three groups ingested approximately the same amount of food, 2.6 gm., the remainder being lost because of scattering. Food consumption figures indicated that the high vitamin group ate about 3 per cent more than the moderate and low vitamin groups.

The experiment proceeded smoothly and was terminated when only three mice (110 weeks of age) were alive and without spontaneous mammary carcinoma. The data on mean body weights, and incidence of tumors and the average time at which they appeared, are given in Table 2. It is obvious that low, moderate, or high dietary content (an over-all fivefold difference) of thiamine, riboflavin, pyridoxine, pantothenate, niacin, and choline, as a group, had no effect upon the incidence of spontaneous mammary carcinoma. The actual relative frequencies of mice with tumors were 86, 86, and 89 per cent, respectively. The tumors appeared, on the average, somewhat earlier in the mice fed the moderate vitamin diet.

Experiment 2.—This study was patterned after the previous one, but with serial gradation of both

TABLE 1

CALCULATED CONTENTS OF B VITAMINS IN THE RATIONS
(μg/gm of diet)

Vitamin	Experiment 1			Experiments 2 and 4			Experiment 3			Per milliliter of yeast extract*
	Low	Moderate	High	Low	Moderate	High	Low	Moderate	High	
Thiamine	4.5	14	23	3.2	10	30	3.3	10	17	660
Riboflavin	1.1	3.5	6	1.4	4	12	0.8	3	5	150
Pyridoxine	3	10	19	2.6	8	24	2.2	8	14	40
Pantothenate	10	30	51	6.6	20	60	7.5	23	38	200
Niacin	12	40	65	3.6	11	33	9	29	49	1,750
Choline	18	690	1,300	600	1,800	5,400	13	500	1,000	2,500

* These are average assay values of Yeast Extract No. 3 (Anheuser-Busch Co.) employed in these experiments. Per milliliter it also contained approximately 1,200 μg. of inositol, 3 of biotin, and 12 of folic acid, plus other factors natural to yeast. The amounts of yeast extract incorporated into the several rations are given under "Methods."

yeast extract and synthetic B vitamins, in the ratio of 1:3:9. Three groups, each made up of 65 DBA female mice 10–13 weeks of age, were given the respective low, moderate, and high vitamin diets; the calculated B-vitamin content of the three rations is detailed in Table 1. The daily ration per mouse was 2.6 gm. In all three groups about 5 per cent of the food was lost by scattering, and average food consumption was nearly equivalent.

The experiment was continued until the mice were 110 weeks of age, at which time there were only two in each group alive and tumor-free. The data on mean body weights, incidence of spontaneous mammary carcinoma, and mean time of tumor appearance are summarized in Table 2. In the three groups of mice ingesting one-, three-, and ninefold concentrations of B vitamins, the tumor incidence was 78, 80, and 84 per cent, respectively. As in Experiment 1, the neoplasms developed at an earlier average time in the mice receiving the intermediate proportions of B vitamins.

The two experiments are in excellent agreement, indicating that dietary B vitamins ranging from minimal requirements to 9 times those amounts had no influence on the incidence of spontaneous mammary carcinoma. The effect upon the rate of appearance of neoplasms will be discussed later.

TABLE 2

INFLUENCE OF VARYING THE DIETARY B VITAMIN CONTENT ON TUMOR FORMATION

Experiment	Dietary vitamin level	Mean body weight (gm.) weeks of age 30	50	70	No. mice*	Per cent mice with tumors weeks of age 50	70	90	110	Mean time of tumor appearance (weeks)	Mice alive and tumor-free at end of experiment
1. Mammary carcinoma	Low	24	25	24	42	5	29	64	86	78±3.0	1
	Moderate	23	26	25	44	2	43	82	86	71±1.9	2
	High	24	27	26	38	0	29	76	89	78±2.5	0
2. Mammary carcinoma	Low	26	27	25	59	2	34	68	78	73±2.1	2
	Moderate	26	29	26	55	13	58	78	80	64±2.1	2
	High	25	28	26	56	13	48	73	84	69±2.6	2
		week of exp.† 0	20	40		week of exp.† 16	28	40	51	65	
3. Skin tumors	Low	32	32	34	47	15	32	53	66		28±2.0	12
	Moderate	33	37	35	48	8	31	60	69		29±1.8	9
	High	34	38	37	48	17	40	58	79		29±2.1	6
4. Skin tumors	Low	29	29	27	56	2	32	50	71	86	37±1.8	6
	Moderate	31	31	29	52	8	67	82	85	85	25±1.0	0
	High	30	30	28	54	7	52	74	80	87	30±1.7	1

* Number of mice adjusted for deaths of nontumor animals (2).
† Weeks after initial application of carcinogen.

Genesis of Skin Tumors Induced by Carcinogens

Experiment 3.—Three groups of 50 C3H male mice, 12–14 weeks of age, were placed on semipurified rations which differed only in their content of B vitamins (Table 1). Five weeks after institution of the diets the animals received the first application of carcinogen, a single drop of a 0.1 per cent acetone solution of methylcholanthrene to the interscapular area. Altogether, they received seventeen applications at twice-weekly intervals.

In this experiment the mice were fed ad libitum, and those ingesting low, moderate, and high proportions of vitamins ate, on the average, 3.6, 3.8, and 3.8 gm/mouse daily during the course of the study. Data on mean body weights of the mice, incidence of skin tumors at the termination of the study (51 weeks after the first carcinogen application), and average time of appearance of these tumors are summarized in Table 2. Based on either total skin tumors (papillomas and carcinomas) or carcinomas alone, there were no significant differences in the incidence of tumors among the three groups. The mean times of appearance of the neoplasms did not vary appreciably.

Experiment 4.—In this study DBA males were utilized, and the three groups each consisted of 65 animals 9–12 weeks of age. They were given 3.1 gm. daily of semipurified diets containing the same 1:3:9 B vitamin proportions as were used in Experiment 2 (Table 1). Small differences in loss of food through scattering resulted in average food intakes of 2.9, 3.0, and 2.95 gm/mouse, for the low, intermediate, and high vitamin groups, respectively.

The carcinogen treatment was begun 11 weeks after institution of the experimental diets and consisted of the application of a single drop of a 0.3 per cent acetone solution of 3,4-benzpyrene to the interscapular area. Eighteen such applications were given at semiweekly intervals. The experi-

ment was concluded 65 weeks after the first treatment with carcinogen. The pertinent data are shown in Table 2. It is evident that the consumption of diets containing B vitamins ranging from minimal requirements to 9 times these amounts had no effect upon the incidence of benzpyrene-induced skin tumors. However, the mice ingesting the moderate concentrations of vitamins developed tumors at a somewhat earlier time.

GROWTH OF TUMORS

Although the actual rate of increase in the size of tumors was not determined, it was considered that an acceptable criterion of tumor growth is the survival time of the host—the interval between appearance of the neoplasm and death of the animal.[1] The average survival time of mice bearing spontaneous mammary carcinomas ranged from 8 to 9 weeks, there being no association with the level of dietary vitamins. A comparable independence of vitamin intake and average survival time existed among the animals with skin tumors. Here the interval measured was that between the appearance of a skin tumor, whether initially a papilloma or carcinoma, and death. The average values were approximately 15 weeks in Experiment 3 and 22 weeks in Experiment 4. These findings are in agreement with the general opinion that variations in vitamin intake—above minimal requirements for body growth—have little effect on the growth of neoplasms.

DISCUSSION

Many investigators have tested the influence of vitamins upon the genesis and growth of neoplasms. Experiments have been performed with both deficiencies and abundances of these food components. The general field has been extensively reviewed (3, 5, 8).

This study had, primarily, a narrower scope—investigation of the possible modifying effects produced by varying the dietary content of B vitamins, as a group, upon carcinogenesis. Furthermore, consideration has been limited to proportions of B vitamins in the range above the minimal requirements of the host. The effects of clear-cut or severe deficiencies have been purposely avoided, since it is well known that these may result in reduced caloric intake and body weight, conditions which themselves may strikingly inhibit carcinogenesis.

Two reports, on the influence of varying the levels of B vitamins from what might be considered just adequate for growth to several times the optimal amounts, are pertinent to the present investigation. In one the effect of multiple B vitamins—in low, control, and excess amounts—on the incidence of spontaneous lung adenoma in strain A mice was tested (13). Differences in tumor formation were observed, but these could easily be accounted for by the variable number of deaths not due to lung tumors.

Perhaps the best study along these lines is that performed by Boutwell and co-workers (1). They examined the formation of skin tumors induced with benzpyrene under conditions in which ten of the B vitamins were varied as a group from levels estimated to be just adequate for maintenance to amounts considerably above the optimal. To other groups, diets were fed in which individual, pairs, and sets of B vitamins were decreased to minimal levels in the presence of adequate amounts of the remaining vitamins. All rations were fed isocalorically. There were no noteworthy differences in the incidence of total skin tumors or carcinomas at 6 months after the first application of carcinogen, except for a somewhat lower incidence of carcinomas in the group fed all vitamins at the lowest level. These mice, however, showed some evidence of pyridoxine deficiency—namely, an acrodynia that was reversed by a single treatment with pyridoxine. In another group which was fed a ration adequate in all B vitamins except pyridoxine, the animals developed nearly as many tumors as did the controls, and at no time did they exhibit clinical evidence of pyridoxine deficiency.

The results of the four experiments reported in the present publication demonstrate that varying B vitamins in the diet, from minimal levels to 5 or 9 times those amounts, had no significant influence on the incidence of spontaneously occurring mammary carcinoma or chemically induced skin tumors. However, in three of these studies—two with mammary carcinoma and one with induced skin tumors—neoplasms developed at a faster rate, on the average, in the mice ingesting moderate or "normal" amounts of vitamins than in those on low or high vitamin intake. The differences were of statistically significant magnitude in the mammary carcinoma experiments if the two were analyzed as duplicates. They were statistically significant in the skin tumor experiment. The studies were long-term, and the finding appears to be valid. It is possible that the results of Boutwell and co-workers (1) may also be interpreted in the same way, inasmuch as the incidence of total skin tumors was higher, at 6 months, in their control group as compared to the low and high vitamin groups. At the present time no great significance is

[1] In a number of experiments in which appropriate data have been gathered, the coefficient of linear correlation between the rate of growth of spontaneous mammary carcinoma and survival time of the host was approximately −0.5.

attached to this finding. Rather, it represents a point for clarification and interpretation in future work in this field.

Although progress has been made in establishing the quantitative dietary requirements of the mouse for B vitamins, much is still unknown and unclear: (*a*) strains differ as to their needs; (*b*) the order of minimal values is known for some of the vitamins, not for others; (*c*) the part played by intestinal synthesis and utilization under a particular set of conditions is not readily defined. Perhaps certain of the vitamins—thiamine, riboflavin, pyridoxine, and pantothenate—are more critical *dietary* components, whereas others can be synthesized in adequate amounts in the intestinal tract. Finally, the requirements for carcinogenesis may not be the same as those for somatic growth.

Despite the difficulties inherent in the choice of vitamin levels, in control of caloric intake and/or body weight, and in the smooth operation of a long-term study, it appears, from our experiments and others cited, that considerable variations in B vitamin intake above minimal requirements have little effect upon the formation of the spontaneous lung adenoma, induced skin tumor, or spontaneous mammary carcinoma. It is likely that this general statement is valid for many tumor types. This in no way negates the fact that there are special situations—due to the particular carcinogen or tissue—in which the dietary vitamin content profoundly affects carcinogenesis. The influence of riboflavin on azo dye-induced hepatic tumors is an excellent example of this category.

It is probable that wide variations in the amounts of B vitamins *fed* are not mirrored proportionately in the actual concentrations in the tissues. In a pilot study accompanying Experiment 3, three groups of C3H males were given equicaloric amounts of the same diet, but differing in vitamin content in the ratio of 1:3:5 (Table 1). After 7 weeks they were sacrificed. Analysis for vitamin concentration revealed an average of 26, 30, and 32 μg. of riboflavin and 8, 10, and 13 μg. of thiamine per gram of liver, in the mice consuming the low, moderate, and high vitamin diets, respectively. Perhaps other tissues, not directly in the path of intestinal absorption (for example, skin and mammary tissue), show even smaller differences. Excluding deficiency levels, wide ranges of *dietary* vitamins may result in *tissue* differences too small to modify carcinogenesis. Or, more likely, once there is an optimal concentration of vitamins in the tissue, increases are excesses of insignificant metabolic importance.

Although not the primary concern of this publication, the question of the possible influence of vitamin B *deficiency* upon carcinogenesis follows naturally. Many investigations designed to enlighten this subject are confounded by uncertainties relating to the level of caloric intake, body weight, and the clinical condition of the animals. However, two relatively well controlled studies by the Wisconsin group (1, 4) strongly suggest that pyridoxine deficiency may inhibit the formation of induced skin tumors. More work along these general lines is clearly warranted.

SUMMARY

Four experiments on the influence of B vitamins in carcinogenesis were performed: two with spontaneous mammary carcinoma and two with carcinogen-induced skin tumors. Three groups of mice were employed in each study, and dietary thiamine, riboflavin, pyridoxine, pantothenate, niacin, and choline were varied, as a group, from levels just adequate for growth to 3, and 5 or 9 times these amounts. The rations were constructed of semipurified components, and the B vitamins were supplied by a yeast extract and, in larger proportion, by synthetic compounds. Caloric intakes and body weights were adequately controlled.

In each experiment, there were no significant differences in the incidence of tumors among the groups fed the minimal, moderate, or high levels of B vitamins. In three of the four experiments, however, the neoplasms developed at a faster rate in the mice ingesting moderate amounts of vitamins than in those on either low or high intake.

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