The Genesis and Growth of Tumors

IV. Effects of Varying the Proportion of Protein (Casein) in the Diet*

ALBERT TANNENBAUM, M.D., AND HERBERT SILVERSTONE

*(From the Department of Cancer Research,** Medical Research Institute, Michael Reese Hospital, Chicago 16, Illinois)*

Proteins are considered to occupy a key position in the structure and function of tissue. As a consequence it might be expected that the level of protein in the diet is a significant modifying factor in the formation and the growth of tumors. This prospect was investigated as part of a broad, integrated study (12, 13, 14) on the relationship of nutrition both to the genesis of tumors and to the growth of tumors once they have become established.

Dietary deficiency of protein, usually measured by decreased body growth, may be the result of too little of a relatively complete protein or of a customary amount of a deficient protein (deficient in one or more essential amino acids). It has been shown that diets in the latter category may inhibit the formation of tumors and retard the growth of tumors. Most of the experiments employed diets so deficient in protein (or essential amino acids) that the food consumption and body growth of the experimental animals were considerably reduced. In a recent review (15) we expressed the opinion that, except for certain special cases, the retarding effects of protein-deficient diets, on tumor formation and tumor growth, were generally due to voluntary caloric restriction or decreased body growth.

In contrast with these protein deficient diets, the present investigations were carried out with quality and quantity of protein that permitted the mice to grow well and appear healthy. In our early experiments the effects of an adequate ration composed of many unpurified commercial components were compared with those produced by the same ration increased in protein content by substituting vitamin-free casein for part of the carbohydrate. In later studies, semipurified diets were used, the protein content ranging from 9 percent to 45 percent casein. In each of the series of experiments, the health and weights of the mice of the individual groups were good and of the same general order.

Because experience has shown that the effects of an experimental procedure may vary with the tumor, four types of mouse tumors were investigated: Spontaneous mammary carcinomas; skin tumors induced by carcinogenic hydrocarbons; subcutaneous sarcomas induced by a carcinogenic hydrocarbon; and spontaneous hepatomas of strain C3H mouse. A brief report on some of these studies has been given previously (16).

METHODS

The mice employed in the present experiments were of inbred strains raised in our laboratories. Litter mates were distributed, as far as possible, among the several groups comprising an experiment. The mice were fed Purina fox chow checkers from weaning until transferred to the experimental diets. They were housed in groups of 5 (except where otherwise indicated) in solid bottom cages with bedding of wood shavings and peat moss. Drinking water was available at all times. Each mouse was numbered and its progress recorded individually.

In each of the investigations reported, the protein content of the individual diets was varied by substituting protein ("vitamin-free" casein[b]) for an equal weight and caloric value of carbohydrate (cornstarch). The other components of the diets—fat, vitamins, and minerals—were identical in source and amount. In the earlier experiments, only two levels of dietary protein were compared. One ration was composed of Purina fox chow meal,[a] skimmed milk powder, and cornstarch.[c] The second ration was similar except that a part of the cornstarch was replaced by an equal weight of "vitamin-free" casein. In later experiments diets composed of partially purified foodstuffs were employed: "Vitamin-free" casein, cornstarch, partially hydrogenated cottonseed-soybean oil,[d] and a salt mixture.[e]

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The B-vitamins were supplied by a small amount of a rice bran extract[f] supplemented by crystalline B-vitamins.[g] Vitamins A, D, and E were given either in cod liver oil and wheat germ oil, or in a concentrate.[h] All the vitamins were supplied in excess of known requirements for growth. In some diets amijel[i] or gelatin[j] was used—principally as a binder.

a. A commercial ration manufactured by Ralston Purina Company. Compounded from a variety of grains, leaf, meat, and fish meals, and supplemented with vitamins. For mice, it is an entirely adequate ration for growth, breeding, and lactation. Protein 25 per cent, fat 5 percent, and ash 6 percent. b. Labco vitamin free casein, Borden Vitamin Co. Such products contain minute quantities of the B vitamins (3). c. Powdered cornstarch, Buffalo No. 126, Corn Products Sales, Argo, Illinois. d. Kremax, generously furnished by Armour and Co., Chicago. e. Wesson's modification of the Osborne-Mendel salt mixture (20). f. Vitab, generously furnished by National Oil Products Co., Harrison, N.J. g. Crystalline vitamins contributed by Merck & Co., Rahway, N.J. h. A concentrate of vitamins A, D, and E, contributed by Distillation Products Inc., Rochester, N.Y. i. A pregelatinized cornstarch. j. Pure food gelatin, Wilson and Company. No. 140.

The composition (protein, fat, carbohydrate, etc.) of the diets was determined from data supplied by the manufacturers. These data were also employed in calculating the caloric values of the rations using 9, 4, and 4 calories per gram of fat, carbohydrate, and portein, respectively.

The diets were prepared by mixing the weighed ingredients with sufficient water to make a mash which was spread in pans and cut into blocks of appropriate size. They were made once a week, stored in a refrigerator at 4° C, and fed daily. At various times the food left in the cages was weighed; since the mice were given a known amount of food, the average food consumption could be approximated.

At two week intervals during the experiment, the mice were weighed and inspected for tumors and other gross changes. All animals were examined post mortem. Tumors were recognized grossly; a considerable number were examined microscopically, including all the questionable lesions.

In reporting the effects of different levels of dietary protein on tumor formation, both the incidence of tumors and the average time of their appearance is given.

EXPERIMENTS

Spontaneous Mammary Tumors

Experiment 1.—Two equivalent groups of 50 dba female mice were placed on the experimental diets when they were 9 to 16 weeks of age. One group, a12, was fed a ration consisting of 35 percent fox chow meal, 24 percent skimmed milk powder, and 41 percent cornstarch; the ration fed the second group, a19, contained the same amounts of fox chow meal and milk powder, and one-third of the cornstarch was replaced by an equal weight of "vitamin-free" casein. The two diets therefore contained the same kinds and amounts of fat (2 per cent), minerals (5 per cent), and vitamins. Diet a12 contained 18 per cent, and diet a19, 32 per cent protein. The diets were fed at a level of 3.4 gm. per mouse daily, and throughout the experiment the animals of both groups consumed, on the average, from 3.0 to 3.4 grams daily. As indicated in Table 1a, the average growth of the mice was satisfactory and equivalent in the two groups. The experiment was ended when the mice were 104 weeks old. The pertinent data are given in Table 1b.

Increasing the dietary protein from 18 per cent to 32 per cent, with casein, appears to have inhibited the formation of mammary tumors. At least part of this apparent inhibition was due to the increased death rate from non-tumor causes in the group fed 32 per cent protein (a19). After the mice reached 50 weeks of age, only 4 of the a12 mice died without mammary tumors (at an average age of 81 weeks), compared with 13 of the a19 mice (at an average age of 73 weeks). A practical method of approximately correcting for the effect of non-tumor deaths on the incidence of tumors is that described by Bryan and Shimkin (2). By using this method for calculating an adjusted total, the difference in incidences of mammary tumors is reduced. Nevertheless, the results were considered suggestive enough to extend the investigation by the following two experiments.

TABLE 1a

GROWTH OF MICE IN EXPERIMENT 1

	Average body weight (gm)				
	Age (weeks)				
Group	14	30	40	60	80
a12–18% protein	22	32	35	32	31
a19–32% protein	23	33	35	33	32

Experiment 2.—In this study a wider range of dietary protein levels was investigated. 250 C3H female mice, born within a 1 week period, were divided into 5 equivalent groups and, when 13 to 14 weeks old, were placed on the experimental rations.

85 per cent of the diet was composed of casein and cornstarch, in the following proportions for the several rations:

Group	Percent casein	Percent cornstarch
n1	9	76
n2	18	67
n3	27	58
n4	36	49
n5	45	40

The remaining 15 per cent of the diets was the same for all 5 rations, consisting of Kremax 3 per cent, cod liver oil 1 per cent, wheat germ oil 1 per cent, salt mixture 4 per cent, Vitab 1 per cent, and

amijel 5 per cent. Synthetic B-vitamins were added in the following amounts in micrograms per gram of ration: thiamin hydrochloride, 2.5; riboflavin, 1.25; calcium pantothenate, 7.5; pyridoxine hydrochloride, 1.25; and choline chloride, 250. The calculated caloric values per gram of diet ranged from 3.73 for n1 to 3.80 for n5.

gms (i.e. 9.4 calories) per mouse per day, and the mice ate all the food given them.

120 C3H female mice born within an 11 day period were divided into 3 equivalent groups. As described in previous publications dealing with animals fed calorie-restricted diets, each set of 5 mice was housed in two cages (13); at each biweekly

TABLE 1b

EFFECT OF CASEIN-ENRICHMENT OF DIET ON THE FORMATION OF SPONTANEOUS MAMMARY CARCINOMAS IN DBA FEMALE MICE

Group	Number of mice: Effective[a]	Number of mice: Adjusted[b]	Mice with mammary tumors: Number	Mice with mammary tumors: Percent of effective total	Mice with mammary tumors: Percent of adjusted total	Age of mice at appearance of tumors (weeks): Range	Age of mice at appearance of tumors (weeks): Mean	Mice tumor free and alive at 104 weeks[c]
a12–18% protein	47	46	37	79	80	52–103	78.6±2.4	6
a19–32% protein	49	40	26	53	65	50–103	80.4±3.0	10

[a] Number of mice alive when 1st tumor was observed in experiment.
[b] Corrected for deaths of non-tumor mice (2).
[c] Age of mice when experiment was terminated.

The diets were fed daily at a level of 4.0 grams per mouse. Accurate food consumption figures could not be obtained, but, on the average, the mice ingested about 3.3 to 3.5 gms. daily. The growth of the mice on the several diets was satisfactory. In Table 2a, the average weights of the mice without tumors are given until 1 year of age; subsequent to this time the numbers of surviving tumor-free mice were too small for the averages to be significant.

TABLE 2a

GROWTH OF MICE IN EXPERIMENT 2

Group	Average body weight (gms) Age (weeks): 14	20	28	36	44	52
n1– 9% casein	25	32	34	38	37	36
n2–18% casein	25	36	39	40	39	40
n3–27% casein	25	34	38	39	36	37
n4–36% casein	25	34	39	39	38	37
n5–45% casein	24	33	38	38	38	37

The experiment proceeded smoothly and there were relatively few deaths of mice not bearing mammary tumors. These non-tumor deaths occurred, in the main, after the 80th week. The experiment was terminated when the mice were 2 years old, at which time only 2 of the remaining mice (in n4) were free of mammary tumors. The data relative to tumor formation are shown in Table 2b. The results will be discussed in connection with Experiment 3.

Experiment 3.—The diets were composed exactly as those of Experiment 2 except that (*a*) only 3 levels of dietary protein were studied, and (*b*) the rations were fed at a restricted caloric level in order to insure equal food consumption. The casein levels of the three rations were 9, 18, and 36 per cent. The rations were fed at a level of 2.5 weighing the 5 mice were divided, placing the heavier animals in one cage, the lighter animals in the other cage. In this way, over the course of the experiment, each animal was given nearly equal access to the available food. The experimental diets were initiated when the mice were 19 weeks old.

The average increases in body weight were not equivalent for the three groups, despite equal caloric intakes (Table 3a). The condition of those animals not bearing mammary tumors was excellent, and only two mice died without tumors by the end of the experiment, when the surviving mice were 110 weeks old. The results with respect to mammary tumor formation are given in Table 3b.

In Experiments 2 and 3, the final incidences of mammary tumors did not vary with the level of dietary protein. However, in both studies, the tumors tended to appear more rapidly in the mice fed 18 per cent casein than in those fed lower or higher proportions of casein. This latter observation is also consistent with the results of Experiment 1 in which both tumor incidence and average time of appearance were somewhat retarded in the group fed the 32 per cent protein diet. Despite the agreement among the results of the 3 experiments, it is apparent that the level of dietary casein did not strikingly affect the formation of mammary tumors.

In these experiments there were incidental findings which are presented without reference to the diets since they were not dependent on the levels of dietary protein.

Growth of mammary tumors.—In the previous 3 experiments, the growth rates of the tumors of the individual mice were determined. At 1 or 2 week

intervals, until the animal died, the major and minor axes of the tumor were measured, by means of a caliper, to the nearest millimeter. The average increase in the sum of these measurements, expressed in 0.1 mm per day, is referred to as the growth index (12). The growth indices of the individual tumors varied considerably—from 0.2 to 15.

TABLE 2b

EFFECT OF PROPORTION OF DIETARY CASEIN ON THE FORMATION OF SPONTANEOUS MAMMARY CARCINOMAS IN C3H FEMALE MICE FED AD LIBITUM

Group	Number of mice	Mice with mammary tumors		Age of mice at appearance of tumors (weeks)	
		Number	Percent	Range	Mean
n1- 9% casein	50	48	96	29- 92	51.5±2.2
n2-18% casein	50	47	94	28- 86	47.0±2.3
n3-27% casein	50	49	98	26- 94	49.5±2.4
n4-36% casein	50	46	92	23-103	50.6±2.9
n5-45% casein	50	42	84	21- 98	48.6±2.6

In Experiment 1 (dba mice), the average growth indices were 7.2 ± 0.6 and 6.2 ± 0.7 for groups a12 and a19 respectively. In Experiment 2 (C3H mice), the average growth indices of the 5 groups ranged from 5.0 ± 0.4 to 6.3 ± 0.5; in Experiment 3, from 4.8 ± 0.4 to 5.4 ± 0.5. There was no regular association between the proportions of dietary protein and the average growth indices of the mammary tumors.

Survival of mice with mammary tumors.—In Experiments 2 and 3, the mice bearing mammary carcinomas were allowed to live until they died—none were sacrificed. The mice of Experiment 2 survived from 1 to 23 weeks and those of Experiment 3 from 1 to 36 weeks after the tumors were first observed, usually when they were about 0.5 cm. in diameter.

There was no association between the proportion of dietary protein and the interval between the detection of the tumor and the death of the mouse: In Experiment 2, the average survival time of the mice of the several groups ranged from 9.2 ± 0.6 to 11.1 ± 0.8 weeks and in Experiment 3, from 11.3 ± 0.9 to 11.9 ± 1.0 weeks. Furthermore, the survival time of the animals was independent of the age at which the carcinomas arose: For example, the animals developing tumors between 30 and 50 weeks of age lived, on the average, the same length of time as those mice developing tumors at 80 to 100 weeks of age.

However, there was a correlation between the rate of growth of the tumor and the survival time of the animal: Those animals in which tumors grew more rapidly, tended to live a shorter time. For 93 mice bearing single mammary carcinomas (Experiment 2), the coefficient of linear correlation between growth index and survival time was −0.51. Although statistically significant, its magnitude suggests that the rate of growth of the tumor is only one of many factors that determine survival time.

Multiple mammary tumors.—C3H female mice often develop more than one mammary carcinoma. In Experiment 2, multiple tumors appeared in 49 to 67 per cent of the tumor-bearing mice of the 5 groups; a few formed as many as 5. In Experiment 3, from 42 to 55 per cent of the tumor-bearing animals in the 3 groups developed more than one mammary carcinoma. In neither experiment was there a consistent correlation between the level of dietary protein and the incidence of multiple mammary tumors.

TABLE 3a

GROWTH OF MICE IN EXPERIMENT 3

Group	Average body weight (gms) Age (weeks)				
	19	28	36	44	52
n51- 9% casein	25	25	23	24	26
n52-18% casein	25	27	26	26	29
n53-36% casein	25	29	27	27	30

Metastases to lung.—In Experiments 2 and 3, an appreciable proportion of the mice with mammary carcinomas had grossly visible tumors of the lungs at autopsy. These were all examined microscopically and, except in a few dubious instances, were ascertained to be metastases from the mammary tumors. There was no relationship between the level of dietary protein and the incidence of metastases in either experiment. Furthermore, the occurrence of metastases did not depend on the age of the mouse. However, these grossly visible lung metastases were found more often in mice that had mammary tumors for a longer time (Table 3c).

This observation—that grossly visible lung metastases occurred more frequently in mice bearing their mammary tumors for more than 2 months—may indicate that metastases derived more frequently from large, partially necrotic tumors. It

may be, on the other hand, that metastases were seen in fewer instances in mice bearing mammary tumors for less than 2 months only because the metastases had less time to grow to a visible size.

Induced Skin Tumors

In the following experiments, tumors of the skin were induced by periodic application, by means of a dropping pipette, of a solution of a carcinogenic hydrocarbon to the skin of the interscapular area. intervals; 17 applications were given beginning two weeks after the experimental diets were instituted.

As shown in Table 4a the mice of the two groups grew at about the same rate. Only 2 of the non-tumor mice in group x12 died during the course of the experiment; on the other hand, 8 of the non-tumor mice of group x19 died before the end of the experiment, principally during the last two months. The study was terminated 54 weeks after

TABLE 3b

Effect of Proportion of Dietary Casein on the Formation of Spontaneous Mammary Carcinomas in C3H Female Mice Fed Isocaloric Diets

Group	Number of mice	Mice with mammary tumors		Age of mice at appearance of tumors (weeks)		Mice tumor free and alive at 110 weeks[a]
		Number	Percent	Range	Mean	
n51– 9% casein	40	36	90	36–99	62.6±2.7	4
n52–18% casein	40	37	93	31–91	55.9±2.7	2
n53–36% casein	40	36	90	38–98	58.1±3.0	3

[a] Age of surviving mice when experiment was ended.

Most of the lesions were first observed as papillomas that generally became carcinomas; occasionally they were carcinomas when first seen. A few sarcomas and myxomas of the skin also appeared. Since the proportions of these categories did not vary from one group to another, and all the lesions were induced by the carcinogen, they are all combined, in the presented data, as skin tumors.

TABLE 3c

Association Between Survival Time and Incidence of Lung Metastases

	Time in weeks between tumor appearance and death. 0 to 9.5 weeks.		More than 10 weeks	
	Mice with mammary tumors	Percent with lung metastases	Mice with mammary tumors	Percent with lung metastases
Experiment 2	110	13	101	36
Experiment 3	44	2	64	27

Experiment 4.—Two groups of dba male mice, born within a 6 week period, were employed. When the mice were 9 weeks old, on the average, they were placed on the same diets as those of Experiment 1. Group x12 was fed the diet composed of Purina fox chow meal, skimmed milk powder, and cornstarch, while group x19 was given the casein-enriched ration. The significant difference in the diets was the protein content: 18 per cent in x12, and 32 per cent in x19. The diets were fed at the level of 4.0 gms. per mouse per day. The two groups consumed approximately equal amounts of food during the course of the experiment, the average daily intake varying from 3.7 to 4.0 gms per mouse.

The carcinogen was a 0.3 per cent solution of 3,4-benzpyrene in benzene, applied at semiweekly the first application of carcinogen, since few new tumors were forming. The data are summarized in Table 4b.

Somewhat fewer of the mice fed the 32 per cent protein ration developed tumors, and these, on the average, appeared at a later time. In x19 (32 per cent protein), 52 per cent of the mice developed skin tumors at the average time of 39.4 weeks after the first application of carcinogen, while in x12 (18

TABLE 4a

Growth of Mice in Experiment 4

Group	Average body weight (gms) Weeks after 1st application of carcinogen.						
	−2	0	6	12	20	30	40
x12–18% protein	24	28	33	37	38	39	38
x19–32% protein	25	29	33	38	38	37	37

per cent protein) 65 per cent developed tumors at the average time of 34.3 weeks. The difference in incidence or in mean time of appearance is not of statistically significant magnitude.

Experiment 5.—250 dba male mice born within a period of 4 weeks were divided into 5 equivalent groups and placed on their respective rations when they were 10 to 14 weeks old. The diets had the same compositions as those employed in Experiment 2. Groups n6, n7, n8, n9, and n10 were fed diets containing 9, 18, 27, 36, and 45 per cent, respectively, of protein (casein); all rations contained the same concentrations of fats (5 per cent), salt (4 per cent), and vitamins. The rations were fed *ad libitum*, and during the course of the experiment the food consumption ranged from an average of 3.4 to 3.8 gms. per mouse daily.

Treatment with carcinogen, 0.3 per cent meth-

ylcholanthrene in acetone, was initiated 4 weeks after the diets were instituted. Five applications of carcinogen were given at 5 day intervals. During the next 14 weeks no tumors arose; consequently 7 more applications were given at 5 day intervals. Thus the mice received 12 drops of the carcinogen solution during the first 21 weeks of the experiment.

basis of effective total), there were respectively 9 and 8 deaths of non-tumor mice compared with only 3 or 4 in the other groups. When the results are calculated on the basis of "adjusted totals," the small differences in incidences become even smaller.

The data of Experiments 4 and 5 indicate that, within limits supporting good body growth, wide variations in the level of dietary protein (casein) have no significant effect on the formation of skin tumors induced by carcinogenic hydrocarbons.

TABLE 4b

EFFECT OF CASEIN-ENRICHMENT OF DIET ON THE FORMATION OF BENZPYRENE-INDUCED SKIN TUMORS

Group	Number of mice (Effective total)	Mice forming skin tumors		Time of tumor appearance (weeks)[a]		Mice tumor free and alive at end of experiment[b]
		Number	Percent	Range	Mean	
x12–18% protein	54	35	65	13–52	34.3±1.6	17
x19–32% protein	48	25	52	13–54	39.4±2.4	15

[a] Time in weeks after 1st application of carcinogen.
[b] 54 weeks after 1st application of carcinogen.

Although the mice of all groups grew well on the several diets, those fed 18 per cent casein (n7) and 27 per cent casein (n8) generally weighed more, on the average, than the mice of the other groups (Table 5a).

TABLE 5a

GROWTH OF MICE IN EXPERIMENT 5

Group	Average body weight (gms) Weeks after 1st carcinogen application						
	−4	0	4	12	20	30	40
n6– 9% casein	27	29	33	38	40	39	37
n7–18% casein	27	32	35	40	41	42	41
n8–27% casein	27	32	36	39	40	41	40
n9–36% casein	26	31	32	37	37	37	39
n10–45% casein	26	31	33	37	38	38	37

The experiment was terminated at the 46th week after the first application of carcinogen when only a few new tumors were appearing and only about 10 per cent of the animals were alive and without tumors. The data are summarized in Table 5b.

There were no significant differences, in either incidence or time of appearance of tumors among the several groups. In groups n9 and n10, which developed the lowest incidences of tumors (on the

INDUCED SARCOMAS

Experiment 6.—Two groups of 50 C57 Black female mice, born within an 8 week period, were employed. When the mice were between 10 and 18 weeks of age they were transferred to rations identical with those utilized in Experiment 1: Group a32 was fed the ration containing 18 per cent protein and group a39 the ration containing 32 per cent protein. At the time the diets were begun, each mouse was injected subcutaneously in the interscapular area with 0.15 mgm. 3,4-benzpyrene in 0.2 cc of a low melting fraction of lard.

During the experiment the mice consumed, on the average, from 2.5 to 3.0 gms per mouse daily and there was no difference between the two groups. The growth of the mice is indicated in Table 6a.

A number of mice—11 in a32 and 14 in a39—were sacrificed because they developed ulcers of the skin or, in a few cases, lymphomata. The skin

TABLE 5b

EFFECT OF PROPORTION OF DIETARY CASEIN ON THE FORMATION OF METHYLCHOLANTHRENE-INDUCED SKIN TUMORS

Group	Number of mice		Mice with skin tumors			Mean time of tumor appearance (weeks)[a]	Mice tumor free and alive at end of experiment[b]
	Effective	Adjusted	Number	Percent of effective total	Percent of adjusted total		
n6– 9% casein	49	47	41	84	87	33.3±0.9	4
n7–18% casein	46	45	37	80	82	35.1±1.1	6
n8–27% casein	48	47	42	87	89	34.7±1.1	2
n9–36% casein	50	45	36	72	80	34.4±1.0	5
n10–45% casein	50	45	38	76	84	35.1±1.1	4

[a] In weeks after 1st application of carcinogen. The range of times of appearance of tumors was from 24 to 46 weeks in all groups.
[b] 46 weeks after 1st application of carcinogen.

ulcerations appeared independent of the site of injection of carcinogen, and a few became so extensive—girding the body and involving the forelegs—that the weights of the affected mice were considerably depressed. Since these ulcerations occurred in about the same number of animals in both groups and at about the same time, these animals were omitted from the data, leaving only 39 mice in group a32 and 36 in group a39. The experiment was ended 39 weeks after the injection of carcinogen since only 1 tumor (in a39) appeared after the 33rd week.

TABLE 6a

GROWTH OF MICE IN EXPERIMENT 6

	Average body weight (gms) Weeks after injection of carcinogen					
Group	0	5	10	20	30	39
a32–18% casein	21	24	26	30	32	34
a39–32% casein	20	24	25	28	30	31

There were no differences, either in incidence or induction time of sarcomas, between the group of mice fed 18 per cent (a32) and that fed 32 per cent protein (a39) (Table 6b). On the average, the growth rates of the sarcomas was the same in both groups, the mean growth indices being 10.7 ± 1.8 and 10.6 ± 1.4 for groups a32 and a39 respectively.

Spontaneous Hepatomas

In Experiment 2, performed with C3H female mice, some of the animals which survived past 60 weeks of age were found to have, at autopsy, grossly visible tumors of the liver. These were grossly and microscopically recognized as benign hepatomas. The data are summarized in Table 7a.

The incidence of hepatomas in the mice fed 9 per cent casein was significantly lower than in those given higher proportions of dietary casein. This incidental finding prompted the following experiment.

Experiment 7.—Three groups, each of approximately 50 C3H male mice, born within a 5 day period, were transferred to their experimental rations when they were 21 weeks old. Groups AK1, AK2, and AK3 were fed rations containing 9, 18, and 45 per cent casein respectively, supplemented with cornstarch to the level of 86 per cent of the diet. The remaining 14 per cent of the diets were identical: Kremax, 5 per cent; salt mixture, 4 per cent; Vitab, 1 per cent; Ruffex[1], 2 per cent; and gelatin, 2 per cent. The vitamin supplements per gram of food were thiamin HCl, 6.7 micrograms; riboflavin, 1.9 micrograms; pyridoxine HCl, 5.7 micrograms; Ca. pantothenate, 15 micrograms; niacin, 19 micrograms; choline chloride, 240 micrograms; and a concentrate containing 2.4 U.S.P. units of A, 0.24 U.S.P. units of D, and 0.1 milligrams of E in 0.001 gm. cottonseed oil.

The mice were fed *ad libitum*. The average food consumption of the mice of AK1 was consistently greater than that of the mice of AK2 which in turn consumed more than the mice of AK3. On the average, the mice of AK1 ate 3.9 gm, and those of AK2 3.7 gms, daily. During the first 10 weeks, the mice of AK3 consumed about 3.0 gms daily and then increased their food intake to 3.5 gms. We can offer no explanation for the unusual food consumption pattern of the mice of group AK3. The growth of the animals is shown in Table 7b.

The individual animals maintained fairly constant weights from the age of 33 weeks until they were sacrificed—at 56 weeks. At this time, the lengths of the animals, from the snout to the base of the tail were measured to the nearest millimeter. In each group the body length ranged from 10.7 to 11.5 cm and averaged 11.0 cm. It may be inferred, then, that the differences in body weight were largely differences in body fat.

At autopsy, when the mice were 13 months of age, hepatomas were noted and measured. The resulting data are given in Table 7c.

The differences in incidences of hepatomas among the 3 groups are statistically significant. The incidences of tumors in the mice of the groups fed 9, 18, and 45 per cent casein were 11, 61, and 38 per cent respectively; these data are in general agreement with those obtained in Experiment 2 (Table 7a). Of the 4 tumors studied in this in-

1. A roughage consisting of celluloses from rice hulls (Fisher Scientific Co.)

TABLE 6b

EFFECT OF CASEIN-ENRICHMENT OF DIET ON THE FORMATION OF BENZPYRENE-INDUCED SARCOMAS

Group	Number of mice	Mice forming sarcomas		Time of appearance[a] of sarcomas (weeks)		Mice tumor free and alive at end of experiment[b]
		Number	Percent	Range	Mean	
a32–18% protein	39	20	51	15–33	24.6±1.0	19
a39–32% protein	36	19	53	19–39	24.4±1.1	17

[a] Weeks after injection of benzpyrene.
[b] 39 weeks after injection of benzypyrene.

vestigation, these results with hepatomas are the most striking.

A number of mice were sacrificed at 8½, 11, and 13 months of age for histological and chemical studies. The livers were examined microscopically; total liver lipids, lipid phosphorus and total cholesterol were determined by standard procedures (10); hepatic riboflavin was determined fluorometrically (1); and liver protein nitrogen was determined by microkjeldahl (6), as the nitrogen of the residue insoluble in 5 per cent trichloracetic acid. Since the results were not associated with the age of the animals, the data obtained at the several times are grouped and summarized in Table 8.

TABLE 7a

INCIDENCE OF HEPATOMAS IN MICE OF EXPERIMENT 2

Percent dietary casein	Number of mice with hepatomas over total number of mice sacrificed between 61 and 110 weeks of age
9	2/20 (10%)
18	6/16 (38%)
27	9/21 (43%)
36	9/18 (50%)
45	7/21 (33%)

None of the factors studied was correlated with the incidences of hepatomas. On the other hand, some of the factors, themselves, were affected by the proportion of dietary protein. Thus, the relative (and absolute) weights of the kidneys, the levels of hepatic protein, and the concentrations of hepatic riboflavin increased with increasing proportions of dietary protein. In contrast, the weights of the livers per hundred grams of mouse, the concentrations of hepatic phospholipid and cholesterol, and the fat-free dry weights of the livers did not differ with the amounts of protein ingested. The higher levels of total liver lipids in the mice of AK1 and AK2 may have been due partly to the low choline content of the diets (about 0.04 per cent) but were related mainly to the body weights of the individual mice. In each group of the present series, and in other experiments, we have found—even in mature animals maintaining their weight or growing slowly—a positive correlation between body weight and total liver lipids: Heavy animals frequently have high total liver fat. Thus the lower average liver lipids of the AK3 mice may have been due to their lower average body weights; of the mice of AK3 analyzed, 7 weighed 37 gms. or less and their total liver lipids averaged 7.4 per cent, while the remaining 4 mice weighed 40 gms. or more with total liver lipids averaging 13.4 per cent.

Histologic examination of the livers of the above mice revealed no significant differences except that mild to moderate fatty infiltration was found in the livers chemically determined to have a high fat content.

TABLE 7b

GROWTH OF MICE IN EXPERIMENT 7

Group	Average body weight (gms) Age (weeks) 21[a]	25	29	33	41	49
AK1– 9% casein	31	34	36	38	38	38
AK2–18% casein	32	37	39	40	40	41
AK3–45% casein	32	33	33	35	37	37

[a] Diets instituted when mice were 21 weeks old.

DISCUSSION

There are many studies on the relationship of the quality and quantity of protein to the incidence and growth of tumors. Most of the early investigators did not dissociate the origin or establishment of a tumor from its subsequent growth; others employed such varying foodstuffs that it is difficult to ascribe the effects specifically to differences in quality or quantity of protein. The literature to 1942 has been reviewed by Stern and Willheim (11). In the following discussion the more substantial findings are reviewed.

Formation of tumors.—White and associates have reported interesting investigations on the effect of diets deficient in either cystine or lysine. Some of these experiments have been performed with the spontaneous mammary carcinoma, others with induced leukemia. They observed that C3H virgin female mice on a low cystine diet (containing 3.9 per cent casein) from the time of weaning did not develop mammary tumors (23). On the

TABLE 7c

EFFECT OF PROPORTION OF DIETARY CASEIN ON THE INCIDENCE OF SPONTANEOUS HEPATOMAS IN C3H MALE MICE

Group	Number[a] of mice	Mice with hepatomas Number	Percent	Number of mice with multiple hepatomas	Size of hepatomas[b] (mm) Range	Mean
AK1– 9% casein	44	5	11	0	3–18	8.4
AK2–18% casein	46	28	61	6	2–16	7.5
AK3–45% casein	45	17	38	2	2–14	8.0

[a] Mice alive at 13 months of age, when the experiment was ended.

[b] In animals with more than one hepatoma, only one (the largest) was used in determining average size.

other hand, mice consuming the same diet, except that 0.5 per cent starch was replaced by 0.5 per cent *l*-cystine, developed 97 per cent spontaneous mammary tumors. It should be pointed out that the mice on the low cystine diet failed to grow, weighing, at 6 months, 12.4 gm. in comparison with 25.3 gm. for those on the cystine supplemented diet.

In another experiment, 25 per cent of a group of mice fed a diet low in lysine (the diet contained 18 per cent gliadin as the principal source of protein) developed spontaneous mammary tumors, compared with 97 per cent in a comparable group of mice fed Purina dog chow (22). At 9 months of age the surviving mice fed the low lysine diet weighed an average of 21 gm., having weighed 16 to 18 gm. at the beginning of the experiment.

These same low-cystine and low-lysine diets also have been investigated with regard to their effects on the production of leukemia induced by the application of methylcholanthrene to the skins of dba mice (24, 25, 26, 27). Mice fed the low lysine ration developed essentially the same incidence of leukemia as those fed adequate diets. On the other hand, the low-cystine diet strikingly inhibited the production of leukemia in comparison with the cystine-supplemented diet or Purina dog chow. The authors state, "The data suggest that cystine played a role in the development of induced leukemia perhaps not associated with its properties as an essential amino acid for growth, but with some other undetermined property."

We are in general agreement with this conclusion, but are of the opinion that the effects obtained with the spontaneous mammary carcinoma are probably due to the voluntary caloric restriction that occurred in those experiments. This inference is supported by the experiments of Larsen and Heston on spontaneous pulmonary tumors (5). They observed that the decreased tumor incidence in mice fed a low cystine ration was of the order produced by the same diet supplemented with cystine, but restricted in amount to that voluntarily ingested by the mice on the unsupplemented ration.

Differing from the above studies are the investigations employing diets that support relatively normal body growth, yet vary in the proportion of protein. Among mice fed diets containing either 20 or 40 per cent protein, Rusch, Johnson and Kline (9) found no significant differences in the incidence of sarcomas induced by injection of 3,4-benzpyrene. For another type of tumor, the azo-dye-induced liver cancer of rats, there is still controversy as to the exact effect of varying the proportion of protein in the diet (8). However, in a single experiment in our laboratory, the incidence of this tumor was significantly inhibited by increasing the casein content of a partially purified ration from 10 to 30 per cent (10).

TABLE 8

EFFECTS OF VARYING PROPORTIONS OF DIETARY CASEIN ON THE RELATIVE WEIGHTS OF KIDNEY AND LIVER, AND ON VARIOUS LIVER COMPONENTS[a]

	AK1 9% Casein	AK2 18% Casein	AK3 45% Casein
Weights of analyzed Mice (gms)	35–43 (38.0)	37–45 (38.3)	32–42 (34.0)
Weights per 100 gm. mouse (gm)			
Liver	4.6–7.3 (5.2)	5.1–6.2 (5.6)	4.4–5.8 (5.2)
Kidneys	1.2–1.4 (1.28)	1.4–1.6 (1.45)	1.6–2.0 (1.84)
Liver Lipids			
Total lipids, percent	10.9–20.8 (15.4)	14.3–23.3 (18.5)	3.9–16.2 (9.5)
Phosphorus, mgm percent	75–108 (86)	67–100 (85)	71–122 (87)
Total cholesterol, percent	.38–.58 (.46)	.33–.63 (.47)	.25–.59 (.41)
Liver protein nitrogen, percent	2.86–2.94 (2.90)	2.96–3.54 (3.12)	3.36–3.73 (3.52)
Liver riboflavin, microgms per gm.	23.5–29.4 (26.0)	27.3–31.5 (29.9)	32.5–35.9 (34.0)
Fat-free dry weight of liver, percent	25–29 (27.4)	24–28 (26.7)	27–30 (28.8)

[a] The data represent analyses on 11 animals in each group except for the protein and riboflavin determinations which were performed only at 13 months on 5 mice in each group. The ranges of individual determinations are given and the figures in parenthesis are the averages; data based on fresh organ weights.

In the present investigations 4 different mouse tumors were studied: Spontaneous mammary carcinoma, induced skin tumor, induced sarcoma, and spontaneous hepatoma. The proportions of dietary protein varied between the limits of 9 and 45 per cent. In 3 experiments with the sponteaneous mammary carcinoma, 2 with the induced skin tumor, and one with the induced sarcoma, the differences in the protein content of the diets did not result in significant differences in the formation of tumors. However, in the mammary tumor experiments there was either a slight increase in the incidence of tumors or a slight acceleration in the average time of appearance of the tumors in the mice fed 18 per cent protein in comparison with those fed either lower or higher levels.

In contrast, in the experiment concerned with the spontaneous hepatoma, the mice fed 9 per cent casein developed significantly fewer tumors than those fed either 18 or 45 per cent; this difference cannot be attributed to a lesser food consumption or body weight. The hepatoma incidence of the group fed 45 per cent casein was lower than that of the group fed 18 per cent. Because the incidence of spontaneous mouse hepatoma is decreased by caloric restriction (15) this observation must be conservatively evaluated since the mice fed 45 per cent casein ate less and weighed less. Since the incidences of hepatomas in the 3 groups were determined only at one time—at autopsy—the observed differences possibly represent variations in the time of tumor appearance only. Nevertheless, the data indicate a striking effect on the rate of formation.

Growth of tumors.—Voegtlin and associates (7, 17, 18, 19) have shown that diets deficient in lysine or cystine and methionine retard the growth of spontaneous mammary tumors. Kocher (4) also investigated the effect of a lysine deficiency. He observed that when the deficient diet was fed for a considerable period (the tumors were approximately 10 to 20 mm. in diameter initially) the inhibiting effect on growth was transient, the tumor resuming normal rapid growth after 30 to 60 days. If the lysine-deficient diet was instituted when the tumors were approximately 25 mm. in diameter the inhibiting effect was either not apparent or of very short duration.

Recently Florence White and Belkin (21), in a model experiment of this kind, reported on the effect of a low-nitrogen diet on the establishment and growth of a transplanted tumor. The experimental diet was restricted in protein to that contained in liver extract (5 per cent of the diet) and stated to be inadequate in at least four essential amino acids. As compared with the control the establishment or "take" of the transplanted tumors was not affected by the experimental diet instituted 1 week previous to inoculation. On the other hand, the tumors of the protein-restricted mice grew at 74 per cent of the rate of growth of the tumors in the controls. The tumors grew by utilizing the tissue protein of the host.

Our data, on the growth rate of the spontaneous mammary carcinoma and induced sarcoma, indicate that variations in the proportion of dietary protein (within limits supporting good body growth) exert little or no effect on the growth rate of the tumors. Although direct measurements were not taken, it was our clinical impression that the same was valid for the induced skin tumors. Moreover, in the experiments with the spontaneous mammary carcinoma there was no association between the level of dietary protein and either the duration of life after the detection of the tumor or the incidence of visible metastases to the lung.

General Comments.—Diets deficient in protein, qualitatively or quantitatively, retard or inhibit both the formation and the growth of many types of tumors. Since, in the main, such diets are voluntarily consumed in suboptimal quantities it seems likely that the resultant inhibition of the genesis and the growth of tumors is due, generally, to the self-imposed caloric restriction rather than specifically to the individual or multiple amino acid deficiency. One demonstrated exception to this interpretation is the effect of a cystine-deficient diet on the induction of leukemia by means of methylcholanthrene (27).

Wide variations in the dietary content of relatively adequate protein (the diets being ingested in relatively equal amounts and producing relatively equal body weights) do not effect significant differences in either the incidence or average time of appearance of some types of tumors. The growth rates of these tumors are also not affected appreciably. However, the rate of formation of one tumor studied, the spontaneous hepatoma of the C3H mouse, appears to be dependent on the proportion of protein (casein) in the ration.

This latter observation, coupled with the fact that a somewhat similar effect has occasionally been reported for rat hepatomas induced by *p*-dimethylaminoazobenzene, suggests that the liver may be unique in its response to variations in the level of dietary protein: The varying proportions of dietary protein, absorbed through the gastrointestinal tract and brought directly to the liver, probably effect wider differences in the concentration of amino acids in that organ than in most tissues. As a consequence, tumor formation in the

liver may be more readily influenced by variations in dietary protein.

More important to the general cancer problem, however, is that the formation of 3 different types of tumors of the mouse was affected to only a slight extent, if at all, by wide variation in the casein content of the diet. Although not necessarily valid for all tumors, this conclusion is probably true for many.

Apart from the studies on tumor formation, there are some interesting observations regarding growth of the mice. When the diets containing from 9 to 45 per cent casein were fed *ad libitum*, the mice of all groups grew well. However, in general, those receiving the 18 per cent ration attained, on the average, a little higher weight level than the mice of the other groups. In 2 studies (Experiment 3 and another nearing completion) in which the groups were restricted to the same caloric intake, the mice fed 9 per cent casein did not grow as well as those fed 18 per cent or more. This may imply some degree of deficiency for the diets containing 9 per cent casein.

SUMMARY

There are few reported investigations on the relationship between the proportion of dietary protein, varying within limits that support good body growth and health, and the formation and growth of tumors. This relationship was studied by utilizing diets compounded of commercial foodstuffs and/or semipurified components and ranging in protein content from 9 to 45 per cent. In each experiment, the proportion of dietary protein was varied by substituting casein for cornstarch, all other components of the diet being left unchanged. Four different mouse tumors were studied: Spontaneous mammary carcinomas, skin tumors and sarcomas induced by carcinogenic hydrocarbons, and spontaneous hepatomas of strain C3H mouse.

No significant effect on either the incidence or average time of appearance was observed for the spontaneous mammary carcinoma, induced skin tumor, or induced sarcoma. In contrast, the incidence of spontaneous hepatomas was significantly lower in mice fed 9 per cent casein than in those given 18 per cent or higher proportions of casein.

The growth rate of spontaneous mammary carcinomas or induced sarcomas was not affected by the proportion of casein fed. In addition, in mice bearing mammary carcinomas, metastases (to the lung) and the duration of life following appearance of the tumors were not influenced by the level of dietary protein.

Under the conditions of these investigations, body growth and general health were generally of the same order among the groups of any one experiment. It is emphasized that these studies differ from those in which rations strikingly deficient in quality or quantity are compared with rations adequate for normal body growth. It is concluded that varying the proportion of protein (casein) in the diet, within limits that permit relatively good body growth, probably has little effect on the formation of many types of tumors, but may have a significant effect upon certain special classes. Incidental findings are discussed.

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