Tumor Incidence Patterns and Nutrition in the Rat [1]

MORRIS H. ROSS AND GERRIT BRAS
Biochemical Research Foundation, Newark, Delaware and Department of Pathology, University of The West Indies, Kingston, Jamaica, West Indies

ABSTRACT Levels of caloric and of protein intake were demonstrated to have a modifying influence on tumor incidence patterns in the male rat. The 5 uniform lifelong dietary regimens used differed only in allotments and intakes of protein (casein), carbohydrate (sucrose) or of total calories. Age-specific rate tables and tumor incidence ratios aided in assessment of the nutritional effects. Total tumor risk was directly and exponentially related to caloric intake, but time differences for development of each of the incidence patterns were related inversely to caloric intake. Among all the groups tumor incidences formed an exponential continuum when related to growth rate in early life and mature body weight. Within each dietary group, rats of heavier weight had greater tumor risk than lighter rats. Occurrence, the proportional incidence and the malignancy of certain tumors correlated with the level of protein intake. Malignant lymphomas were predominant in rats with high protein intake, whereas fibromas and fibrosarcomas predominated in rats with low protein intake. Tumor incidence patterns differed quantitatively, qualitatively or both. Thus, in 2 groups with identical caloric intake, risk for all tumor types was similar but the group with higher protein had a greater risk for malignancy. Rate patterns for benign tumors, but not for malignant tumors, were dependent upon the mortality rate patterns of their respective populations. Lowest incidence, greatest delay in time of occurrence, absence of malignant epithelial tumors and greatest life expectancy, were observed when intakes of protein, carbohydrate and total calories were low.

Different nutritional regimens affect the prevalence of many age-associated diseases, the life span of the individual and, as a result, mortality patterns of a population. A multidiscipline study, of which some aspects are presented here, was devised to assess the influence of diet upon degenerative disease and mortality in the rat. Particular emphasis was placed upon the chronological sequence of biochemical, biological and pathological events and processes over the entire life span. The interrelationships among diet, age and hepatic enzyme activity (1–6) suggested that the enzyme activity levels and patterns were related to processes of ageing and that modification of the rate of change of these and other tissue constituents by nutritional means would result in changing patterns of mortality and disease. It was learned that length of life was influenced not only by the degree of dietary restriction but also by change in the ratio of the protein and carbohydrate components of the diets (7). The shortest life expectancy was observed to be associated with the highest incidence of glomerulonephrosis, a condition noted commonly in rats fed a commercial diet ad libitum, whereas the greatest life expectancy and the lowest incidence of kidney lesions were found among rats whose intakes were low in protein, carbohydrate and calories (8).

The influence of long-term caloric restriction in reducing the incidence of tumors is well documented for the rat and the mouse (see reviews by Rusch (9), Tannenbaum and Silverstone (10), and White (11)). Experimental studies of the influence of diet on tumorigenesis have, for the most part, been concerned with induced or transplanted tumors, or with highly inbred mice predisposed to specific tumor types. In the rat, certain deficiencies have been shown to result in the appearance of specific tumors not normally observed in the strain of rats used, or to produce an increase in the incidence of specific tumors. The most notable of these

Received for publication February 8, 1965.
[1] This investigation was supported in part by Public Health Research Grants HD 00490 and HD 00086 from the National Institute of Child Health and Human Development.

involve the thyroid gland (12), forestomach (13, 14), maxilla (15), and liver (16).

Relatively few data are available on lifelong tumor incidence patterns of large closed or non-migrant populations of rats where non-food substances or known carcinogens have not been purposely added to the diet. It is the notable exception when pertinent information is cited in the literature regarding environmental conditions, size of population, or when statistical or actuarial analysis has been performed (17, 18).

This paper presents an evaluation using actuarial methods of analysis of the effects of 5 different lifelong, dietary regimens upon tumorigenesis and is particularly concerned with age-specific incidence, tumor type, and the quantitative contribution of these tumors to death patterns in the same population of rats reported upon earlier (7, 8).

MATERIALS AND METHODS

The rat. Approximately 1000 male rats of the Charles River S.D. strain were used in these studies. Breeders had been routinely vaccinated for *Salmonella enteriditis* and *Salmonella typhimurium* and the female breeders were never older than 7 to 8 months of age. The number in their litters averaged 12 to 14 at birth, but was reduced to, at most, 10 in the third day of life. Mortality among littermates, without respect to sex, was 5% during the first 20 days. On the twentieth day the animals were weaned, sexed and shipped; upon arrival on the twenty-first day they were immediately weighed and divided at random into 5 groups. The number of rats used in each of the 5 dietary groups, termed control, A, B, C and D, were 210, 210, 120, 210 and 195, respectively.

A detailed description of the care, environment and housing has been presented previously (7, 8).

Diet and feeding regimens. The "control" group was maintained with a commercial diet[2] ad libitum and consumption was measured daily. The 4 "experimental" groups were alloted purified diets (table 1) on a restricted basis. The components [3] of the purified diets were identical and the only variables were in intake of protein (casein), of carbohydrate (sucrose) and of total calories.

The dietary allotments permitted: Intakes high in protein — high in carbohydrate (dietary group A); intakes high in protein — low in carbohydrate (dietary group B); intakes low in protein — high in carbohydrate (dietary group C); intakes low in protein — low in carbohydrate (dietary group D).

The intake of protein was identical in rats of groups A and B, and in rats of groups C and D. In groups A and C, the individual daily intake was isocaloric. The carbohydrate intake was identical for rats receiving diets B and D. Rats of group D had the lowest caloric intake. The daily intake of all other constituents was identical. The feeding schedule was such that

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[3] The authors acknowledge the generosity of the following organizations for their donations of ingredients used in the diets: sugar from the Sugar Research Foundation, New York; Mazola Oil from the Corn Products Company, New York; vitamins from Hoffmann-LaRoche, Inc., Nutley, New Jersey; Lederle Laboratories (Division of American Cyanamid Company), Princeton, New Jersey, and Merck Institute for Therapeutic Research, West Point, Pennsylvania.

TABLE 1

Composition of purified diets

	Diet A	Diet B	Diet C	Diet D
	%	%	%	%
Casein [1]	30.0	50.85	8.0	21.62
Sucrose	61.0	33.90	83.0	54.05
Corn oil [2]	5.0	8.47	5.0	13.52
Salt mixture (USP 12)	4.0	6.78	4.0	10.81
Vitamins and trace elements [3]				
Kilocalories/g	4.09	4.15	4.09	4.24
Kilocalories supplied by casein, %	29.3	49.0	7.8	20.4

[1] Vitamin-Free Test Casein, 89% protein nitrogen, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Mazola Oil, Corn Products Company, New York.
[3] For vitamin and trace element content of diets, see reference (7).

the amount allotted differed according to the diet fed and to the age of the rat. The detailed feeding schedule and its rationale have been described earlier (5, 7).

The precise amounts of each ingredient consumed when maximal food allotments had been reached are given in table 2, and these amounts were maintained for the remainder of the rats' lives. The cumulative caloric intake for each of the groups is shown in table 3.

Pathological examination. Two hundred and ninety rats were chosen at random at 7 different age periods (between 100 to 995 days) and were killed for biochemical, pathological and cytological studies. All other rats were observed until their "natural" death dates.

A thorough necropsy was performed on every rat as soon after death as was practicable; pieces representing normal and diseased tissue (at least 25) were routinely taken from all animals and fixed in Bouin's fluid, as well as occasionally in buffered formalin. Histological sections obtained from the paraffin-blocked tissues were routinely cut at 6 μ and stained with hematoxylin and eosin, and special staining procedures were used as required. The gross necropsy observations accompanied the sections submitted to the histopathologist but data as to age and diet were withheld.

The nomenclature used conforms to the International Classification of Disease (19).

Analysis of data. Tumor data were organized so as to permit actuarial analysis according to the methods described by Dublin et al. (20), as well as to those

TABLE 2

Maximal food allotments of purified diets [1]

	Diet A	Diet B	Diet C	Diet D
	g/day/rat	*g/day/rat*	*g/day/rat*	*g/day/rat*
Casein	4.3	4.3	1.1	1.1
Sucrose	8.7	2.8	11.9	2.8
Corn oil	0.7	0.7	0.7	0.7
Salt mixture (USP 12)	0.6	0.6	0.6	0.6
Vitamins and trace elements [2]	—	—	—	—
Total food allotted	14.3	8.4	14.3	5.2

[1] Allotments from 685 days of age.
[2] Daily allotments of vitamins and trace elements identical for each rat.

TABLE 3

Cumulative caloric intake

Age of rats [1]	Dietary group				
	Commercial	A	B	C	D
days	*kcal*	*kcal*	*kcal*	*kcal*	*kcal*
99	5174	2344	1410	2344	898
199	13028	6690	4013	6690	2569
299	20828	11527	6907	11527	4422
399	28517	16894	10121	16894	6477
499	36256	22599	13505	22599	8669
599	43463	28325	16991	28325	10873
699	51159	34077	20447	34077	13090
799	58971	39925	23933	39925	15337
899	66262	45774	27419	45774	17584
999	72586	51623	30905	51623	19831
1099		57471	34391	57471	22079
1199		63320	37877	63320	24326
1299		69169	41363	69169	26573
1399		75018	44849	75018	28820
1499			48335		31067
1599					33315
1699					35562

[1] Exclusive of first 21 days.

methods recommended by our actuarial consultants.[4]

All data and derived computations are presented on the basis of the presence of a tumor at time of death. Tumor data obtained from the 290 animals killed were not used in the actuarial treatment, and population size was adjusted accordingly.

All age-specific tumor incidences were calculated on the basis of 100-day periods, using the number of rats having histologically confirmed neoplasia at time of death against the number of living rats entering that age period.

The relative tumor incidence ratios were derived because such values offer a valid basis for comparisons of incidence patterns of one dietary group of animals with those of others, particularly where initial population size differs among the groups or where, due to differences in mortality patterns, the size of the population at risk differs at different periods.

Computation of the tumor incidence ratio value (TIR)[5] was made by dividing the actual number of tumors observed at each age period in an experimental group by the expected number of tumors that would have occurred had that group experienced the same risk as a standard population. The age-specific tumor rates of the group of rats fed the commercial diet were used as "standard rates." Such ratios are determined for individual age periods to ascertain changes in trend with the passage of time; a broader age scale was also used where TIR values were calculated for the first 10 periods and for the remaining periods. To emphasize similarities or differences between total tumor incidence patterns of the different groups, however, ratios were obtained from the sum of the complete data for all age periods; namely, total, actual tumor incidence and total, expected incidence. This single value allowed quantitative expression of the difference in tumor incidence patterns as they are affected by different dietary regimens. All ratio values were expressed relative to the value for the control group which was 100. The "ratio of the corrected rates" used by Murray and Hoffman (21) were also applied.

Instantaneous relative growth rates were computed according to the method described by Brody (22) at age periods of 21 to 49, 98 and 189 days of age.

RESULTS

1. Tumor incidence in the total population. One hundred and forty-eight tumors were found in 138 rats among the 934 used for this study. Ten of these rats had more than one type of tumor. Of the 290 rats killed during the course of the experiment, six had tumors. Among those rats that were permitted to live out their lives, 132 rats (20.5%) showed histopathological evidence of neoplasia.

The largest number of cases, 25, occurred in the 900- to 999-day period, representing 19% of the total number of tumor-bearing rats (fig. 1).

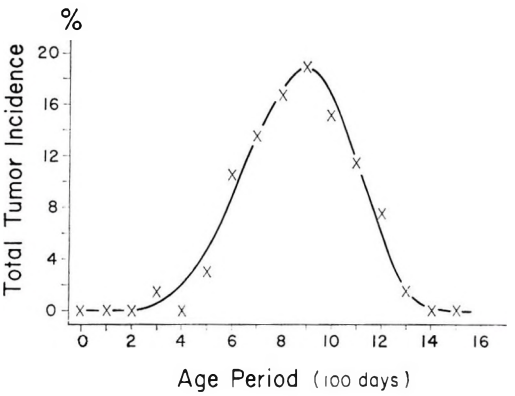

Fig. 1 Tumor incidence as percentage of total population (corrected for rats killed).

Except for the last period, the increase in age-specific incidence rate was exponential in character, with the acceleration being a function of the logarithm of age. Thus, to maintain the trend in acceleration of tumor incidence of this total population, progressively longer time-periods were required to maintain the same rate changes in chronologically older rats.

[4] Actuary Department, New York Life Insurance Company, New York.

[5] $\text{TIR} = \frac{(c_x)}{(c'_x)} \times 100$

where

$c'_x = (\bar{C}_{x_1} \times 1_{x_1}) + (\bar{C}_{x_2} \times 1_{x_2}) + (\bar{C}_{x_3} \times 1_{x_3}) \ldots\ldots$

$c =$ actual number of tumor-bearing rats.

$c' =$ expected number of tumor-bearing rats.

$\bar{C}_{x_{1,2,3}\ldots} =$ age-specific standard tumor rate, consecutive periods.

$1_{x_{1,2,3}\ldots} =$ exposure (i.e., number of living rats entering period), consecutive periods.

2. *Influence of diet upon tumor incidence.* A. Total incidence: the largest number of rats bearing tumors and the greatest number of tumors (10 rats had more than one tumor) were observed in that group whose intake was high in both carbohydrate and protein (group A), (see table 4). Considerably fewer tumors were noted in that group whose total intake was isocaloric to group A but which had a greatly reduced protein intake (group C). On the basis of an equivalent, initial population size, the smallest number of rats bearing tumors was found among that group whose intake was low in both protein and carbohydrate and which concomitantly had reduced intakes of calories (group D). Nearly twice as many tumors were found, however, in that group (B) whose intake of carbohydrate was similar to group D but whose intake of calories and protein was higher. Similar numbers of rats with tumors were found in that group of rats fed the commercial ration and in those of group C.

B. Age specificity: In each group the first tumor found was malignant, and the first tumor in any group was found at 345 days of age in a rat fed the commercial ration. In rats fed diets A and C, tumors were observed first in the 500- to 599-day period, whereas in rats fed diets B and D there was a delay in tumor appearance until the sixth period. Differences in time of occurrence among these groups became accentuated with advancing age, so that the time period in which 50% of the tumors in each group had been found was period 7, 8, and 9, and 10 for the control, A, B, C and D groups, respectively.

The distribution curves of deaths of rats with tumors at all age periods could be obtained only for the control, A and C groups and their maximal incidences occurred during the eighth, ninth and tenth 100-day periods, respectively. Because of the low incidence in tumors in groups B and D, significant distribution assessments cannot be made.

Figure 2 shows the tumor incidences arranged on an accumulated basis and adjusted for equal, initial population sizes and expressed as percentage of the total number of tumor-bearing rats. The individual curves were generally similar in contour but differed from each other in slope and in their displacement in time. The proportion of the total incidence was 22.5, 29.5, 15.8, 23.7 and 8.6% for the control, A, B, C and D groups, respectively.

The displacement in time for each of the curves (fig. 2) afforded an estimate of the time differences between groups in the development of each incidence curve: the curve for the control rats was used as a baseline, or standard, and was assigned a value of 1 (fig. 2); the remaining curves were adjusted so as to afford their superimposition upon the control curve by the use of the displacement factors 0.81, 0.72, 0.85, 0.62 for A, B, C and D, respectively. Although there was a large difference in the total number of tumor-bearing animals in groups A and C, the factors were nearly the same for the 2 groups, the members of which were fed on an isocaloric basis.

TABLE 4

Size of populations and total number of tumors

Dietary group	No. in initial population	No. in initial corrected population [1]	No. of rats killed	No. of rats bearing tumors	No. of tumors observed	No. of rats with 2 or more tumor types	No. of rats with metastatic tumors	No. of killed rats bearing tumors	No. of rats with tumors used in actuarial treatment of data
Commercial	210	208.6	50	32	34	2	11	1	31
A	210	209.3	60	49	53	4	19	2	47
B	120	119.4	60	10	10	—	2	1	9
C	210	208.4	60	36	40	4	11	2	34
D	195	188.7	60	11	11	—	5	–	11
Total	945	934.4	290	138	148	10	48	6	132

[1] Adjusted for accidental deaths and proportion of time alive in population.

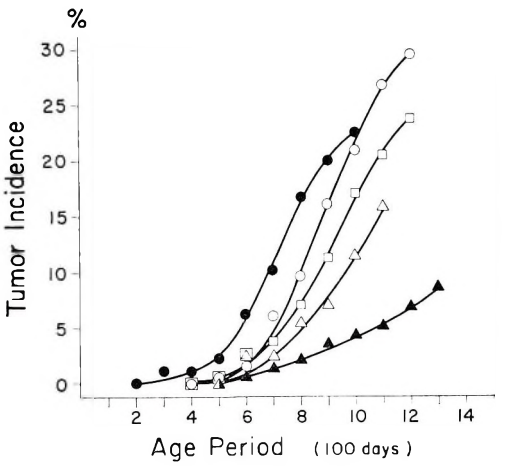

Fig. 2 Influence of diet on cumulative increase in tumor incidence with age, expressed as percentage of the total number of tumors. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

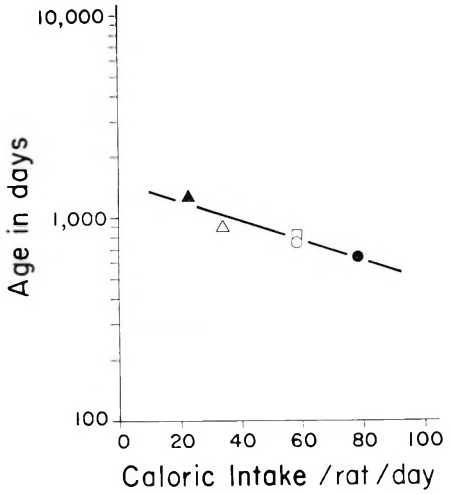

Fig. 3 Correlation between age when tumor incidences were identical, and caloric intake among the different dietary groups. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

However, 2 groups which have approximately the same number of tumor-bearing rats (controls and C) show a difference of about 15%. Not only did dietary group D have the smallest number of tumors, but it had the longest interval between time of occurrence of each tumor.

The age when each group contributed the same percentage (7.5%) to the total number of tumors in the entire population ranged from 630 days for the control group to 1260 days for group D. These times also correlated with the caloric intake of the animals as an inverse power function of age (fig. 3), namely, the lower the caloric intake the greater the delay in appearance of each tumor.

C. Age-specific tumor rate: Complete age-specific tumor rates are shown in table 5, and the tumor rate curves of all 5 groups were non-linear. The change in tumor rate for the control group increased exponentially and uniformly with age. The other tumor rate curves, by contrast, were not only displaced toward later ages but each of these groups had its own characteristic and significantly different tumor rate pattern. With the exception of period 9 for group C, at no single time-period was there an age-specific rate among the experimental groups higher than that observed in the control group, even though the largest total number of tumors was found among the rats fed diet A.

D. Caloric intake relationship: There was an exponential relationship between tumor incidence ratio (TIR) values (all periods) and the daily caloric intake of rats in each of the 5 groups. Rats of group D consumed approximately one-third of the total calories consumed by those rats fed the commercial diet but their TIR values were only 7% that of the latter group. The relative increase in tumor risk, therefore, became progressively larger with increasing levels of caloric intake. The similarity of the relationship between TIR values for all periods and caloric intake was also evident in the relationship between the TIR values and caloric intake for the first 10 age-periods (table 6).

Although there were nearly 40% more tumors in rats fed diet A than in rats fed diet C, the TIR values for these 2 groups was almost the same. The larger number of tumors of group A is explained by the difference in life expectancy. This is shown in table 7 where the population of group C was expanded at each age period to equal that of group A. This recalculation resulted in an expected total number of tu-

TABLE 5

Diet and age-specific tumor rates ($\bar{C}_x$) [1]

Age periods	Dietary group				
	Commercial	A	B	C	D
days	$\bar{C}_x$	$\bar{C}_x$	$\bar{C}_x$	$\bar{C}_x$	$\bar{C}_x$
21–99	0	0	0	0	0
100–199	0	0	0	0	0
200–299	0	0	0	0	0
300–399	0.012	0	0	0	0
400–499	0	0	0	0	0
500–599	0.014	0.006	0	0.006	0
600–699	0.050	0.013	0.027	0.021	0.008
700–799	0.065	0.055	0	0.024	0.008
800–899	0.191	0.052	0.041	0.053	0.010
900–999	0.267	0.124	0.026	0.104	0.025
1000–1099	0.500	0.143	0.100	0.250	0.020
1100–1199	—	0.276	0.143	0.267	0.027
1200–1299		0.444	0	0.800	0.091
1300–1399		0	0	—	0.250
1400–1499		—	0		0
1500–1599			—		0
1600–1699					0
1700–1799					—

[1] $\bar{C}_x$ (spontaneous tumor rate) is the ratio of the number of rats demonstrating neoplasia at death to the number of living rats entering that period. The exposure is corrected for accidental deaths and those killed. Dash indicates entire population dead.

TABLE 6

Diet and relative tumor incidence ratios (TIR)

Dietary group	TIR	
	First 10 periods	All periods
	(× 100)	(× 100)
Commercial	100	100
A	37.9	35.8
B	17.6	13.9
C	37.3	37.9
D	7.2	7.0

mor-bearing rats nearly equivalent to that observed for group A. The TIR values of each dietary group did not correlate consistently with the percentage level of, the proportion of, or the intake of any dietary constituent consumed by the rats in that group.

The relationship between tumor risk and caloric intake can be extended to tumor risk and body weight parameters. The complete growth pattern for each group from day 21 of life of all rats, to the death of the last rat in each group, is shown in figure 4. Cessation of growth occurred at approximately 33 weeks of age for rats fed the commercial diet. Average mature weight at this time was 530 g and was maintained until the fiftieth week. Subsequently, the animals became more obese until their average weight reached a maximum of 612 g at 85 weeks. Body weights as high as 800 g were recorded for individual rats that did not have tumors. Food intake diminished for this group of rats after 85 weeks of age and roughly paralleled weight loss.

The growth rates of each of the experimental groups were different over a selected, early period of time (table 8) but the rate of change of these values also differed with increasing age. The growth curve for rats in group C was nearly linear for more than the first 60 weeks. Although consistent differences between the average body weight of rats in groups A and C were evident, these differences became much less after 145 weeks of age.

At any age period, individual weights of rats in each of the 5 dietary groups varied sufficiently to produce overlaps between adjacent groups. Although the amount of food offered was held constant for a considerable period of time after the sixtieth week, accumulation of body mass continued. Subsequently, nearly every individual rat in all 5 groups showed a decrease in weight and a linear proportionality was found among the 5 groups between the maximal weight and weight at death.

TABLE 7

Tumor incidence on an adjusted population basis

Age periods	Group A		Group C				
	No. of rats	No. of rats with tumors	No. of rats	No. of rats with tumors	Age-specific tumor rate	Adjusted [1] no. of rats	Expected no. of tumors
days					(× 100)		
21–99							
100–199							
200–299							
300–399							
400–499							
500–599	176	1	165	1	0.06	176	1.1
600–699	157	2	140.1	3	2.1	157	3.3
700–799	144.5	8	123.5	3	2.4	144.5	3.5
800–899	116	6	95.0	5	5.3	116	6.2
900–999	89.1	11	67.2	7	10.4	89.1	9.3
1000–1099	49	7	28.0	7	25.0	49	12.3
1100–1199	29	8	15	4	26.7	29	7.7
1200–1299	9	4	5	4	80.0	9	7.2
1300–1399	1	0	0	0	0	1	—
Total		47		34			50.6

[1] Adjusted to number of rats in group A.

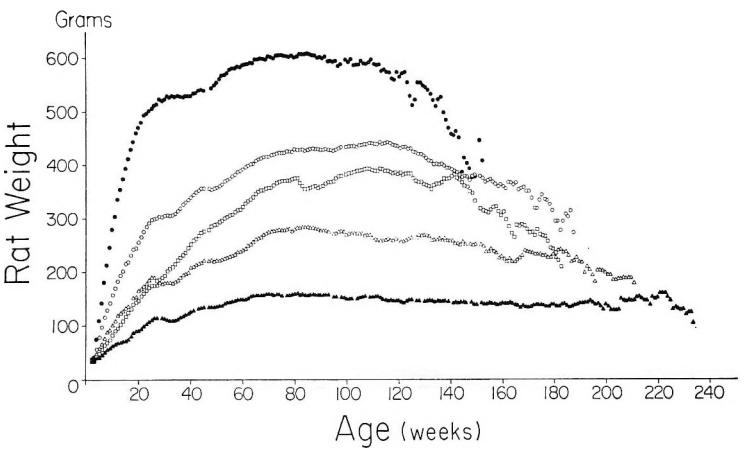

Fig. 4 Influence of diet on growth of rats and subsequent body weight changes with age. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

When the average weights of each of the experimental groups had reached a maximum (table 8) they were found to be related linearly to the cumulative caloric intake of that group although the time required to reach these weights differed. An exponential relationship existed between the maximal body weight of the group and its tumor prevalence.

The tumor incidence data were reorganized so that "new groups" were formed using the maximal body weights of the rats as the basis of segregation rather than dietary history. Age-specific tumor rate tables were derived, arbitrarily using that group of rats whose maximal weights ranged between 400 and 499 g as the standard for calculation of TIR values. The risk of tumor formation for the rats in the lowest weight class was less than 2% that of those in the heaviest weight class (fig. 5). Rats with maximal body weights approxi-

TABLE 8

Diet and body weight

Dietary group	Avg initial wt	Avg wt, 119 days	Growth rate [1]	Maximal avg wt [2]
	g	*g*	*K*	*g*
Commercial	39.4	429	0.024	610
A	37.5	232	0.019	440
B	36.6	145	0.014	282
C	38.2	130	0.013	394
D	39.3	80	0.007	167

[1] Computed as: Instantaneous relative growth rate (K)

$$K = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$ where

W = weight in grams
t = age in weeks ($t_2 = 17$, $t_1 = 3$).

[2] Excluding rats that were ill and showed marked and moribund weight loss at that time.

mately 12% less than that of the heaviest group had a tumor risk at least 50% lower.

In addition, since rats within each group, consuming a single diet, attained different weights, they were divided into 2 subgroups on the basis of body weight, namely, a heavier and a lighter subgroup (at a time when maximal weight of each group had been reached). TIR values were calculated, using the age-specific rates of the entire control group as a standard. In every group but that fed diet C, the heavier subgroup had a greater TIR value than the lighter subgroup (table 9). Although each set of TIR values was exponentially related to the corresponding mean body weight of the subgroup, the slopes of these curves differed; the lighter members of each dietary group showed greater rate of increase in risk with increase in body weight than did the heavier members. That there was variation in response to the different dietary regimens was also evident (table 9)

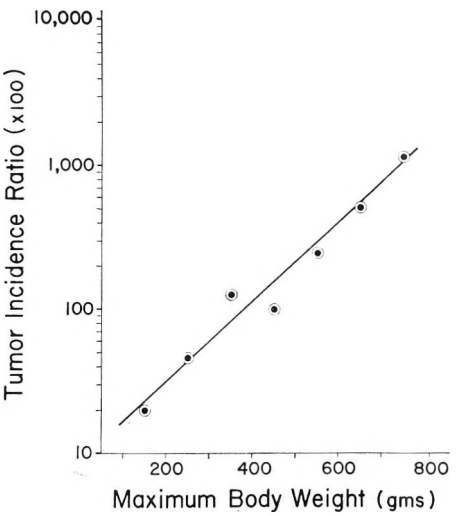

Fig. 5 Correlation between maximal body weight and tumor incidence ratio. (Rats with maximal body weights, 400 to 499 g, used as standard population for computation of TIR values.) Compared with the standard population, TIR values lower than 100 indicate the degree of beneficial effect and more than 100, the degree of deleterious effect.

since the 2 subclasses of group C showed no significant difference in TIR values while there was more than 109% difference in TIR values between the heavier and lighter members of group D.

Tumor prevalence was related to growth rate (fig. 6). When longer periods (first 200 days) were used in computing growth rates the deviation of group C in this relationship became negligible, even though these rats had attained only 50% of their growth.

TABLE 9

Body weights [1] and tumor incidence ratios (TIR) [2]

Dietary group	(1) Lighter wt subgroup		(2) Heavier wt subgroup		Increase in TIR values of subgroup 2 over 1
	wt range	TIR	wt range	TIR	
	g	(× *100*)	*g*	(× *100*)	%
Commercial	316–596	74.4	597–842	107.0	44
A	255–425	30.4	426–543	35.8	18
B	146–278	11.4	279–324	18.8	65
C	146–383	38.9	384–498	38.4	−1 [3]
D	100–158	4.7	159–192	9.8	109

[1] Division of groups made on the basis of individual rat weight at a time when average maximal weight of all rats had been reached.
[2] Computed, using age-specific tumor rates of the commercial dietary group as "standard rates."
[3] Negligible.

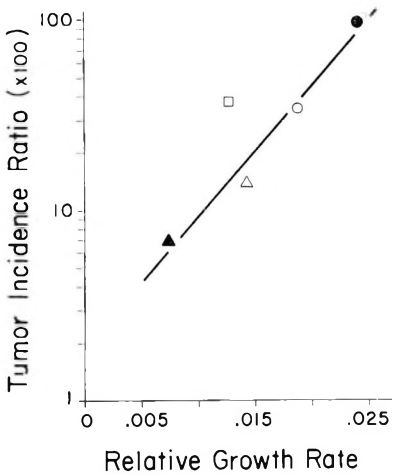

Fig. 6 Correlation between growth rate in early life and tumor risk (TIR) in later life. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

3. *Influence of diet upon tumor type.* Among the 148 tumors found in the total population, including the 6 observed in the animals killed, there were 28 taxonomic types (table 10). Malignant lymphoma was the predominant tumor found, with fibrosarcomas, fibromas and pancreatic islet cell tumors found in decreasing numbers in that order.

The major observations which indicated that nutrition has a marked influence upon tumor type, were: 1) occurrence in certain dietary groups of significant numbers of tumor types not observed in the other groups; 2) difference in "proportional" incidence of tumor types between groups; 3) difference in proportion of malignant-to-benign tumors; 4) difference in age-specific incidence patterns of specific tumor types.

Among the experimental groups, the greatest variety of tumor types was observed in those rats whose caloric intake was highest (groups A and C). Of all 5 groups, primary tumors of the lung were noted in rats of group A only, and accounted for 9.4% of the total number of tumors occurring in this group. Those groups with intakes high in carbohydrate (control, A and C) had malignant epithelial tumors to the extent of some 12% of the total number of tumors. However, malignant epithelial tumors were not found in groups B and D.

Malignant lymphomas predominated in the 3 groups of rats having high intakes of protein, whereas in the 2 groups with low intakes of protein (C and D), tumors of connective tissue, fibromas and fibrosarcomas were predominant (table 11). In addition, the incidence of pancreatic islet cell tumors was significantly higher in those groups of rats whose intake of protein was high.

Malignant lymphomas, tumors of subcutaneous tissue, pancreatic islet cell tumors and primary tumors of the lung showed an increase in rate with increasing age in those groups in which there were sufficient data to permit determination of age-specific rates. In the control group, malignant lymphomas, tumors of the subcutaneous tissue and pancreatic islet cell tumors were consistently displaced to earlier ages than in the other groups. The malignant lymphoma rate patterns of group C were displaced to later ages than that of group A, but for tumors of the subcutaneous tissue, this relationship was reversed.

The rate pattern for each tumor type conformed to a general trend within each dietary group with the exception of malignant lymphomas of rats in group A. The age-specific rate pattern for malignant lymphomas in these rats was significantly displaced to earlier ages compared with the patterns of the other tumor types of this group of rats.

A relatively high tumor risk for tumors of connective tissue (accounting for approximately 50% of the total TIR value for that group) was observed among rats with low protein intake, regardless of caloric intake (table 12). A relatively high tumor risk for malignant lymphomas (accounting for between one-third to one-fourth of the total TIR value) was noted for those groups (control, A and B) whose intake of protein was high regardless of the caloric intake. Tumors of the pancreas account for approximately 20% of the total risk incurred by those groups whose caloric intake was low regardless of the intake of protein.

Among the 142 tumors observed in rats permitted to live out their lives, 62 were

TABLE 10

Diet and tumor type

Tumor type	Total	Number of tumors				
		Dietary group [1]				
		Commercial	A	B	C	D
Malignant lymphoma						
Lymphosarcoma	4 } 34	—	3	—	—	1
Reticulum cell sarcoma	30 }	11	11	2	5	1
Connective tissue						
Fibrosarcoma	28	3	11	1	9 [2]	4
Fibroma	22	8 [2]	2 [2]	1	9	2
Skin						
Papilloma	2	1	—	—	1	—
Squamous cell carcinoma	5	—	3	—	2	—
Appendix	1	1 [3]	—	—	—	—
Pancreas						
Islet cell tumor	18	4	8	3 [2]	1	2
Adenocarcinoma	1	—	—	—	1	—
Adrenal cortical adenoma	3	—	—	2	—	1
Thymoma	2	—	—	—	2 [2]	—
Lung						
Adenoma	3 } 5	—	3 [2]	—	—	—
Carcinoma	2 }	—	2	—	—	—
Salivary gland adenocarcinoma	1	—	1	—	—	—
Liver						
Hepatoma	3	1	1	—	1	—
Cholangioma	1	—	1	—	—	—
Stomach adenocarcinoma	1	—	—	—	1	—
Kidney cortical adenoma	1	—	1	—	—	—
Testes	7	3	3	—	1	—
Mammary fibroadenoma	1	1	—	—	—	—
Retinoblastoma	1	—	—	1	—	—
Glioma	1	—	—	—	1	—
Lipoma	2	1	—	—	1	—
Rhabdomyosarcoma	2	—	2 [4,5]	—	—	—
Lymphangioma	3	—	1 [4]	—	2	—
Hemangioma	1	—	—	—	1 [6]	—
Unknown origin						
Anaplastic carcinoma	1	—	—	—	1	—
Carcinoma	1	—	—	—	1	—
Total	148	34	53	10	40	11

[1] Number of tumors not adjusted for differences in initial population size or for the number of rats killed at various ages.

[2] One case found in a rat that was killed.

[3] Hair follicles or hair matrix.

[4] Mesentery.

[5] Voluntary muscle.

[6] Mesenchymal tissues.

benign whereas 80 were malignant. The difference in proportional incidence of malignant to benign tumors (table 13) was most striking between groups A and C; there were twice as many malignant tumors as benign in rats of group A, whereas the incidences were equal in group C. Furthermore, the age-specific rate pattern of malignant tumors of group A was nearly consistently displaced to earlier ages than the benign pattern of this group or the malignant or benign pattern of group C. Since the numbers of benign tumors in each of these 2 groups were nearly equal, this difference in ratio emphasizes the greater malignant tumor risk suffered by

TABLE 11

Proportionate tumor incidence [1] and diet

Dietary group	Malignant lymphoma		Tumors of subcutaneous tissue	
	No. specific/ total tumors		No. specific/ total tumors	
		%		%
Commercial	10/33	30.3	11/33	33.3
A	14/51	27.5	12/51	23.5
B	2/9	22.2	2/9	22.2
C	5/38	13.2	17/38	44.7
D	1/11	9.1	6/11	54.6

[1] Tumor data from animals permitted to live out their lives.

TABLE 12

Tumor incidence ratios for specified tumor type

Tumor type [1]	Tumor incidence ratios [2]				
	Dietary group				
	Commercial	A	B	C	D
	(× 100)	(× 100)	(× 100)	(× 100)	(× 100)
Malignant lymphoma	35.6	10.7	3.1	5.6	1.3
Tumors of subcutaneous tissue	32.3	9.1	3.1	19.0	3.8
Pancreatic islet cell tumors	12.9	6.1	3.1	1.1	1.3
Primary tumors of lung	0	3.0	0	0	0
Others	25.9	9.9	4.6	16.7	0.6
Total	106.8	38.8	13.9	42.4	7.0

[1] All tumors included if more than one type was present in a rat.
[2] "Standard rates" = age-specific tumor rates of all tumor-bearing rats in the commercial dietary groups permitted to live out their lives.

TABLE 13

Influence of diet on proportion of malignant to benign tumors

Dietary group	No. of tumors [1]		Tumor incidence ratio [2]		Tumor incidence ratio
	Benign	Malignant	Benign	Malignant	Malignant-to-benign
			(× 100)	(× 100)	
Commercial	16	17	51.6	54.8	1.06
A	17	34	12.9	25.9	2.00
B	5	4	7.7	6.2	0.81
C	19	19	21.2	21.2	1.00
D	5	6	3.2	3.8	1.19
Total	62	80			

[1] In rats permitted to live out their lives.
[2] New age-specific rate tables organized for each group on the basis of malignancy. Tumor incidence ratio values computed using age-specific rates of entire commercial dietary group as standard rates. Values may exceed 100 since standard rates are based on incidence of tumor bearing animals, whereas these data consider every tumor type.

rats of group A. The TIR values obtained for the benign tumors and for the malignant tumors of each of these groups not only confirm this observation but also show a considerable decrease in relative benign tumor risk. When age-specific rates of the benign tumors and of malignant tumors of group C were used as the standard rates in deriving the corresponding TIR values of group A, the value obtained for the benign tumors of group A was less than that of group C by 57%, whereas the malignant tumor TIR value of group A was higher than that of the group C rats by 24%. The age-specific rate patterns of the benign tumors show an exponential trend related to age. For malignant tumors, however, the rate trends for groups A and C are directly and linearly related to age.

DISCUSSION

The exponential character of the relationship between tumor incidence (as expressed in TIR values) and caloric intake, indicates that the level of incidence for rats on any dietary regimen forms a continuum. A separation, therefore, cannot be made between the level of caloric intake at which caloric over-consumption, with its attendant, deleterious effects ends, and the level at which caloric restriction with its beneficial effects begins. The extremes in caloric restriction required under the conditions of the this study rule out the possibility that increasing beneficial effects could be expected by further restriction. As also reported by others (23, 24) the reduction in incidence achieved by caloric restriction is accompanied by a displacement of the tumor incidence pattern to later ages. It appears that these salutary effects upon tumor incidence are, in a large measure, attributable to a delay in time of onset of the incidence pattern to ages which chronologically approach or are well within the senescent period of life.

Group D rats not only had the greatest life expectancy (7) and the lowest tumor incidence, but had the greatest delay in time of development of the age-specific incidence pattern. Despite this, however, far fewer total calories were consumed by this group by the time the first tumor appeared than by any other group. In fact, all the tumors observed in this group had occurred when the cumulative caloric intake was less than that of the control group or of the 2 experimental groups with high caloric intake at the time when the first tumor death in any of these 3 groups was recorded. Those tumors that did occur in rats of group D may have had an altered dependency on caloric intake, or the caloric intake may have been so severely reduced that the effect of other "factors" became dominant.

The inseparable effect of long-term restriction in caloric intake upon body weight and upon tumor incidence has prompted the suggestion (24, 25) that inhibition of tumorigenesis may be mediated through mechanisms which are intimately involved with growth. Our results lend further emphasis to this correlation in that the TIR values were related exponentially to maximal body weight attained by each dietary group. The relationship between tumor incidence and maximal body weight exists irrespective of distinct dietary groups. In fact, within all dietary groups, except group C, higher tumor risks were noted for the heavier rats than for the lighter rats even though their caloric intake was identical. The rate of increase in risk with increasing caloric intake accelerates more rapidly for the lighter weight series than for the heavier series. Each subgroup with its different tumor risk may conceivably represent a somewhat different portion of the genetic spectrum of the entire group (26); the variation in their mature body weights suggests different abilities early in life to convert food substances into body mass. These variations in body weight characteristics, however, need not be solely of genetic origin since it has been shown that the nutritive condition in utero (27) and during the suckling period (28) has a marked effect on postweaning rate of development and on body weight.

The modification in variety and incidence of specific tumor types by the level of protein of the diet consumed is definite and striking with no two dietary groups having similar patterns. It is remarkable, however, that change in total number of specific tumor types and their TIR values is uniquely balanced and conforms

strictly to limits set by the total caloric intake only. The modification, by dietary means, of the incidence and type of specific tumors has also been shown for the rat in the data of Saxton et al. (24), and of Gilbert et al. (18); and for the mouse in the data of White and Andervont (29), Tannenbaum and Silverstone (30), and Silverstone and Tannenbaum (31).

The changes in incidence of different degenerative diseases, including specific tumor types brought about by a single measure, caloric restriction, can best be explained by postulating the existence of a common underlying mechanism:

Different, lifelong, dietary regimens have been shown to influence age-specific mortality rates differently at different age periods (7). The factors required to adjust the life expectancy pattern of each of the experimental groups to that of the control group were nearly identical to the factors necessary to make the same adjustment in total tumor risk patterns. Within any dietary population there is a signal uniformity in the sequence and proportion of time required for biological, biochemical and pathological events to occur (5–8), and the numerical factors in actuality must represent a quantitative expression of the difference in physiological conditions of the populations. Thus, the major differences at any one chronological age period, for each of these parameters, are most likely the result of the different rates of physiological ageing.

The contribution made by tumors toward mortality risk may be assessed by comparing the number of deaths of rats bearing tumors with total number of deaths or of number of rats dying without tumors. Differences in actual number of tumors may, in part, be due to: (a) the consequence of a "true" reduction in prevalence of tumors, (b) the result of a delay in time of appearance which resulted in a concomitant, subsequent reduction in prevalence, or (c) the result of more members of a population reaching older ages where the risk of incurring tumors was greater.

The increase in age-specific tumor rate was found to be proportional, logarithmically, to the existing mortality rate of that population (6). The patterns were nearly identical for four of the dietary groups but not for group D. Thus, the displacement of mortality patterns in time, and the differences in population size, led to differences in number of tumors but not in tumor probability at death.

In comparison of groups A and C. both having the same tumor risk, rats of group A with their longer life span, allowed more of its members a longer period of time in which to develop tumors. Similarly, when group C is compared with the control group there is a significantly lower risk for the longer-lived rats in group C even though numbers of tumors were equivalent between groups and tumor rate patterns had the same dependency upon mortality rate patterns.

Even though rats of group B were relatively long-lived, there was an apparent reduction in prevalence of tumors due to delay in time of onset, a subsequent difference in age-specific tumor rates and a difference in the duration of time in which tumors were found.

However, rats of group D that had the longest life expectancy, showed only one-third the tumor probability at death of the other groups. This may have been the result of time differences in rate of tumorigenesis. The relatively large number of deaths due to trichobezoars in this group, however, may have so altered this relationship that, had these rats survived, the entire group might have suffered tumor morbidity rates that were similar to those of the other groups.

The benign tumor morbidity rate increases strictly as a function of the mortality rate for each of the 5 groups. Each of these coincides one with the other. Ultimately, regardless of diet, all rats suffer a benign tumor risk of the same magnitude, although at different times. The discrete and consistent effects of the level of caloric intake of each group, therefore, disappears when the calendar is replaced as a basis of calculation by the age-specific mortality rate pattern. This also indicates that animals bearing benign tumors do not generally have their life spans shortened any more than the other members of their group.

Age-specific, malignant tumor morbidity rate, however, differs markedly in its rela-

tionship to age-specific mortality rate for each of the 5 groups, with the greatest malignant tumor risk at death in rats of group A and the lowest in rats of group D. Thus, with increasing mortality rate, after a rate of 0.1 has been reached, the corresponding increase in malignant morbidity rate is more than 3 times greater for rats of group A than that of rats in group D. Unlike the observations based upon specific rates or relative tumor incidence, that rats fed the control diet suffered the most deleterious effects, the above relationship places these rats in a position of risk of dying with malignant tumors, intermediate to those of groups C and D. This deviation cannot be explained on the basis that these control rats maintained their malignant tumors and their lives for longer periods of time than did the rats fed the other diets, since the age-specific morbidity-mortality rate pattern is consistently displaced to earlier ages. Regardless of the method of expression, the basis of calculation, the type of tumors that occur, or the degenerative disease under consideration, a regimen low in total calories, carbohydrate and protein consistently showed the most beneficial effects of any of the dietary groups.

ACKNOWLEDGMENTS

Acknowledgment is gratefully made to the late James B. Duffy, to Joseph Gregg and to James Flaharty for their technical assistance. The authors are indebted for the advice and encouragement generously given by Doctor Paul György, Philadelphia, Pennsylvania; Joseph Sibigtroth, New York Life Insurance Company, New York; and Doctors William Batt and Arthur Valk, Biochemical Research Foundation, Newark, Delaware.

LITERATURE CITED

1. Ross, M. H., and J. O. Ely 1954 Ageing and enzyme activity. J. Franklin Inst., *258:* 63.
2. Ross, M. H., and J. O. Ely 1954 Protein depletion and age. J. Franklin Inst., *258:* 241.
3. Ross, M. H., and W. G. Batt 1956 Alkaline phosphatase activity and diet. J. Nutrition, *60:* 137.
4. Ross, M. H., and W. G. Batt 1957 Diet-age pattern for hepatic enzyme activity. J. Nutrition, *61:* 39.
5. Ross, M. H. 1959 Protein, calories and life expectancy. Federation Proc., *18:* 1190.
6. Ross, M. H. 1964 Nutrition, disease and length of life. Ciba Foundation Study Group no. 17, Diet and Bodily Constitution. Little, Brown and Company, Boston.
7. Ross, M. H. 1961 Length of life and nutrition in the rat. J. Nutrition, *75:* 197.
8. Bras, G., and M. H. Ross 1964 Kidney disease and nutrition in the rat. Toxicol. Applied Pharmacol., *6:* 247.
9. Rusch, H. P. 1944 Extrinsic factors that influence carcinogenesis. Physiol. Rev., *24:* 177.
10. Tannenbaum, A., and H. Silverstone 1953 Nutrition in relation to cancer. In: Advances in Cancer Research, vol. 1, eds., J. B. Greenstein and A. Haddow, Academic Press, New York, p. 451.
11. White, F. R. 1961 The relationship between underfeeding and tumor formation, transplantation, and growth in rats and mice. Cancer Res., *21:* 281.
12. Axelrad, A. A., and C. P. Leblond 1955 Induction of thyroid tumors in rats by a low iodine diet. Cancer, *8:* 339.
13. Brunschwig, A., and R. A. Rasmussen 1941 The relation of diet to benign neoplasia (ulcero-papillomas) of the rat's stomach. Cancer Res., *1:* 371.
14. Sugiura, K. 1942 The relation of diet to the development of gastric lesions in the rat. Cancer Res., *2:* 770.
15. Orten, A. U., C. G. Burn and A. H. Smith 1937 Effects of prolonged chronic vitamin A deficiency in the rat with special reference to odontomas. Proc. Soc. Exp. Biol. Med., *36:* 82.
16. Schaefer, A. E., D. H. Copeland, W. D. Salmon and O. M. Hale 1950 The influence of riboflavin, pyridoxine, inositol and protein depletion-repletion upon the induction of neoplasm by choline deficiency. Cancer Res., *10:* 786.
17. Saxton, J. A., G. A. Sperling, L. L. Barnes and C. M. McCay 1948 The influence of nutrition upon the incidence of spontaneous tumors of the Albino rat. Acta Unio. Internat. Contra Cancrum, *6:* 423.
18. Gilbert, C., J. Gillman, P. Loustalot and W. Lutz 1958 The modifying influence of diet and the physical environment on spontaneous tumour frequency in rats. Brit. J. Cancer, *12:* 565.
19. International Classification of Diseases 1957 Manual of the International Statistical Classification of Diseases, Injuries and Causes of Death. World Health Organization, vol. 1, 1955 Rev., Geneva.
20. Dublin, L. I., A. J. Lotka and M. Spiegelman 1949 Length of life. The Ronald Press Company, New York.
21. Murray, W. S., and J. G. Hoffman 1941 Physiological age as a basis for the comparison of strains of mice subject to spontaneous mammary cancer. Cancer Res., *1:* 298.

22. Brody, S. 1945 Bioenergetics and Growth. Reinhold Publishing Corporation, New York, New York.
23. Tannenbaum, A. 1942 The genesis and growth of tumors. II. Effects of caloric restriction per se. Cancer Res., *2*: 460.
24. Saxton, J. A., Jr. 1945 Nutrition and growth and their influence on longevity in rats. Biol. Symp., *11*: 177.
25. Tannenbaum, A. 1940 Relationship of body weight to cancer incidence. Arch. Pathol., *30*: 509.
26. Heston, W. E., and G. Vlahakis 1962 Genetic obesity and neoplasia. J. Nat. Cancer Inst., *29*: 197.
27. Chow, B. F., and C-J. Lee 1964 Effect of dietary restriction of pregnant rats on body weight gain of the offspring. J. Nutrition, *82*: 10.
28. Widdowson, E. M., and R. A. McCance 1960 Some effects of accelerating growth. I. General somatic development. Proc. Royal Soc., *B 152*: 188.
29. White, J., and H. B. Andervont 1943 Effect of a diet relatively low in cystine on the production of spontaneous mammary-gland tumors in strain C_3H female mice. J. Nat. Cancer Inst., *3*: 449.
30. Tannenbaum, A., and H. Silverstone 1949 The genesis and growth of tumors. IV. Effect of varying the proportion of protein (casein) in the diet. Cancer Res., *9*: 162.
31. Silverstone, H., and A. Tannenbaum 1951 Proportion of dietary protein and the formation of spontaneous hepatomas in the mouse. Cancer Res., *11*: 442.

Shift in the Relative Distribution of Body Nitrogen between Skin and Carcass in Mouse Radiation Chimeras [1]

A. L. KRETCHMAR, W. H. McARTHUR AND C. C. CONGDON
Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee, Knoxville College, Knoxville, Tennessee and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

ABSTRACT Earlier work on nitrogen balance of radiation chimeras suggested the possibility that the relative distribution of kinds of body nitrogen in tissues of these animals might be altered. The purpose of this study was to determine whether measurable changes in nitrogen content of tissues occur in radiation chimeras. A total of 108 male mice was used. The experimental group, 36 mice, were given 950 r total-body x-irradiation and then 20×10^6 bone marrow cells by intravenous injection; the cells were taken from normal, unirradiated mice of a different strain (homologous bone marrow cells). The 2 control groups were: 36 normal untreated mice; and, 36 mice that were given 950 r x-irradiation and then injected with 20×10^6 bone marrow cells from donors of the same strain (isologous bone marrow cells). The irradiated mice treated with homologous bone marrow cells had a decreased fraction of total-body nitrogen in the carcass and an increased fraction in skin. This shift in nitrogen distribution was detectable as early as 4 days after treatment, but was more definite after 25 days. The results are biochemical evidence that some integumentary structure or structures may be a major target of the graft-versus-host reaction in radiation chimeras.

Lethally irradiated mice that would die if untreated, can recover if injected intravenously with living bone marrow cells taken from unirradiated normal donor mice. However, if the injected cells are from donor animals of a different strain or species and therefore genetically incompatible, the treated irradiated mice show only temporary recovery, followed in 20 to 60 days by many fatalities (1). The mortality is associated with a "secondary syndrome" (homologous disease), not related to the acute effects of irradiation but believed to be a consequence of destructive immunological interaction of the proliferating grafted cells and the recovering irradiated host (1). The nature of the biochemical mechanisms underlying the pathogenesis of the secondary syndrome are unknown but the idea of "metabolic starvation" has been put forth in this connection (2). This imprecise concept has been useful in that it directs attention to metabolic parameters in the radiation chimera [2] for an explanation of the loss in body weight that is a clinically prominent aspect of the secondary syndrome. The weight loss occurs despite an adequate intake of food comparable to that of the 2 control groups; namely, normal mice and irradiated but isologous (same strain) marrow-treated animals (3).

We have reported previously (4) that the nitrogen balance of radiation chimeras is positive and that these animals retain as much nitrogen as do irradiated mice treated with isologous cells. The latter control animals do not show the secondary loss of weight that occurs in similarly irradiated mice given homologous (different strain) marrow. It was concluded that the nitrogen retained by isologous marrow-treated animals is utilized for regeneration of the hemopoietic system, other repair processes and growth. Since the homologous chimeras retain as much nitrogen but failed to gain in body weight, we speculated that there was a change in the distribution of kinds of body nitrogen in

Received for publication June 4, 1965.
[1] Research sponsored by U. S. Atomic Energy Commission.
[2] "Radiation chimera" is used to describe an animal whose tissues contain living cells of more than one genotype as a consequence of introducing living foreign cells into an irradiated host animal.

tissues of mice with homologous marrow grafts (4).

The objective of the present study was to determine whether measurable changes in nitrogen content of tissues of radiation chimeras were present. The results suggest that nitrogen may be deposited in skin at the expense of the other tissues.

MATERIALS AND METHODS

Irradiated animals were all [101 cum ♀ × C3H/Auf Cum ♂] F_1 male mice 11 to 14 weeks old. Donors of homologous bone marrow were [C57BL/6 cum ♀ × DBA/2 Cum ♂] F_1 male mice from 79 to 194 days old. The details of the bone marrow treatment have been reported (5, 6); 20×10^6 isologous (IBM) or homologous (HBM) cells were injected intravenously on the day of irradiation. There were 18 mice in each group (normal, IBM and HBM) at 4 and 25 days after treatment (total 108 animals).

Nine hundred and fifty rads total-body irradiation were delivered with a constant potential x-ray machine; 250 kv, 15 ma. Inherent filtration was 1 mm Al; half-value layer, 0.5 mm Cu; target-object distance 60 cm; dose rate in air approximately 157 r/min. Animals, kept 10 to a cage before and after irradiation, had free access to food [3] and water. Some animals were maintained, in order to measure nitrogen balance, in the special metabolism cage described previously (4) for 48 or 96 hours before killing.

All animals were decapitated, the blood being collected in a round-bottomed flask, and after exsanguination the thoracic viscera were dissected and added to its contents. Similarly, the abdominal viscera, skin, tail and head, carcass, and excreta were put into separate appropriately labeled vessels. Fifty to 150 ml of 6 N H_2SO_4 were added. A reflux condenser was adjusted over each and digestion at boiling temperature carried overnight or to completion. The fat which overlay the digested specimens was extracted with chloroform and measured gravimetrically. The extracted samples were then diluted to give approximately 1 mg N/ml and nitrogen was determined in aliquots by the Technicon Autodigester and Autoanalyzer system (7). The total nitrogen of the animal was thus partitioned into 4 gross compartments: I, blood and thoracic viscera; II, abdominal viscera; III, skin, tail and head; and IV, carcass.

RESULTS

Nitrogen balance was variable and, as reported previously (4), no significant difference between normal, isologous, or homologous marrow-treated irradiated mice was noted; the data are therefore not included.

The most significant observations were in the nitrogen of "skin" (compartment III) and "carcass" (compartment IV). Figure 1 (a and b) illustrates the correlation

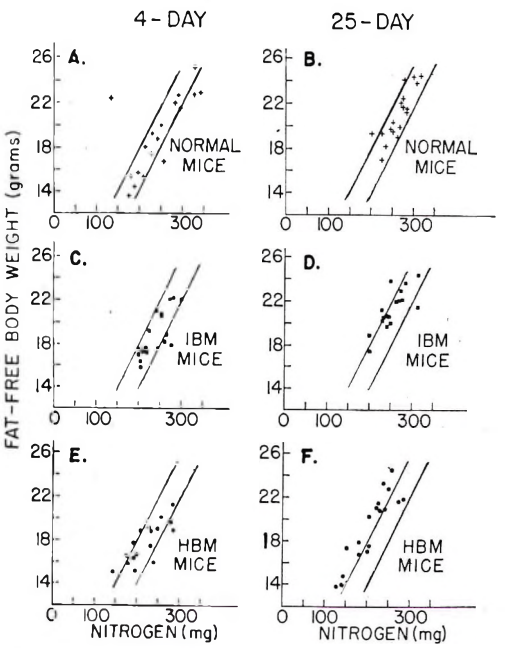

Fig. 1 Plot of nitrogen content of "carcass" against body weight for normal (a and b) and irradiated mice given isologous (c and d) or homologous bone marrow cells (e and f). In 6 groups (6 mice/group) of normal mice the carcass content was 38.3 to 39.2% of the total nitrogen of the animal. The lines in all the graphs are drawn with a slope of 1/11.25 and 1/13.75 g fat-free body weight/mg nitrogen. These lines indicate the area on the graph corresponding to the statement that 12.5 ± 1.25 mg of nitrogen/g body weight is contained in the carcass of normal mice.

[3] Purina Mouse Chow, Ralston Purina Company, St. Louis.

between body weight [4] and "carcass nitrogen" in unirradiated control mice 4 and 25 days after 1 ml Tyrode's solution injected intravenously. There were 12.5 ± 1.25 mg nitrogen/g body weight in this fraction. In irradiated mice 4 and 25 days after 20×10^6 isologous bone marrow cells (fig. 1, c and d) the same relationship fits the data. If irradiated mice were given 20×10^6 homologous cells, however, (fig. 1, e and f) a significant number of the animals had less nitrogen in the "carcass." This low carcass nitrogen, although it occurred in some of the 4-day mice, characterizes the majority of the animals 25 days after homologous marrow. The data also show that about half of the mice in the latter group had not recovered body weight and weighed less than 18 g.

Figure 2 (a and b) shows that "skin nitrogen" is also correlated with body weight in normal mice and that approximately the same amount of nitrogen per gram of body weight is contained in this fraction as in the "carcass." Again, the data for irradiated mice given isologous bone marrow cells (fig. 2, c and d) is comparable to normal animals. Four of these animals had "skin nitrogen" content that was higher than expected for their body weight. When the ratio of skin-to-carcass nitrogen content was computed, however, the ratio was normal, indicating that relative distribution of body nitrogen was normal.

A number of the irradiated mice given homologous marrow (fig. 2, e and f) had more "skin nitrogen" than expected for their body weight. Seven of the 18 animals at 4 days had high nitrogen content in this fraction. Of the 8 mice that weighed less than 18 g at 25 days and presumably, therefore, were suffering most severely from homologous disease, 5 had high skin nitrogen content. In contrast with the isologous marrow-treated groups, when the ratio of skin-to-carcass nitrogen

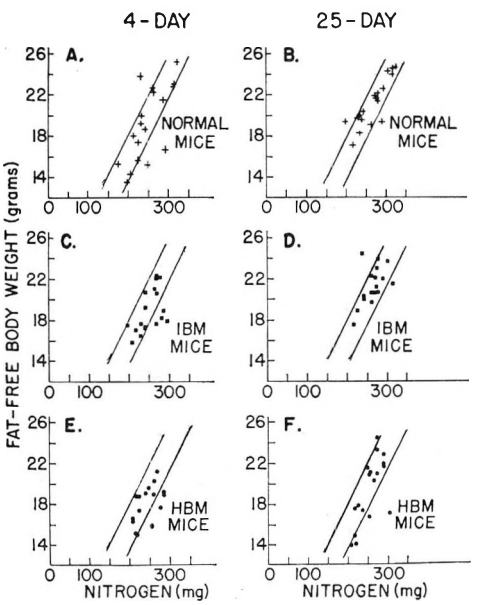

Fig. 2 Plot of nitrogen content of "skin" against body weight for normal (a and b), and irradiated mice given isologous (c and d) or homologous bone marrow cells (e and f). Refer to legend of figure 1 for explanation of lines. Nitrogen content of "skin" was 38.5 to 40.2% of the total nitrogen of the normal animals, 12.53 ± 1.38 mg/g of body weight.

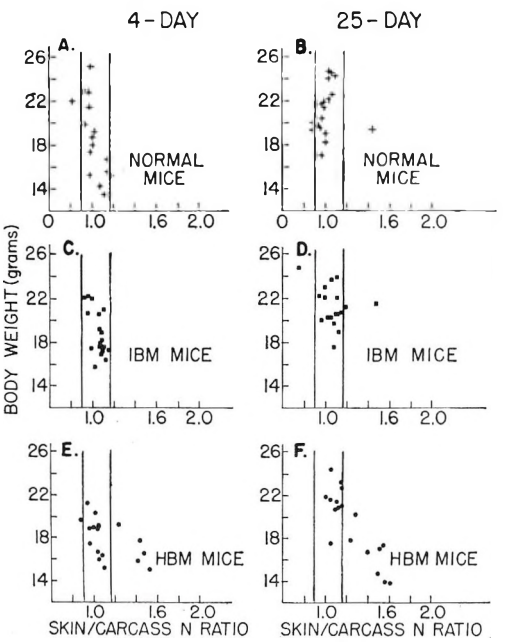

Fig. 3 Plot of the ratio of "skin" to "carcass" nitrogen against body weight for normal (a and b), and irradiated mice given isologous (c and d) or homologous bone marrow cells (e and f). The lines indicate that this ratio is 1.03 ± 0.13 in normal mice.

[4] The animals used varied in age from 11 to 14 weeks of age and were not selected for body weight. The range in body weights at the start of the experiment was the same in the "normal," "IBM" and "HBM" groups.

content was computed it was higher than for normal mice, indicating that there was a change in relative distribution of body nitrogen.

The ratio of skin to carcass nitrogen content was computed for all animals and is shown in figure 3. There is no correlation between the ratio of skin-to-carcass nitrogen and body weight in normal (fig. 3, a and b) and irradiated mice given isologous bone marrow cells (fig. 1, c and d). This ratio is slightly over 1.0. However, in irradiated mice given homologous cells (fig. 3, e and f), the ratio of skin-to-carcass nitrogen is greater than 1.0 in most and there is a negative correlation with body weight. This trend, present as early as 4 days after treatment, is especially clear at 25 days. Thus, in irradiated mice given homologous marrow, low carcass nitrogen is associated with increased skin nitrogen and this association is especially characteristic of animals weighing less than 18 g.

DISCUSSION

The skin-to-carcass ratios for the homologous marrow-treated animals demonstrate clearly a shift in distribution of body nitrogen from carcass to skin. There is, indeed, a definite negative correlation between this ratio and body weight in chimeras at 25 days. The negative correlation indicates that the animals with severest secondary disease (as judged by body weight) show the greatest shift in nitrogen distribution but that the tendency is present in all the animals over the whole range of clinical severity present in this experiment.

The method used to divide the whole animal had the advantage of technical simplicity but the disadvantage of grossness. This disadvantage is particularly important with respect to skin where no attempt was made to separate dermis, epidermis and fur. Since absent or delayed hair growth is a part of the abnormality in radiation chimeras, it appears likely that the amount of nitrogen included in our "skin" that is due to the protein content of the fur would be less in animals given homologous marrow than in control or isologous marrow-treated mice. Despite this factor tending to decrease the nitrogen content of "skin," the results show that the degree of shift of nitrogen from the general tissues to "skin" is sufficient to be detectable with a simple and relatively crude partition of the animals.

These results could be explained if the skin were a major target organ of the antihost activity of the grafted foreign immunologically competent cells that are present in the bone marrow cell inoculum injected after irradiation. It could be suggested that the antihost activity of these cells causes deposition of nitrogen in skin and constitutes a significant drain on the animal's metabolic resources as is evident from the decreased fraction of the total nitrogen of the chimera that is present in the carcass. If the extra nitrogen were deposited in a noncellular integumentary compartment at the expense of cellular protein of the general tissues, a loss of body weight could result even though the net nitrogen balance was positive. The evidence is, therefore, consistent with the speculative explanation (4) of positive nitrogen balance without weight gain or in the presence of loss in body weight (cf. fig. 1D, ref. (4)) exhibited by radiation chimeras.

The discussion can be illustrated by reference to a simple model system not intended to simulate in detail the animals in these experiments but only to illustrate the concept involved. Consider a system of only 3 compartments: 1, PP = plasma and extracellular nitrogen pool, 300 mg; 2, BWM = muscle pool (18 to 20% protein), 10 g as body weight; and 3, BWP = noncellular protein pool, 1.5 g as body weight. Assume a constant daily input of 100 mg N into PP; from this pool 50 mg N/day are retained as muscle protein with a nitrogen-to-body weight conversion factor of about 30 (since muscle is about 19% by weight protein) and a small amount, 3 mg N/day retained as nonprotoplasmic nitrogen in BWP with a nitrogen-to-body weight conversion factor of 6.25.[5] At balance, 100 mg N/day are excreted from PP and 50 mg and 3 mg N/day are returned to PP from BWM and

[5] This weight conversion factor would only be valid for a pure protein of 16% nitrogen. Protein in solution would have a weight conversion factor of about 8. However, if "nonprotoplasmic protein" was precipitated extracellularly as, for instance, collagen (8) or bound to cell surfaces, it would contain less "bound" water and, therefore, have a nitrogen-to-weight conversion factor between 8 and 6.25.

BWP, respectively. Figures 4 and 5 are analogue computer outputs for this model. Figure 4 shows that the system is in nitrogen balance for the first 20 days with body weight 11.5 g (BWM + BWP). Figure 5 shows that the equilibrium PP size is 300 mg, BWM is 10 g and BWP is 1.5 g as assumed in setting up the model. At approximately 20 days the only change introduced into the system (automatically by the computer program) is a shift to 43 mg N/day retained as muscle protein and 10 mg N/day as nonprotoplasmic protein. This is the same total retention, 53 mg N/day, but at a slightly different ratio in the direction of nonprotoplasmic protein. The system goes into slight positive nitrogen balance, loses 1.3 g (about 9%) body weight (fig. 4), mobilizes muscle protein and accumulates nonprotoplasmic protein (fig. 5).

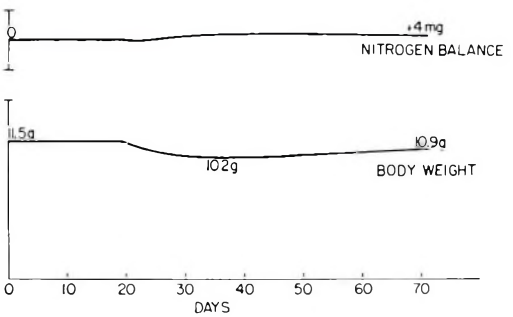

Fig. 4 Analogue computer output for the model system described in the text. The traces show the effect on nitrogen balance and body weight of a change in the ratio, *nitrogen retained as "muscle protein"/nitrogen retained as "noncellular protein,"* without any change in total nitrogen retained by the system.

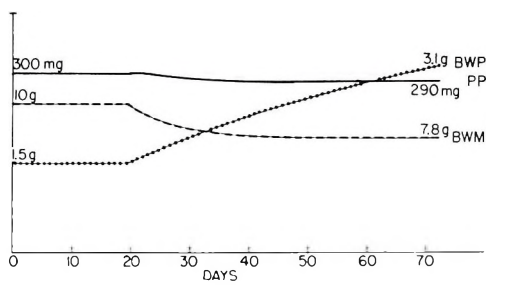

Fig. 5 Analogue computer output for the model system described in the text. The traces show the effect of the change indicated in figure 4 on the size of the plasma and extracellular nitrogen pool, PP; muscle pool, BWM; and noncellular protein pool, BWP.

The molecular form of the extra nitrogen deposited in "skin" might be the proteins of antigen-antibody complexes. Hager et al. (9) have presented direct evidence for antibody protein on the surface of cells of a homograft during rejection and have further indicated that antibody is also present on the host's cells as well. There is clear evidence for location of transplantation antigens on cell surfaces (10). The antibody bound to cells by surface antigens could constitute a large reservoir of extracellular protein. It would not be unexpected if the grafted immunological apparatus of the chimera were reacting against skin since histopathologic evidence of damage to integumentary structures is frequently observed (11) in radiation chimeras with homologous disease.

ACKNOWLEDGMENT

The interest and suggestions of Dr. J. R. Totter of the staff of the Division of Biology and Medicine, U. S. Atomic Energy Commission, are gratefully acknowledged.

ADDENDUM

We have recently found a large size (excluded from Sephadex G-200) material with UV absorption at 280 mμ (presumably protein) in the soluble fraction of homogenates of skin of the body (head, tail, and limbs amputated) of radiation chimeras. This material migrates toward the cathode at pH 8.6 on cellulose acetate strips.

LITERATURE CITED

1. Congdon, C. C. 1959 Recovery from radiation with special consideration of the use of bone marrow transplantation. In: Progress in Hematology, vol. 2, ed., L. M. Tocantins. Grune, New York, p. 21.
2. Urso, I. S., C. C. Congdon and R. D. Owen 1959 Effect of foreign fetal and newborn blood-forming tissues on survival of lethally irradiated mice. Proc. Soc. Exp. Biol. Med., *100:* 395.
3. McArthur, W. H., A. Kretchmar and C. C. Congdon 1963 Study of nitrogen balance in irradiated mice given foreign bone marrow. Blood, 22: 502.
4. McArthur, W. H., C. C. Congdon and A. L. Kretchmar 1964 Nitrogen balance in radiation chimeras. Proc. Soc. Exp. Biol. Med., *117:* 171.
5. Kretchmar, A. L., and C. C. Congdon 1961 Biochemical changes and increased liver weight in bone marrow chimeras. Am. J. Physiol., 200: 102.

6. Suu, V-T., C. C. Congdon and A. L. Kretchmar 1963 Increase in lysozyme activity in kidneys of irradiation chimeras. Proc. Soc. Exp. Biol. Med., *113:* 481.
7. Ferrari, A. 1960 Nitrogen determination by a continuous digestion and analysis system. Ann. N. Y. Acad. Sci., *87:* 792.
8. Kendrew, J. C. 1954 Structure proteins In: The Proteins, vol. 2, part B, eds., H. Neurath and K. C. Bailey. Academic Press, New York, p. 845.
9. Hager, E. B., M. P. DuPuy and D. F. H. Wallach 1964 Immunologic suicide and studies on the role of antibody and complement in canine kidney homograft rejection. Ann. N. Y. Acad. Sci., *120:* 447.
10. Davies, D. A. L. 1964 Transplantation antigens: studies in the mouse. In: Centre National De La Recherche Scientifique Conference on the Transplantation of Allogeneic Hemopoietic Cells, in press.
11. DeVries, M. J. 1964 The histopathological signs of secondary disease in different species. In: Centre National De La Recherche Scientifique Conference on the Transplantation of Allogeneic Hemopoietic Cells, in press.

Amino Acid Diets and Maximal Growth in the Rat [1]

Q. R. ROGERS AND A. E. HARPER
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

ABSTRACT Amino acid diets fed in an agar gel have been found to support weight gains of rats as great or greater than those obtained with diets containing an equivalent quantity of casein supplemented with methionine. Over 1% arginine, 0.6% asparagine and feeding the diet in gel form were necessary to obtain maximal weight gain. The diet contained amino acids in excess of the requirements of the rat, and hence it should be possible to improve the efficiency of utilization of the amino acid mixture.

It was not until after the extensive investigations of Rose and co-workers had culminated in the discovery of threonine that amino acid diets of known composition could be prepared which would support growth. At the close of their work, Rose and associates (1) had developed amino acid diets that would support a moderate rate of growth but not a rate equivalent to that obtained with diets containing intact protein. When it was observed that intact protein increased weight gain of animals fed amino acid diets, the growth-stimulating factor was for some time considered to be peptide in nature. For example, Woolley (2) observed that a supplement of 2% of casein improved the growth of mice fed an amino acid diet and attributed the increased growth rate to a peptide growth factor which he isolated from enzymatic digests and designated strepogenin. A few years later Maddy and Elvehjem (3) reported equivalent growth rates for mice fed whole casein or an amino acid diet. Still later, however, (1952) Maddy and Swift (4) were not able to achieve maximal weight gains in rats fed amino acid diets.

The quantity of protein required in amino acid diets for maximal growth of the rat is in fact, large. In table 1 are listed the weight gains of rats fed a complete amino acid diet, supplemented with increasing increments of casein. Twelve per cent of casein was required before weight gain was equivalent to that of rats fed a diet containing 20% casein. (The amino acids in the diet did not prevent maximal weight gain from being attained.)

From the late 1940's until the present there has been considerable controversy over the question of whether there is a specific compound in protein which increases the growth rate of animals fed an amino acid diet. There have been several reports that amino acid diets supported growth rates equivalent to these obtained with diets containing intact protein and an even greater number indicating that protein would stimulate the growth of rats fed amino acid diets. An examination of the literature indicates that maximal growth rate has, in fact, never been obtained routinely.

One question has been the importance of using only the L-isomers of amino acids as compared with adding appreciable quantities of D-amino acids to the diets.

TABLE 1

Effect of increasing increments of casein on the growth of rats receiving an amino acid diet

Diet no.	Amino acids	Casein	Weight gain [1]
	%	%	*g/14 days*
1	22 [2]	0	73±3.3
2	22	4	80±3.8
3	22	8	91±2.6
4	22	12	100±4.0
5	0.3 L-cystine	20	102±4.4

[1] Average of 5 rats ± SE.
[2] A mixture of L- and DL-amino acids formulated (5) to provide 1.5 times the requirement of each indispensable amino acid plus a mixture of dispensable amino acids to give a total of 22% amino acids in the diet.

Received for publication May 17, 1965.
[1] With technical assistance of Robert Matz. Contribution no. 687 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts. Supported in part by a grant from the National Live Stock and Meat Board, Chicago, and by NASA Grant NsG-496.

Greenstein and associates (6) were the first to prepare an all L-amino acid diet from crystalline amino acids. They were thus able to avoid the problem of the effect of the D-isomers. They obtained good growth but again the growth was not equivalent to that obtained with intact protein (7).

One of the major problems has revolved around the nutritional interrelationships among the dispensable amino acids, arginine, proline and glutamic acid, and perhaps others. If one of these amino acids is low in the diet, the quantity of the others needed for rapid growth is markedly increased (8). Hepburn et al. (9) observed an improvement in the growth of rats fed an amino acid diet when arginine was increased to 1% or more of the diets. They also observed a growth response to high levels (4 to 6%) of glutamine.

A partial solution to this problem has been reported recently by Breuer et al. (10). These investigators observed that asparagine was an important factor accounting for the response of rats fed amino acid diets to intact protein. They showed that asparagine stimulated growth of rats fed either acid-hydrolyzed protein or a protein-free amino acid diet. Their growth rates were *nearly* equivalent to that obtained with diets containing intact protein. Even though asparagine had been used frequently in amino acid diets prior to this report, its peculiar requirement for maximal growth had not been pointed out (10).

Stimulation of food intake has always been an important factor in obtaining normal growth of rats fed an amino acid diet. The efficiency of utilization of diets that did not support maximal growth has been shown to be as high as that observed for diets containing casein (11).

We decided to reinvestigate the problem with an effort to overcome the osmotic effect of the low molecular weight amino acids. We had observed a very severe depression in food intake when rats, trained to eat one meal a day, were offered an amino acid diet (12). The reduction in food intake appeared to be related to the osmotic effect of the amino acids — similar to osmotic effects of low molecular weight carbohydrates (13) — and not to a deficiency of any amino acid (5). We began by studying the effect of feeding the diet as an agar gel containing 50% of water, on the assumption that this might reduce osmotic effects in the gastrointestinal tract.

EXPERIMENTAL

Male wealing rats of either the Holtzman or Sprague-Dawley strain were fed a purified diet containing 10% amino acids and 10% casein for 2 days to allow the rats to adjust to the animal laboratory. The rats were then separated into groups (6 to 10 rats/group) whose average weight differed by less than one gram. The animal laboratory was maintained at 25° and 50% relative humidity and the rats were placed in suspended galvanized cages with wire-mesh bottoms and were given water and food ad libitum.

The proportions of the amino acid mixtures were based upon the requirements as determined by Rao et al. (14). The specific compositions used are listed at the bottom of each table or figure. Unless otherwise stated, all amino acids used were L-isomers.[2] Spot purity checks were run, and it was found that certain amino acids were contaminated with up to 1% of other amino acids. Amino acid analyses of amino acid mixtures used checks within 5% of the theoretical value for over half of the amino acids; the rest checked within 10% except for proline (30% high) which was present on the chromatogram in so small an amount that the inherent error was large. Since our objective was to obtain maximal growth and not to determine requirements, these figures were considered satisfactory.

The composition of the diet was as follows: (in per cent) corn oil, 10.0; salts,[3]

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland or General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] The salt mixture contained: (in per cent) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.0025; $Na_2SeO_3 \cdot 5H_2O$, 0.0015. The salt mix was prepared by and purchased from General Biochemicals, Inc. The salts at 5% of the diet provided in per cent of element: Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; Se, 0.0000025.

5.0; vitamin mix,[4] 0.5; choline chloride, 0.2; amino acid mixture or protein, as indicated in each table or figure; and dextrin and sucrose at a ratio of 2:1 to make 100%. Diets were made into gel form by mixing vigorously into the dry diet an equal quantity of boiling 3% agar solution. When the diet had been blended evenly, it was poured into trays with airtight covers and allowed to cool before the covers were replaced. All diets were refrigerated until used. Food cups containing the dry diets were filled every other day. The agar gel diets were fed daily. The rats were weighed daily for 5 days and at 7, 10 and 14 days after the beginning of the experiment.

RESULTS

The effect of feeding the amino acid and casein diets in the gel form is shown in figure 1. The rate of weight gain was less for rats fed the dry diets than for those fed the same diets in gel form. The difference was greater with the amino acid diet than with the casein diet. Also shown in figure 1 is the effect on weight gain of adding arginine (1% arg·HCl) to the amino acid diet. The additional arginine increased weight gain an average of 1 g/day during the 2-week period. Weight gain of the rats fed the agar gel-high arginine-amino acid diet was still considerably less than that of rats fed the casein diet.

[4] 0.5% of the vitamin mixture in the diet provided the animals with 0.44% sucrose plus the following vitamins in mg/kg diet. Thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% in mannitol), 30; ascorbic acid, 50 (added to help prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of A/g and 32,500 USP units of D_2/g in a mixture of gelatin, sugar and starch), 12.31. (The thiamine, niacinamide, folic acid, menadione and vitamin B_{12} were purchased from Nutritional Biochemicals Corporation, and the rest were purchased from Hoffmann-La Roche, Inc., Nutley, New Jersey.)

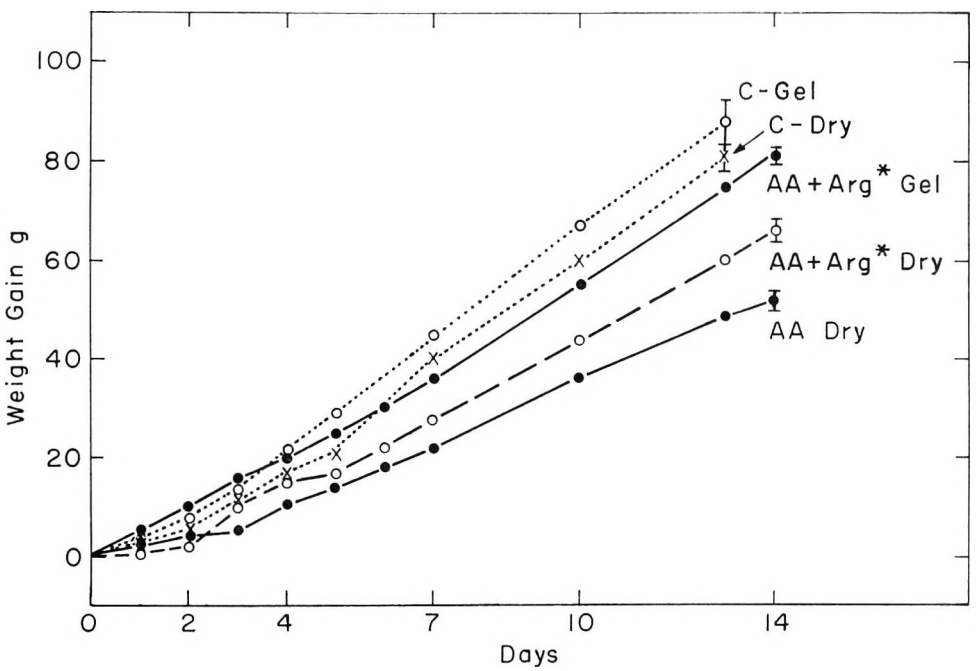

Fig. 1 Effect of additional arginine and feeding diets in agar gel form on weight gain of rats fed casein or L-amino acid diets. Each point represents the average of 6 to 8 rats. The amino acid diets contained: (in %) arg·HCl, 0.42; his·HCl, 0.46; ileu, 0.95; leu, 1.21; lys·HCl, 2.01; met, 0.88; phe, 1.27; thr, 0.88; trp, 0.19; val, 0.95; ala, 0.19; asp, 0.19; cys, 0.19; glu, 2.43; gly, 1.46; pro, 0.19; ser, 0.19; tyr, 0.19; and sodium acetate (NaAc), 1.80. Key: asterisk (*) indicates addition of amino acid mix providing: (in %) Arg·HCl, 1.00; tyr, 0.30; cys, 0.20; and NaAc, 0.39. A.A. = amino acid diet; C = casein diet.

TABLE 2

Effect of asparagine and agar gel on growth of rats fed an L-*amino acid diet*

Diet no.	Nitrogenous components				Weight gain [1]	
	Egg albumin	L-Amino acids	Asp·NH_2	Arg·HCl	Dry	Gel
	%	%	%	%	*g/14 days*	*g/14 days*
1	—	16.6 [2]	0.6	1.35	83±2.5	100±3.2
2	—	16.6	—	1.35	66±4.0	80±3.4
3	—	16.6	0.6	0.35	—	69±4.0
4	18.4	—	—	—	68±2.6	83±3.8

[1] Average of 6 rats ± SE (exp. 4).
[2] The amino acid mix provided in the diet: (in %) his·HCl, 0.40; ileu, 0.80; leu, 1.08; lys·HCl, 1.76; met, 0.80; phe, 1.14; thr, 0.80; trp, 0.17; val, 0.80; ala, 0.34; asp, 0.34; glu, 3.41; gly, 2.28; pro, 0.34; cys, 0.34; ser, 0.34; tyr, 0.34; and NaAc, 1.09.

The effect of adding asparagine to our best previous amino acid diet is shown in table 2. For the first time in our laboratory we obtained a weight gain of rats fed the amino acid diet which was as high or higher than that obtained with a diet containing protein. With the amino acid diet used both asparagine and high arginine were required. Also, weight gain was less with each diet when the diet was fed dry.

In table 3 are listed the results of an experiment in which the best amino acid diet and a casein control diet were compared. As in the previous experiment, the rats fed the amino acid-agar gel diet gained somewhat more weight than rats fed the casein-agar gel diet ($P > 0.1$). When nine of the L-amino acids were replaced by DL-amino acids (see footnote to table 3), the weight gain was reduced from 97 to 81 g. This difference was statistically significant ($P < 0.01$). Also, casein was added to the amino acid diet to ascertain whether this would stimulate growth. As shown in table 3, no stimulation occurred.

TABLE 3

Effect of casein and DL-*amino acids on growth of rats fed an* L-*amino acid diet*

Diet no.	Component	Weight gain [1]
		g/14 days
1	19.1% Amino acid diet [2] +0.6% asp·NH_2 and 1.34% arg·HCl	97±3.4
2	L- replaced by DL-amino acids [3]	81±3.3
3	Diet 1+7.6% casein	96±6.0
4	19% Casein+0.3% met	91±1.9

[1] Average ± SE for at least 10 rats.
[2] The amino acid mix provided in the diet: (in %) his, 0.45; ileu, 0.96; leu, 1.24; lys, 2.02; met, 0.91; phe, 1.30; thr, 0.91; trp, 0.19; val, 0.98; ala, 0.39; asp, 0.39; cys, 0.39; glu, 3.90; gly, 2.61; pro, 0.39; ser, 0.39; tyr, 0.39; and NaAc, 1.25.
[3] Ala, asp, ileu, met, phe, ser, thr, trp, and val added in DL-form, all at twice the concentration except as follows: met, 1.33 ×; phe, 1.33 ×; and trp, 1.5 ×.

In the final experiment (table 4) the amino acid mixture was formulated such that arginine·hydrochloride was reduced from 1.34 to 1.08% of the diet. This reduction resulted in a lower weight gain (87 g/2 weeks) of the rats fed the amino acid diet as compared with the casein control (98 g/2 weeks). Again rats fed the diets in gel form (table 4) gained more weight than those fed dry diets, but glutamine substituted for asparagine on an equal weight basis did not stimulate growth. The difference between the weight gains of the groups fed asparagine and glutamine was significant ($P < 0.01$). Also, when alanine and glutamic acid replaced serine, glycine, aspartic acid, proline, cystine and tyrosine on an equal nitrogen basis, weight gain was reduced from 87 g/2 weeks to 73 g/2 weeks. This difference was significant ($P < 0.01$). When the fat content of the diet was increased to 50% (calorie/N remaining constant), the weight gain was reduced from 87 g/2 weeks to 76 g/2 weeks.

DISCUSSION

As indicated in the results, the rate of weight gain of rats fed each agar gel diet was greater than that of rats fed the comparable dry diet. The difference was somewhat greater for groups fed the amino acid diet than for those fed the casein diet (fig. 1). Water fed in purified diets has been shown by Keane et al. (15) to in-

TABLE 4

Effect of asparagine and glutamine on the growth of rats fed an L-amino acid diet

Diet no.	Amino acid source	Asp·NH_2	Glu·NH_2	Weight gain [1]	
				Dry	Gel
	%	%	%	*g/14 days*	*g/14 days*
1	16.0 Casein + 0.3 met			90 ± 2.2	98 ± 2.2
2	19.1 Amino acids [2]	0.6	—	78 ± 1.5	87 ± 2.3
3	19.1 Amino acids	—	0.6		77 ± 1.6
4	19.1 Amino acids	0.3	0.3		88 ± 2.3
5	19.1 Special mix [3]	0.6	—		73 ± 2.4
6	29.0 Amino acids [4]	0.9	—		76 ± 2.1

[1] Average of 9 to 10 rats ± SE.

[2] The amino acid mix provided in the diet: (in %) arg·HCl, 1.08; his·HCl, 0.42; ileu, 0.90; leu, 1.14; lys·HCl, 1.86; met, 0.84; phe, 1.20; thr, 0.84; trp, 0.18; val, 0.90; ala, 0.36; asp, 0.36; glu, 3.61; gly, 2.41; pro, 0.36; cys, 0.36; ser, 0.36; tyr, 0.36; and NaAc, 1.57.

[3] Ser, gly, asp, pro, cys, and tyr were deleted and equal quantities of ala and glu were added to provide equal nitrogen intakes.

[4] A 50% fat diet (40% lard and 10% corn oil). Amino acids increased proportionally because of additional fat.

crease weight gain and protein efficiency ratio. These authors indicated that the increased weight gain could not be explained solely on the basis of increased food intake. In the present experiments only group food intake measurements were taken. Rats fed the best amino acid diet (table 2, group 1) consumed 11.0 g of the dry diet/day but 13.0 g of the agar gel diet/day (dry weight). Likewise, when diet 2 (table 2) was fed in gel form, food intake increased from 8.8 to 11.2 g/rat/day. These results indicate that the increased weight gain was due primarily to increased food intake.

When rats are fed daily in a single 2-hour period, they will eat 10 to 14 g of a starch-casein diet (5, 12) and remain healthy and of normal appearance. However, rats fed an amino acid diet for only 2 hours a day ate only 3 to 5 g and several rats developed stomach ulcers and died (12). Czajka et al.[5] have observed that infant rats are much more sensitive to osmotic effects of either carbohydrates or amino acids than older rats, indicating that the osmotic effects may be most important early in life. A depression in the food intake of rats fed once a day was also observed when glucose or sucrose replaced starch, but it was not as severe as when amino acids replaced casein (16, 12). In the present experiments feeding the amino acid diets in gel form increased food intake and weight gain. This appears to be a more marked effect than that reported for the addition of water to purified diets (15). We would suggest that this is due to the gel preventing the severe osmotic effect, namely, preventing water from being drawn into the stomach and activating the stretch receptors and thereby reducing food intake. This effect may be the cause for the slightly lower weight gain observed when amino acid diets have been compared with protein diets (9, 10, 17). It appears that this is not the only factor, however, since amino acids are not inhibitory when fed with protein (table 1). The beneficial effects of feeding diets in agar gel form appear to be greatest when the diet is inadequate in some respect.

Weight gain increased when asparagine was added to either the dry or gel amino acid diet. Breuer et al. (10) reported that a supplement of asparagine increased the weight gain of rats fed amino acid diets. Their best weight gain for rats fed an amino acid diet was *nearly* as high as that of a control group fed casein. In retrospect it should be noted that some of the amino acid mixtures which had supported the best weight gain in the past (namely, Maddy and Elvehjem (3) and Sauberlich (17)) contained asparagine. Its necessity for most rapid weight gain, however, had not been emphasized. Hepburn and Bradley (9) have shown recently that glutamine fed at 5 to 6% of the diet increased the weight gain of rats fed an amino acid diet to nearly that observed when protein was fed. In the present study when glu-

[5] Czajka, D. M., A. M. Browning, H. A. Dymsza and S. A. Miller 1964 Dietary osmolarity and survival of neonatal rats. Federation Proc., 23: 503 (abstract).

tamine was tested at the same level as asparagine (0.6%), there was no stimulation of weight gain (see table 4).

Over 1% of arginine was required in our studies to obtain maximal weight gain (see tables 2–4). Hepburn and Bradley (9) have shown that at least 4% glutamic acid and at least 0.9% arginine are required for maximal growth. Our unusually high requirement for arginine may be the result of the marginal level of glutamic acid added (3.6%). Womack and Rose (8) have shown a relationship among arginine, proline and glutamic acid in rapidly growing rats fed amino acid diets. When the amino acid composition of the casein and amino acid diets are compared (table 5), the greatest discrepancy is noted among the dispensable amino acids, in particular among arginine, proline and glycine with unknown discrepancies in the amides. Considerably more work needs to be carried out to determine the minimal amounts of each of the above amino acids and asparagine and glutamine required for maximal growth of rats fed a diet that contains an excess of all other dispensable amino acids.

TABLE 5

Amino acid composition

	Amino acid diet	Casein diet
	%	%
L-Arginine	1.12 [1]	0.49
L-Histidine	0.33 [1]	0.49
L-Isoleucine	0.82	0.64
L-Leucine	1.11	1.19
L-Lysine	1.44 [1]	1.04
L-Methionine	0.82	0.60 [2]
L-Phenylalanine	1.16	0.67
L-Threonine	0.82	0.53
L-Tryptophan	0.174	0.15
L-Valine	0.82	0.88
L-Alanine	0.35	0.37
L-Aspartic acid	0.35	0.95
L-Glutamic acid	3.50	2.94
Glycine	2.33	0.25
L-Proline	0.35	1.58
L-Cystine	0.35	0.07
L-Serine	0.35	0.68
L-Tyrosine	0.35	0.74
L-Asparagine	0.60	?
L-Glutamine	—	?

[1] Reported here as the free base but added to the diets as the hydrochlorides.
[2] 0.3% methionine added to casein.

When about half of the L-amino acids were replaced by DL-amino acids, the weight gain was reduced (table 3). A reduction in weight gain when certain D-amino acids are added has been noted earlier (18). In the present experiments asparagine and high arginine did not prevent the depression.

Group 3 in table 3 was included to find out whether maximal weight gain had been reached and whether intact protein would increase weight gain of rats fed the amino acid diet. As shown, no increase was observed, indicating that maximal rate of weight gain had been obtained. Confirming the work of Breuer et al. (10) group 5 (table 4), receiving only the indispensable amino acids and alanine, glutamic acid and asparagine, gained only 73 g/14 days as compared with 87 g/14 days for the group receiving also serine, glycine, aspartic acid, proline, cystine and tyrosine. The lower weight gain for group 2 in this experiment was apparently the result of the lower quantity of arginine present in the amino acid mix (1.08% arg·HCl as compared with 1.34% arg·HCl in tables 2 and 3). Group 6 was included to learn whether a high fat diet containing only amino acids as a source of nitrogen would support a rapid rate of growth. The weight gain of 76 g/14 days indicates that no special problem of the type described earlier by Rao and co-workers [6] should be encountered in using this amino acid mixture in high fat diets. Therriault[7] used the amino acid diet shown in table 5 in acute and chronic cold adaptation studies and observed that weight gain and mortality were the same for rats fed this diet as for those fed a diet containing casein. This is a further indication of the adequacy of the amino acid diet.

A question may be raised as to whether a reproduction study over several generations would not be the best test for the adequacy of an amino acid diet. Schultze (19, 20) has carried rats fed amino acid diets through 4 generations "without evidence of gradual deterioration of the reproductive or lactation performance;" yet, "none of the rations supported maximum

[6] Rao, P. B R., and B. C. Johnson 1958 Amino acid mixture as dietary source of nitrogen for rat growth. Federation Proc., *17*: 489 (abstract).
[7] Unpublished results, D. Therriault.

post-weaning weight gains." It would therefore be unexpected if a diet that supports rapid postweaning weight gain would not support reproduction and lactation.

LITERATURE CITED

1. Rose, W. C., M. J. Oesterling and M. Womack 1948 Comparative growth on diets containing ten and nineteen amino acids, with further observations upon the role of glutamic and aspartic acids. J. Biol. Chem., *176:* 753.
2. Woolley, D. W. 1945 Observations on the growth-stimulating action of certain proteins added to protein-free diets compounded with amino acids. J. Biol. Chem., *159:* 753.
3. Maddy, K. H., and C. A. Elvehjem 1949 Studies on growth of mice fed rations containing free amino acids. J. Biol. Chem., *177:* 577.
4. Maddy, K. H., and R. W. Swift 1952 Growth and metabolism studies with rats fed rations containing purified amino acids. J. Nutrition, 47: 243.
5. Chen, M-L., Q. R. Rogers and A. E. Harper 1962 Observations on protein digestion in vivo. IV. Further observations on the gastrointestinal contents of rats fed different dietary proteins. J. Nutrition, 76: 235.
6. Greenstein, J. P., S. M. Birnbaum and M. Winitz 1956 A water-soluble synthetic diet. Arch. Biochem. Biophys., 63: 266.
7. Greenstein, J. P., and M. Winitz 1961 The Chemistry of the Amino Acids, vol. 1, chapt. 3. John Wiley and Sons, New York.
8. Womack, M., and W. C. Rose 1947 The role of proline, hydroxypyroline and glutamic acid in growth. J. Biol. Chem., *171:* 37.
9. Hepburn, F. N., and W. B. Bradley 1964 The glutamic acid and arginine requirements for high growth rate of rats fed amino acid diets. J. Nutrition, 84: 305.
10. Breuer, L. H., Jr., W. G. Pond, R. G. Warner and J. K. Loosli 1964 The role of dispensable amino acids in the nutrition of the rat. J. Nutrition, 82: 499.
11. Stucki, W. P., and A. E. Harper 1962 Effects of altering the ratio of indispensable to dispensable amino acids in diets for rats. J. Nutrition, 78: 278.
12. Rogers, Q. R., M-L. Chen, C. Peraino and A. E. Harper 1960 Observations on protein digestion in vivo. III. Recovery of nitrogen from the stomach and small intestine at intervals after feeding diets containing different proteins. J. Nutrition, 72: 331.
13. Harper, A. E., and H. E. Spivey 1958 Relationship between food intake and osmotic effect of dietary carbohydrate. Am. J. Physiol., *193:* 483.
14. Rao, P. B., V. C. Metta and B. C. Johnson 1959 The amino acid composition and the nutritive value of proteins. I. Essential amino acid requirements of the growing rat. J. Nutrition, 69: 387.
15. Keane, K. W., C. J. Smutko, C. H. Krieger and A. E. Denton 1961 Water addition to purified diets. II. Effects of water and carbohydrate on protein efficiency ratio in rats. J. Nutrition, *81:* 87.
16. Peraino, C., Q. R. Rogers, M. Yoshida, M-L. Chen and A. E. Harper 1959 Observations on protein digestion in vivo. II. Dietary factors affecting the rate of disappearance of casein from the gastrointestinal tract. Canad. J. Biochem. Physiol., 37: 1475.
17. Sauberlich, H. 1961 Growth of rats fed protein-free diets supplemented with purified amino acid mixtures. J. Nutrition, 74: 298.
18. Berg, C. P. 1953 Physiology of the D-amino acids. Physiol. Rev., 33: 145.
19. Schultze, M. O. 1956 Reproduction of rats fed protein-free amino acid rations. J. Nutrition, 60: 35.
20. Schultze, M. O. 1957 Nutrition of rats with compounds of known chemical structure. J. Nutrition, 61: 585.

Histopathology of Mice Fed Irradiated Foods [1]

SAMUEL W. THOMPSON, II,[2] RONALD D. HUNT,[3] JOHN FERRELL,[4] EDWARD D. JENKINS[5] AND HARRY MONSEN[6]

Pathology Division, U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado and Department of Anatomy, College of Medicine, University of Illinois, Chicago, Illinois

ABSTRACT A total in excess of 4000 mice of the Cb and Strong A strains were fed a composite diet composed of pork loin, whole boned chicken, evaporated milk, blanched carrots and white potatoes, which was 100% irradiated, the same diet non-irradiated, a similar diet in which only one component was irradiated, or a commercial pellet diet. Representative groups of mice were killed at periodic intervals after receiving the diet for from zero to 600 days, and tissue sections were prepared from specimens of all organs and tissues which were fixed in formalin at the time of necropsy. The fixed heart of each mouse was serially sectioned and mounted on film strips. All tissue sections and film strip mounts were examined by bright field microscopy. Cardiac auricular dilation and rupture reported to have been produced in these strains of mice, by the irradiated diets used, were not observed in any mouse in any diet group of this study. Extensive statistical evaluation of all lesions observed in the various phases of this study indicated that no lesion occured with significantly increased frequency in the irradiated diet groups.

In 1960, Monsen (1) reported on the occurrence of a lesion which affected the left auricular appendage of the hearts of some mice of the Cb (BALB/C w/o Milk Factor) and Strong A strains. A similar lesion has been described by Fry et al.[7] and Meier and Hoag (2), to occur in old inactive breeders of the BALB/C strain of mice. Monsen's preliminary report implicated the irradiated foods which were fed and provoked considerable comment in the lay press in the latter part of 1959.

The study conducted at the United States Army Medical Research and Nutrition Laboratory,[8] as reported here, was designed to evaluate further the reproducibility, pathogenesis and role of irradiated food as a cause of this particular lesion of the left auricular appendage of the heart. Although this is essentially a report of negative data it is felt that it should be of broad general interest due to its magnitude and experimental design.

METHODS

All of the foods used in this study were procured from the same geographic area and source as those used originally by Monsen. All items were procured in one time period of 2 weeks, with the exception of potatoes, and contract specifications were written to insure that the production methods were the same as those used in the preparation of the foods used by Monsen. United States Army Veterinary Corps personnel supervised all phases of this procurement operation to insure strict compliance with the contract specifications.

Items for the control diets, with the exception of potatoes, were preserved by quick-freezing. They were subsequently transported from the procurement source to Denver, Colorado, and were subsequently stored at −23° until used. Items for the irradiated diets were initially preserved by quick-freezing, with the exception of potatoes, and were transported to the irradiation site at −23°. The irradiation site and reactor were the same as used

Received for publication February 10, 1965.

[1] Supported by project no. 3A012501A803, U.S. Army Medical Research and Development Command, and Army Medical Research Contract no. D-49-007-MD-794.

[2] Present address: Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

[3] Present address: Animal Research Center, Harvard Medical School, Boston.

[4] Present address: U.S. Army Medical Research Unit, Fort Detrick, Maryland.

[5] Present address: U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado.

[6] Present address: College of Medicine, University of Illinois, Chicago.

[7] Fry, J. M., K. Hamilton and H. Lisco 1960 An unusual spontaneous cardiac lesion of unknown etiology in mice. Federation Proc., *19:* 109 (abstract).

[8] The principles of laboratory animal care, as promulgated by the National Society for Medical Research, were observed.

for the irradiation of foods used by Monsen. All foods scheduled for irradiation were processed through the reactor during a single time period of 30 days. To accomplish this, it was necessary to run three 8-hour shifts per day at the reactor site. With the exception of potatoes, all irradiated foods were exposed to 5.58 megarads. Subsequent to irradiation, the irradiated foods, with the exception of potatoes, were transported from the irradiation site to Denver, Colorado, and were subsequently stored until used at 24°.

The white potatoes used in this study were procured in the same geographic area as those used by Monsen, but it was necessary to procure them as available during the several years of this study. Potatoes irradiated at 10,000 to 12,000 rep and non-irradiated, were stored at 5° to 10°, in transit, and at our laboratory.

Phase 1. Breeding of Cb and Strong A inbred strains of mice. The breeding stock was obtained from Monsen. Upon receipt, all breeding stock was quarantined for 2 weeks, and during this period veterinarians maintained daily surveillance. At the end of the quarantine period, the surviving healthy mice were divided into breeding pairs. Monogamy was practiced in the breeding program, and each animal was identified with an ear tag. Breeders were mated at 2.5 months of age and maintained for as long as they would produce satisfactory litters. When satisfactory litters were not being produced by breeding pairs, the male and female were caged separately and allowed to live until the completion of the experimental phase.

When for reasons of spontaneous neoplasms, or other disease, the breeders died or were killed, complete necrospy and histopathological examinations were performed as outlined for experimental mice in phase 2. At the termination of the experimental diet studies, all live breeding animals were killed, necropsied, and examined histopathologically.

Phase 2. Repetition of Monsen's original protocol. Monsen's original protocol was repeated with such modifications as were required to conform to existing facilities.

In this phase, a total of 2412 mice were fed various diets. All mice were identified with an individual numbered ear tag and were housed in groups of 6 in stainless steel cages with wire-mesh floors in air conditioned, positive pressure quarters (24° ± 1°) with 100% turnover of air. Both the Cb and Strong A strains of mice (1206 of each) were used in this phase. Each group of 1206 mice was subdivided into 3 diet groups (201 males, 201 females each): irradiated composite diet, non-irradiated composite diet, and laboratory chow.[9] Each group of animals of a given strain, fed each of the diets, was composed of replicate units, separated by sex, composed of paired littermates. In this fashion, littermates of both sexes were distributed in equal numbers through each diet group. The animals were fed the specific diets starting at 28 to 30 days of age.

These mice were maintained with the diet and were killed in accordance with a fixed schedule. This schedule was developed by our statistician on a random distribution basis, and each animal and its prescribed date for termination in the study was established at the time it started to receive the diet. One hundred and ninety-five males and 195 females of each strain of mice were killed after 200 days on the experiment, and 102 males and 102 females of each strain were killed on days 300, 400, 500, and 600 of the experiment. Throughout the 600 days of the experiment, the animals were under constant daily observation of a veterinarian. Clinically sick animals were killed by CO_2 asphyxiation when, in the opinion of the attending veterinarian, their illness was of such nature that survival overnight or through the weekend was unlikely.

The irradiated and non-irradiated diets used in this phase were composed of the following foods on a wet-weight basis: pork loin (8.8%); boned whole chicken (6.0%); evaporated milk (19.3%); Idaho, brown skin, white potatoes (19.3%), and blanched carrots (43.0%). Each food was pressure-cooked separately at 120° for 20 to 30 minutes before being incorporated into the composite diet. The meat products were ground in a Hobart commercial food grinder after being cooked. Each food item was then separately homoge-

[9] Laboratory Chow (1957 formula), Ralston Purina Company, St. Louis.

nized in a Waring blender. The homogenized components were then blended into a composite diet using a Hobart 56.78-liter food blender. Sufficient composite diets for a 5-day period were made at one time and stored in sterile, fiber, disposable quart containers or in individual disposable aluminum foil feeding dishes. Diet which was stored for feeding on days 2 to 5 was held at −23°. Periodic analyses were made on the diets for protein, carbohydrate, fat, and ash content.

Two and one-half grams of a vitamin mixture [10] and 2 g of a salt mixture [11] were added to 100 g (dry weight) of each composite diet. This supplement was added to the diet daily and only to the amount of the diet required to fill the food dishes.

In the feeding of mice on test, fresh diet, in disposable aluminum foil dishes, was supplied every day to all animals receiving either composite diet. No record of food consumption was kept since the food and water were furnished ad libitum. Food was provided for each animal on the basis of 13.5 g/animal/day or 81 g/feeding dish/cage of 6 animals. Advantage was taken of the nocturnal feeding habits of mice. They were fed in the late afternoon, and the food dishes were left in the cages until the following morning when they were removed and discarded. One hundred mice of each strain (50 mice of each sex) fed each of the 3 diets used in this phase, were weighed twice a week for the first 60 days of life.

Phase 3. *Pathogenesis.* The purpose of this phase, which was carried out concurrently with phase 2, was to determine whether there were any early manifestations of the cardiac lesion which were detectable histopathologically and possibly grossly. It was designed to determine whether the lesion was present prior to the mice being placed on test, and if not, when and how the lesion manifested itself prior to becoming fully developed.

Only the Cb strain of mice was used in this phase. This strain was considered to be more suitable for this purpose than the Strong A strain since the incidence of cardiac lesions, as reported by Monsen, was much higher in mice of the Cb strain.

At the time the mice were selected to be fed the various diets described in phase 2, littermates, in excess of those required for phase 2, were segregated into 2 groups according to sex. A total of 108 mice was placed on test in small increments as dictated by availability. These animals were identified by ear tags.

All mice used in this phase were housed, fed, and cared for in a fashion similar to those of phase 2 with the exception that only the irradiated, vitamin-supplemented, diet was used. At zero days and every 25 days thereafter up to and including 100 days on test, 6 females and 6 males were killed by CO_2 asphyxiation. Beginning at 125 days and every 25 days thereafter, up to and including 300 days on test, 3 females and 3 males were killed.

Phase 4. *Etiology.* This phase was designed to ascertain the cause of the heart lesions originally observed by Monsen. Unless otherwise indicated all animal management practices were as described for phase 2.

Study 1. Since it was possible that irradiated foods were the cause of the heart lesion, the possible role of the individual components of the composite diet were investigated. This was accomplished by feeding the composite diet in which only one of the components was irradiated. The composition of the basic composite diet was as described in phase 2.

A total of 1600 mice of the Cb strain was used in this study. Littermate groups of 5 mice each were used. These were separated into 5 groups, of 320 mice each. Each group received a different basic composite diet in which the following components were irradiated: group 1, pork; group 2, chicken; group 3, evaporated milk; group 4, white potatoes; and group 5, blanched carrots.

Each of the basic diet groups was subdivided into subgroups of 80 mice each. Each of these subgroups received the basic

---

[10] Vitamin mixture contained: (mg/kg of diet) vitamin A concentrate (200,000 units/g), 45.0; vitamin D conc (400,000 units/g), 2.5; α-tocopherol, 50.0; ascorbic acid, 450.0; inositol, 50.0; choline chloride, 750.0; menadione, 22.5; *p*-aminobenzoic acid, 50.0; niacin, 45.0; riboflavin, 10.0; pyridoxine·HCl, 10.0; thiamine·HCl, 10.0; Ca pantothenate, 30.0; biotin, 0.2; folic acid, 0.9; and vitamin B_{12}, 0.0135 (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation, Cleveland).

[11] Hawk-Oser salt mixture no. 3; Hawk, P. B., and B. L. Oser 1954 Practical Physiological Chemistry, ed. 13. McGraw-Hill Book Company, Blakiston Division, New York, p. 1374; obtained from Nutritional Biochemicals Corporation.

diet, but the manner in which it was prepared varied as follows: (a) all components cooked and the diet supplemented with vitamins; (b) all components cooked and the diet not supplemented with vitamins; (c) the irradiated component not cooked and the diet supplemented with vitamins; and (d) the irradiated component not cooked and the diet not supplemented with vitamins.

All mice were maintained with the various diets and were killed in accordance with a fixed schedule. This schedule was developed by our statistician on a random distribution basis, and each animal and its prescribed date for termination in the study was established at the time it was given the diet. Twenty mice from each subgroup of each of the 5 major diet groups, for a total of 400 mice, were killed by CO_2 asphyxiation after 100, 200, 400, and 600 days on test.

Study 2. From the results of Monsen's original studies, there was evidence to suggest that irradiated evaporated milk could be involved in the production of the cardiac lesion. To further evaluate the correlation between irradiated milk and the occurrence of heart lesions, 72 mice (36 males and 36 females) of the Cb strain were fed the milk diet. Paired littermates were used. This irradiated milk was obtained from Monsen's original stocks. It was kept at 24° until used. Prior to feeding it was fortified with the vitamin and mineral mixtures as described for phase 2. The mice in this study were divided into 4 diet treatment groups, composed of 9 males and 9 females each, as follows: 1) uncooked irradiated milk, 2) cooked irradiated milk, 3) uncooked non-irradiated milk, and 4) cooked non-irradiated milk. All mice were permitted to live out their life span.

Study 3. To evaluate the possible effect of variations in the storage temperature of irradiated evaporated milk, as these might affect the occurrence of cardiac lesions, 42 males and 42 females (paired littermates) of the Cb strain were fed an irradiated diet similar to that used in phase 2, with the exception that the evaporated milk component was stored at −23° rather than at 24°. These mice were allowed to live out their life span or 600 days on test if they did not die prior to that time.

Study 4. To investigate the possible role of sanitation, as related to feeding methods, in the etiology of the heart lesions, 36 males and 36 females (paired littermates) of the Cb strain of mice were fed from bean pots rather than disposable containers. One-half of the these animals were fed the irradiated composite diet, and one-half received the non-irradiated diet described for phase 2. These animals were fed in the afternoon, but the containers were not removed until the following afternoon. All of these mice were permitted to live out their life span or up to 600 days on test.

Gross pathology. All mice used in phases 1 through 4 that died or were killed during the test period were necropsied. Each carcass was weighed prior to being necropsied, and the heart, spleen, liver and kidneys were separately weighed at the time of necropsy. All grossly recognizable lesions were recorded on necropsy forms especially prepared for this project. The left side of the heart of each mouse was photographed prior to fixation.

Gross specimens of the following organs and tissues were taken at the time of necropsy and were fixed in neutral buffered 10% formalin (3): salivary gland, cervical lymph node, thyroid and parathyroid glands, heart and great vessels, mediastinal lymph node, pancreas, stomach, 3 levels of the small intestine, large intestine, mesenteric lymph nodes, lung, spleen, liver, gallbladder, muscle, bone marrow, adrenals, kidney, bladder, gonads, accessory sex organs and brain.

Histopathology. Tissues obtained at necropsy were processed and paraffin-embedded according to routine schedules used in the U. S. Army Medical Research and Nutrition Laboratory (USAMRNL) (3) and stored as paraffin blocks and wet tissues. With the exception of the heart, paraffin-embedded tissue sections were cut from all embedded specimens on a rotary microtome at a setting of 8 μ, were affixed to glass microscope slides, and were stained with Harris' hematoxylin and eosin (3). A total in excess of 125,000 tissue sections was prepared from this material. These were reviewed by a pathologist and further histopathological procedures were carried out as required.

The entire heart from each mouse was processed separately and embedded in toto and was then serially sectioned with a rotary microtome set at 5 μ section thickness. The ribbons of serial sections were mounted on 35-mm film stock, according to Pickett and Sommer's technique (3), and stained with Harris' hematoxylin and eosin. Every fortieth tissue section of each heart, representing approximately 200 μ intervals, was affixed to a glass microscope slide. A total in excess of 800,000 tissue sections of the heart specimens was prepared and examined microscopically by our staff pathologists.

Records. A detailed records system was designed prior to commencing any phase of this study and was rigidly adhered to and constantly inspected and reviewed throughout the course of the study.

For each mouse, when it was placed on experiment and given an ear tag number, an individual mouse identification card was placed on file. On this card the ear tag number, sex, sire, dam, birth date, date on experiment, phase, diet and necropsy date were recorded.

On the day when a particular mouse was to be killed and necropsied, our records clerk assigned the animal a MRNL pathology accession number, entered this number on the identification card prior to filing it, and prepared a necropsy work sheet for the pathologist. The latter form was placed in an individual case folder which was prepared for each accessioned case. Following the necropsy, a completed necropsy form, identified by MRNL pathology accession number, was prepared and filed in the case folder prior to further processing of the selected specimens by our laboratory staff.

Upon completion of the histopathological examination of the tissue sections prepared for each case, a complete histopathology report and check list identified by MRNL pathology accession number, was prepared and filed in the appropriate case folder. At this time the pathologist also prepared and punched a pathology master code card, and supplemental cards if required, according to the coding system of Thompson et al. (4). These cards were sent to our statistician who punched in the information related to the diet and experimental treatment which the mouse had received, by reference to the previously filed mouse identification card. At this time the statistician completed the genetics record code card for each such case. This latter card carried face entries and marginal punched code which identified each mouse by sire and dam, litter number, dates of birth and death, sex, days on experiment, and principal types of lesions noted on the pathology master code card. It was initiated for each animal at the time it was placed on experiment and was maintained throughout the experiment in our statistician's office. It allowed for correlation of the more frequent lesions observed between related animals (sire, dam, littermates) and between unrelated animals.

Our pathologists and laboratory staff did not have access to the mouse identification card nor the genetics record card. They identified the specimens from each mouse only by its MRNL pathology accession number, which had no relation to the experimental treatment the animal received. Therefore, our technicians were not aware of the actual identity of any particular mouse, and our pathologists read more than 925,000 tissue sections "blind."

The data entered in the pathology master code cards, supplemental code cards and genetic record code cards were subjected to extensive statistical analyses and evaluation. However, these analyses were not commenced until the last case had been examined by our pathologists, and all case folders were completed and returned to the central files.

RESULTS AND DISCUSSION

The averages of 8 separate analyses of the proximate composition, expressed as a percentage of dry weight, of the irradiated and *non-irradiated* composite diets used in phase 2 and 3 are shown in table 1.

A variety of histopathological lesions were noted in a sizeable number of the mice used in this study. The morphology and distribution of these lesions have been described elsewhere (5). We shall limit our consideration to the statistical analyses of the distribution and incidence rates of these lesions.

Phase 2. Growth data were accumulated on 300 mice of each strain (50 mice

TABLE 1

Proximate composition of the composite diets used in phases 2 and 3

Diet	Protein	Fat	Carbohydrate	Ash
		% moisture-free wt		
	Phase 2			
Irradiated	23.77	21.44	44.57	10.22
Non-irradiated	24.56	21.68	43.08	10.68
	Phase 3			
1	19.62	20.55	48.98	10.85
2	19.10	21.69	48.53	10.68
3	21.27	20.76	46.88	11.09
4	20.92	18.94	49.70	10.44
5	22.23	19.51	47.80	10.46

of each sex on each of 3 diets) for the first 60 days of life. At 20 days of age, at the time that the feeding of the experimental diets was commenced, all of the mice weighed between 7.5 and 8.5 g and the variance in body weight at this time was an individual characteristic rather than one related to sex or strain characteristics. During the ensuing 40 days that growth data were collected no essential differences in growth rate were noted between the 2 strains of mice or between the groups on the 3 different diets. During the period of 20 to 38 days of age the male mice of each strain gained 11 $\pm$0.5 g for an average of 0.63 g daily gain in weight. The females of each strain gained 8.5 $\pm$ 0.5 g for an average of 0.44 g daily gain in weight, during the same period of time. From the thirty-eighth to the sixtieth day of age the males of each strain gained 4.5 g for an average of 0.21 g per day, and attained a total body weight of 23.5 g. During this same period the females of each strain gained 3.5 $\pm$ 0.5 g, for an average of 0.17 g daily gain in weight, and attained a total body weight of 19.5 g.

Of the 1203 mice of each strain originally used in this phase, data were available for analysis from 1086 Cb and 1033 Strong A mice. A variable number of mice were lost from each diet group throughout the study as a result of cannibalism and advanced autolysis. The mice upon which the analyses were performed were divided as follows: irradiated diet, 161 male and 193 female Cb strain and 171 male and 183 female Strong A strain; non-irradiated diet, 160 male and 186 female Cb strain and 175 male and 189 female Strong A strain; and pellet diet, 175 male and 211 female Cb strain and 149 male and 166 female Strong A strain.

When comparing the overall incidence of a lesion between diet groups, it became important to also compare the distribution of animals within age groups, as many of the lesions we observed increased in incidence with advancing age. There were no significant differences in population distribution, by sex, between the various diet and age groups. On the basis of this uniform distribution, statistical analysis of each lesion was performed using the total of each lesion per diet group. In the majority of cases, if the lesions were compared by age group, the number of lesions per group would have been diluted to the extent that accurate statistical analysis could not have been made.

Statistical evaluation employing the Chi-square method of analysis was made comparing the incidence of lesions in the irradiated diet group with both the non-irradiated and pellet control groups. A valid probability level of 0.05 was used. In many instances there were too few lesions per group (less than 5) to permit accurate statistical analysis.

The most frequently observed non-neoplastic lesions were myocarditis, calcification of the myocardium and epicardium, necrosis of the caudate lobe of the liver, hepatitis, urolithiasis, adrenal cortical hyperplasia, reticuloendothelial hyperplasia of the lymph nodes, lymphocytic infiltration of the kidney and urinary bladder, calcification of the brain, testicular atrophy, epididymitis and nematodiasis. With the exception of the last 3 lesions, no significantly higher incidence of any of these lesions occurred in any diet group,

sex, or strain of mice, used in this phase. We observed 5 instances of testicular atrophy in mice of the Cb strain and an equal number of instances in mice of the Strong A strain. Although there was an apparent significantly higher incidence (4:1) of this lesion in the Cb strain of mice fed the irradiated diet, the total number of such lesions observed in the various diet groups was not sufficient to allow accurate comparison or to implicate irradiated food in the production of testicular atrophy. Chronic epididymitis occurred only in mice of the Cb strain, and did not occur with increased frequency in the irradiated diet group as compared with the non-irradiated diet group. However, it occurred more frequently in both these diet groups than in the pellet diet control group.

Adult nematodes occurred in the large intestine of mice of all diet groups. Although there was a significantly higher incidence of parasitism in the irradiated diet group, as compared with the non-irradiated diet group, the incidence in the pellet diet control group was significantly higher than in the irradiated diet group. These differences are probably related to the composition of the diet rather than to an effect of the irradiated food.

Other types of non-neoplastic lesions were sporadically observed throughout all diet groups, in addition to those just described. However, their number was too small to permit statistical analysis.

Lung tumors were the most frequently noted neoplasms in either strain of mice used in this phase. However, there was no significant difference in the incidence of lung tumors between the irradiated diet and control diet groups. In addition to lung tumors, a variety of other neoplasms were observed. In no instance was there a significantly higher incidence of any tumor in the irradiated diet group as compared with the control diet groups. However, in most instances, the number of neoplasms observed, of any particular type, was too few to permit accurate analysis. In some instances, single tumors were observed in the irradiated diet group and none in the control group (mast cell tumor, metastatic carcinoma, sebaceous gland adenoma, and ependynoma). It is felt that irradiated food should not be implicated on the basis of the singular occurrence of a lesion. In other instances, single or multiple tumors occurred in control animals with no corresponding tumors in the irradiated diet group.

From the data obtained in phase 2, irradiated food could not be implicated in the production of any of the lesions observed. However, in a few instances the number of lesions was too small to permit statistical analysis.

The mice fed the composite irradiated diet in phase 2 were apparently as healthy as the mice fed the composite non-irradiated diet or the commercial laboratory mouse pellet diet.

Phase 3. Since heart lesions similar to those originally observed by Monsen were not observed in any of the mice used in this phase, its original purpose was not fulfilled. A variety of lesions similar to those described in phase 2 were noted in the mice of this phase. The following lesions were observed in the 53 male and 56 female mice from this phase which were available for analysis: necrosis of the caudate lobe of the liver, nematodiasis of the large intestine, lymphocytic infiltration of the urinary bladder, reticuloendothelial hyperplasia of the lymph nodes, sebaceous gland adenoma, urolithiasis, adrenal cortical hyperplasia, and calcification of skeletal muscle and myocardium.

The only lesions which occurred with any frequency and warrant discussion are calcification of the myocardium, adrenal cortical hyperplasia, and renal urolithiasis. Calcification of the myocardium occurred with approximately the same incidence in the mice of phase 3 and the Cb control mice of phase 2. Adrenal cortical hyperplasia occurred only in the female mice and significantly less frequently than in any diet group of Cb mice in phase 2. This observation is directly related to the younger age distribution of the mice in phase 3. Urolithiasis occurred only in the female mice (16.8%) of this phase and primarily in mice 130 days of age or less. The fact that the incidence was considerably lower in phase 2 Cb mice (3.6%) may be related to the fact that phase 2 animals were killed at 200 days of age or older. It is doubtful that the increased incidence

of urolithiasis in phase 2 can be related to the feeding of irradiated food.

Phase 4. The purpose of this phase was to determine which, if any, component of the irradiated diet might be responsible for the production of the heart lesion originally described by Monsen. Since no heart lesions of this type were observed, the primary purpose of this phase of the study was not fulfilled. However, a variety of lesions were observed in the phase 4 mice which were similar to those noted in phase 2.

Study 1. Of the 1600 mice of the Cb strain which were originally fed diets used in this study, data were available for analysis from 1391 mice which were fed the composite diet in which only one of the components was irradiated.

In the analysis of lesions in study 1 of phase 4, comparisons were made between total lesions per group (identified by irradiated component of the diet). Comparisons were also made with the incidence of lesions observed in the experimental and control groups of the Cb mice in phase 2. An attempt was made to compare the lesion incidence within the major groups (between subgroups a, b, c, d); however, in most instances the number of lesions became too diluted to permit accurate statistical comparison. For the same reason, in the analyses of these data, it was not possible to subdivide the lesions into age groups.

A variety of non-neoplastic lesions were observed in the mice used in this study; however, only the following occurred with sufficient frequency to permit statistical analysis: epididymitis, urolithiasis, calcification of the myocardium, necrosis of the caudate lobe of the liver, hepatitis, myocarditis, nematodiasis of the large intestine, adrenal cortical hyperplasia, reticuloendothelial hyperplasia of lymph nodes, and calcification of the brain. No significant differences in incidence of epididymitis, urolithiasis, calcification of the myocardium, necrosis of the caudate lobe of the liver, or hepatitis, occurred in any of the diet groups of this study, or between this study and the results obtained with the Cb strain of mice in phase 2. However, the incidence of certain types of lesions did vary significantly between diet groups of this study or between this study and phase 2, and we shall limit our discussion to these lesions.

The incidence of myocarditis in male mice in groups 4 (potatoes) and 5 (carrots) was significantly lower than in the other groups and corresponded to the incidence of myocarditis observed in experimental and control Cb mice of phase 2. In the male mice of groups 1 (pork), 2 (chicken), and 3 (evaporated milk), the incidence was significantly higher than in the phase 2 mice. There were no significant differences in the incidence of myocarditis in the female mice of the 5 groups of this study. Similarly there was no significant difference between the female mice of any group of this study and the 2 control groups of Cb mice in phase 2. There were no cases of myocarditis in the female Cb mice fed the irradiated diet in phase 2.

Nematodiasis of the large intestine occurred with a variable distribution in both the male and female mice of this study. The incidence of nematodiasis in group 1 (pork) male mice and groups 2 (chicken) and 4 (potatoes) female mice was higher than in the Cb strain of the non-irradiated diet group of phase 2; however, it was not significantly higher than in the irradiated and pellet diet groups of phase 2.

Although there was a significant difference in the incidence of adrenal cortical hyperplasia between the group 2 (chicken) and group 5 (carrots) males, in no instance did this lesion occur with greater frequency than in phase 2 irradiated and non-irradiated diet groups. There was no significant difference in the incidence of adrenal cortical hyperplasia between the various female diet groups of this study.

A significantly higher incidence of reticuloendothelial hyperplasia of the lymph nodes occurred in the group 2 (chicken) male mice when compared with the male mice of group 3 (evaporated milk). However, in no group of this study was the incidence of this lesion significantly higher than the irradiated or non-irradiated diet groups of phase 2. There were no significant differences in the incidence of this lesion between the various female diet groups of this study.

Although the incidence (2.7%) of calcification of the brain in the group 1 (pork)

males was higher than that observed in the other groups and the diet groups of phase 2, the small number of cases (4) makes it difficult to attach any significance to this difference. No significant difference in the incidence of this lesion was observed between the various female diet groups.

Lung tumors were the most frequently encountered neoplasms in study 1 of phase 4. A significantly lowered incidence of lung tumors occurred in group 4 (potatoes) male mice; however, this finding was not observed in the female mice of this group. In no case was there a significantly higher incidence of lung tumors in any group of this study when compared with the experimental and control Cb mice of phase 2. No other type of tumor occurred with great enough frequency to allow statistical analysis. The general types and distribution of neoplasms within the various groups of this phase were not dissimilar from those observed in phase 2.

In only 2 instances in study 1 of phase 4 was the incidence of a particular lesion higher than that observed in the Cb mice of phase 2: myocarditis and calcification of the brain. The significance of these differences is not understood. Because of the random distribution of these differences within this study and because there were no lesions which occurred with increased frequency in the irradiated diet group of phase 2 when compared with its controls, it is felt that these data do not implicate irradiated food in the production of any of these lesions.

Study 2. The purpose of this study was to evaluate further the possible role of irradiated evaporated milk in the production of the heart lesion originally observed by Monsen. No such lesion was observed in this study.

The mice within this study available for analysis were divided as follows: cooked irradiated milk, 8 males and 2 females; cooked non-irradiated milk, 6 males and 6 females; uncooked irradiated milk, 6 males and 4 females; and uncooked non-irradiated milk, 9 males and 9 females. The lesions observed in the mice of these various groups were limited to thrombosis of the heart, lymphocytic infiltration of the kidney, adrenal cortical hyperplasia, granulosa cell tumor, urolithiasis, and calcification of myocardial or skeletal muscle, or both. However, the only lesions which occurred with any frequency were urolithiasis (9%), and calcification of the myocardium (18.5%) or skeletal muscle (8%), or both. The high incidence of these lesions, as compared with similar lesions occurring among the Cb mice in phase 2, is explained on the basis of the extremely high calcium intake of these mice. That these lesions bear no relation to the feeding of irradiated milk was amply borne out by the fact that the incidence rate for each type of lesion was greater among the mice fed the non-irradiated milk diets than in the irradiated milk diets.

The inability to produce the heart lesion, described by Monsen, in this study cannot be explained even though both the mice and milk used in this study were obtained directly from Monsen. Monsen was able to produce a very high incidence of his typical heart lesion in mice fed a diet consisting solely of milk.

Study 3. The purpose of this study was to determine whether there was any correlation between the temperature at which irradiated milk was stored and the occurrence of the heart lesion described by Monsen. A total of 18 male and 25 female Cb mice was available for analysis. The lesions which were observed were as follows: myocarditis, calcification of the heart, lymphocytic infiltration of the kidney, adrenal cortical hyperplasia, reticuloendothelial hyperplasia of the lymph node, calcification of the brain, epididymitis, hepatitis, necrosis of the caudate lobe of the liver, nematodiasis of the large intestine and lung tumors.

Since no heart lesions, as described by Monsen, were observed in this study or the irradiated diet group of phases 2 and 3, it can be concluded that the temperature at which the irradiated evaporated milk was stored did not influence the production of the heart lesion.

The incidence of some lesions observed in study 3 of phase 4 was lower, but in many cases was higher, than that observed in the irradiated diet group of phase 2. Direct comparisons between this study and phase 2, with 1086 surviving Cb mice, cannot be made since the mice in this study were allowed to live out their lifespan and

therefore represented a much older population. Such comparisons would also be misleading since in study 3 of phase 4 the occurrence of a single lesion constitutes an incidence of 5.6% among the 18 surviving males or 4.0% among the 25 surviving females.

Study 4. Of the 72 Cb mice fed from bean pots, data for statistical analyses were available from 65. These surviving mice were distributed within the study as follows: irradiated composite diet, 15 males and 17 females; and non-irradiated composite diet, 15 males and 18 females. The lesions observed among these mice were limited to myocarditis, calcification of the myocardium, necrosis of the caudate lobe of the liver, nematodiasis, reticuloendothelial hyperplasia of the lymph nodes, and adrenal cortical hyperplasia.

The incidence of most lesions was too low to allow any analysis or comparison between the irradiated and non-irradiated diet groups or between these groups and the other diet groups in phase 2. Lymph node hyperplasia and adrenal cortical hyperplasia in the female mice were the only lesions which occurred with great enough frequency to allow statistical analysis. There were no significant differences in the incidence of these 2 lesions between the irradiated and non-irradiated diet groups of the bean pot study. When compared with the other diet groups of phase 2, no difference in incidence of lymph node hyperplasia existed. The incidence of adrenal cortical hyperplasia in the female mice in the irradiated diet group of the bean pot study was significantly higher than in any group of female Cb mice in phase 2. This observation may indicate that the lesion occurs with greater frequency when female mice are fed under unsanitary conditions; however, in view of the known hormonal relationship to the lesion, this does not appear plausible. As adrenal cortical hyperplasia did not occur with a significantly increased frequency in female mice fed irradiated food under sanitary conditions, it is felt that this observation in the bean pot study is not of any great importance. The fact that no other lesion occurred with greater frequency in the bean pot study lends support to this conclusion.

ACKNOWLEDGMENTS

A number of consultants participated in the execution of this study. Since their participation was pre-planned and constituted a vital portion of the overall design of the study, it is felt that their participation constituted a more integral part of our methods than would be implied by a mere acknowledgment. These consultants and their role in this study are as follows:

William Manion, Armed Forces Institute of Pathology, Washington, D. C., reviewed selected gross specimens and tissue sections of lesions obtained from Monsen. This review was conducted with one of the authors (Thompson) and served to characterize the gross and microscopic manifestations of the lesion as originally seen by Monsen. Based upon this review, plans were formulated as to the overall approach that would be undertaken in the study reported here.

Frank Johnson, Armed Forces Institute of Pathology, Washington, D. C., advised us on special histopathological procedures which were adopted and used throughout this study. He also trained several of our technicians in these special techniques by providing several weeks of training in his laboratory.

Harry Monsen, University of Illinois, Chicago, served as a special consultant to the group in Denver. He provided the necessary data from his own experiments which were used by our statistician (Jenkins) in developing our approach to this study.

Norman Witt, University of Colorado, Boulder, Colorado, served as a special consultant in chemical microscopy and conducted a special training course in microscopy for our technicians and pathologists with a view to developing the capabilities that would be required to expediently handle and study the excess of 925,000 tissue sections that were generated in the course of this study.

Robert Huseby, American Medical Center, Denver, Colorado, provided consultant services by orienting our pathologists as to the nature of the lesions, and incidence rates of these lesions, which are known to occur in the strains of mice used in this study. This information was also used by our statistician (Jenkins) in planning the

statistical design of the study. Dr. Huseby also assisted us in the procurement, review and classification of reference sets of pathologic lesions most commonly reported to occur in the strains of mice used in this study.

Thelma Dunn, National Institutes of Health, Washington, D. C., reviewed those lesions, observed in the mice used in this study, which were of reticuloendothelial origin, and advised us in classifying these lesions for purposes of statistical analysis.

Charles L. Davis, National Jewish Hospital, Denver, Colorado, served as our general consultant in pathology and reviewed, at weekly intervals, the tissue sections from lesions which had been accumulated during the week by our respective staff pathologists and residents. He served as the arbiter in the resolving of differences in interpretation by our staff of particular problems or unusual lesions.

Richard H. Follis, Jr., Armed Forces Institute of Pathology, Washington, D. C., reviewed those lesions, observed in mice used in this study, which were of the urinary system, and advised us in classifying these lesions for purposes of statistical analysis.

Lt. Colonel Clinton Gould, VC, Quartermaster Food and Container Institute, Chicago, advised us on the problems related to the procurement, irradiation and storage of the foods used in this study during the planning phases of the project. During the actual experimental phases, he supervised the purchase, processing, irradiation, transportation, and local storage of all foods used in our study.

LITERATURE CITED

1. Monsen, H. 1960 Heart lesions in mice induced by feeding irradiated foods. Federation Proc., *19:* 1031.
2. Meier, H., and W. G. Hoag 1961 Studies on left auricular thrombosis in mice. Exp. Med. Surg., *19:* 317.
3. Thompson, S. W. 1965 Selected Histochemical and Histopathological Methods. Charles C Thomas, Springfield, Illinois, pp. 9–11, 29–40, 117–125, 763–772.
4. Thompson, S. W., E. D. Jenkins and M. A. Fox 1961 A Histopathological and Histologic Coding System for Tissue Collections. Charles C Thomas, Springfield, Illinois.
5. Thompson, S. W., R. D. Hunt and J. F. Ferrell 1963 Report of histopathology of mice fed irradiated foods. U. S. Army Medical Research and Nutrition Laboratory, Report 279. U. S. Army Medical Research and Nutrition Laboratory, Denver, Colorado.

Influence of Diet on the Hypocholesterolemic Action of Methyltestosterone in Dogs [1]

LIESE L. ABELL, ERWIN H. MOSBACH AND FORREST E. KENDALL
Columbia University Research Service, Goldwater Memorial Hospital, Welfare Island; Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University and The Public Health Research Institute of the City of New York, Inc., New York, New York

ABSTRACT The effect of altering the relationship between the major constituents of the diet (protein, fat, carbohydrate) upon the hypocholesterolemic activity of 17α-methyltestosterone in dogs was studied. In diets containing casein, lard, salt mixture, vitamins, 1% cholesterol and either sucrose or cornstarch, the amounts of fat or protein were varied at the expense of carbohydrate. Groups of 5 dogs were maintained on each regimen for 4 weeks, and the cholesterol content of the serum, high density (α-) and low density (β-) lipoprotein fractions was determined at regular intervals. An increase in the percentage of calories derived from lard from 5 to 48% enhanced the hypercholesterolemia resulting from cholesterol feeding. Administration of methyltestosterone to dogs fed the cholesterol-containing experimental diets consistently produced a decrease in the cholesterol content of the α-lipoprotein fraction that was not affected by varying the proportions of the 3 dietary components. Methyltestosterone decreased the β-lipoprotein cholesterol in dogs fed diets low in fat or diets containing cornstarch and increased it in dogs fed diets high in fat or containing sucrose.

The synthetic androgen 17α-methyltestosterone has been found to lower serum cholesterol levels in human beings (1, 2), in rabbits (3), in dogs (4, 5), in chickens (6, 7) and in rats (8). We have reported previously (5) that methyltestosterone exerts its hypocholesterolemic action in dogs maintained with low fat-high cholesterol diets and with high fat-low cholesterol diets, but fails to be effective in dogs kept on high fat-high cholesterol regimens. These observations indicate that the effect of this androgen was modified in some way by changes in the composition of the diets. In the present study, diets of known composition were used to evaluate the influence of each of the 3 major dietary constituents (protein, fat, carbohydrate) upon the hypocholesterolemic effect of methyltestosterone in dogs fed high cholesterol diets. The effects of high fat versus low fat, of high protein versus low protein and of the substitution of sucrose for cornstarch were studied. Diets were used containing these 3 variables in the 8 possible combinations.

METHODS

Two groups of 5 adult mongrel dogs (4 males, 1 female; and 3 males, 2 females) varying in weight from 3.2 to 14.6 kg were used for the experiments. Each dog was housed separately. Each group of dogs was maintained for 4 weeks throughout the following sequence of feeding periods: 1) control diet; 2) experimental diet; 3) experimental diet plus 1% cholesterol; 4) experimental diet plus 1% cholesterol plus 200 mg of 17α-methyltestosterone (1 capsule 6 times/week). Following the completion of each sequence, the dogs were returned to the control diet for periods of from 4 to 17 weeks before being used for a subsequent study. The control diet consisted of commercial dog bisquits [2] and contained 20 to 60 mg/100 g of Δ^5,3β-OH sterol, (estimated as digitonin - precipitable, Liebermann - Burchard positive material) and 2 to 5% of total lipid. Eight experimental diets were used.[3] The diets were analyzed for fat content by weighing the hexane-extractable material; for cholesterol by hydrolyzing the diet with

Received for publication June 23, 1965.
[1] Supported by Public Health Research Grants no. HE-52 and no. AM-05222 from the National Institutes of Health.
[2] Big Red Kibbled Dog Cakes, GLF Marketing Company, Canandaigua, New York.
[3] Purchased from Nutritional Biochemicals Corporation, Cleveland, and from General Biochemicals Incorporated, Chagrin Falls, Ohio.

TABLE 1
Composition of diets [1]

	Diet [2]							
	FPS	FpS	FPC	FpC	fPS	fpS	fPC	fpC
	% of total calories							
Protein (casein)	19	11	19	11	23	12	23	12
Carbohydrate (sucrose)	38	41	—	—	72	83	—	—
Carbohydrate (cornstarch)	—	—	38	41	—	—	72	82
Fat (lard)	43	48	43	48	5	5	5	6

[1] All diets contained 4% salt mixture (10) and vitamins (11).
[2] F = high fat; P = high protein; S = sucrose; f = low fat; p = low protein; and C = cornstarch.

10% alcoholic KOH, extracting the nonsaponifiable material into petroleum ether and analyzing dried aliquots of the petroleum ether solution for cholesterol (9). Protein estimations were performed using Kjeldahl digestion and multiplying the figures obtained for nitrogen by 6.25.

The composition of the diets used is listed in table 1. α-Cellulose was added when needed to adjust the available caloric content of the diet to approximately 4 kcal/g. The diets furnished from 396 to 473 kcal/100 g and were fed in weighed amounts sufficient to maintain constant body weight. No differences in response to the various regimens were noted that could be attributed to differences in either sex or body size of the animals. The diets were so designed that the calories available from either fat or protein were varied at the expense of carbohydrate; 2 kinds of carbohydrate (sucrose and cornstarch) were fed at 2 different levels of fat and protein content. In this way it was possible to estimate separately the influence of the different foodstuffs upon serum cholesterol concentrations. Diets FPS, FpS, FPC and FpC, whose fat content ranged from 43 to 48% of the calories, are defined as "high fat" diets; diets fPS, fpS, fPC, and fpC, whose fat content ranged from 5 to 6% of the calories, are defined as "low fat" diets. These diets were used to assess the influence of dietary fat upon cholesterol levels. Likewise, the influence of dietary protein can be estimated by considering diets FPS, FPC, fPS, fPC (protein content from 19 to 23% of the calories) as "high protein" diets and diets FpS, FpC, fpS, and fpC (protein content from 11 to 12% of the calories) as "low protein" diets. The influence of either sucrose or cornstarch was demonstrated by comparing the results obtained when feeding diets FPS, FpS, fPS, fpS (sucrose content 38 to 83% of the calories) with those obtained when feeding diets FPC, FpC, fPC, fpC (cornstarch content 38 to 82% of the calories). During pertinent periods 1% cholesterol was added to the experimental diets in the manner described previously (12); 200 mg of 17α-methyltestosterone in gelatin capsules were administered orally 6 times per week. Serum taken from each dog at 2-week intervals was analyzed for total cholesterol by the method of Abell et al. (13) and fractionated in the preparative ultracentrifuge (Spinco Model L) at a specific gravity of 1.063. The low density (β-) and high density (α-) lipoprotein fractions were isolated by the method of Havel et al. (14) and analyzed for cholesterol as described previously (5).

Table 2 presents the average serum total cholesterol concentrations together with the cholesterol content of the α- and β-lipoprotein fractions for each group of 5 dogs during each feeding period. Each figure represents the average of from 8 to 10 determinations. The upper part of the table summarizes the cholesterol concentrations of the serum, and of the α- and β-lipoprotein fractions of the dogs during a control period with the stock diet, which preceded each of the 8 experimental dietary sequences. The lower part of the table presents corresponding data determined during the experimental regimens. The data are given in detail, because they form the basis on which the subsequent tables have been constructed.

Table 3 presents the serum total cholesterol levels of the dogs grouped according to the 3 dietary variables, and tables 4 and 5 present such data for the α- and β-lipoprotein fractions, respectively. When the

TABLE 2

Average serum cholesterol pattern of dogs on each regimen

Feeding period		Cholesterol							
		mg/100 ml			Stock diet		*mg/100 ml*		
Control	Serum	157 ± 11 [1]	134 ± 8	111 ± 5	187 ± 7	131 ± 12	139 ± 14	163 ± 13	150 ± 8
	α-lipoprotein	124 ± 8	109 ± 6	91 ± 4	138 ± 5	101 ± 9	108 ± 10	120 ± 13	122 ± 5
	β-lipoprotein	33 ± 4	25 ± 2	20 ± 2	49 ± 3	30 ± 3	31 ± 4	43 ± 8	28 ± 3
		Diets [2]							
		FPS	FpS	FPC	FpC	fPS	fpS	fPC	fpC
Experimental	Serum	212 ± 12	220 ± 8	191 ± 9	256 ± 11	164 ± 12	189 ± 13	148 ± 14	159 ± 8
	α-lipoprotein	157 ± 6	156 ± 5	134 ± 6	170 ± 7	120 ± 9	140 ± 14	111 ± 10	121 ± 5
	β-lipoprotein	55 ± 7	64 ± 8	57 ± 5	86 ± 6	44 ± 4	49 ± 5	37 ± 4	38 ± 4
Experimental + 1% cholesterol	Serum	408 ± 96	492 ± 42	469 ± 38	387 ± 27	316 ± 24	481 ± 59	363 ± 50	309 ± 18
	α-lipoprotein	151 ± 8	136 ± 8	152 ± 4	175 ± 8	159 ± 6	140 ± 11	145 ± 7	142 ± 5
	β-lipoprotein	257 ± 103	356 ± 49	317 ± 38	212 ± 30	157 ± 23	341 ± 66	218 ± 50	167 ± 23
Experimental + 1% cholesterol + 200 mg methyl-testosterone	Serum	482 ± 134	620 ± 66	349 ± 19	217 ± 19	239 ± 30	349 ± 59	255 ± 20	215 ± 28
	α-lipoprotein	84 ± 6	72 ± 4	111 ± 3	95 ± 3	84 ± 6	91 ± 6	85 ± 6	76 ± 5
	β-lipoprotein	398 ± 137	548 ± 69	238 ± 17	122 ± 28	155 ± 31	258 ± 59	170 ± 23	139 ± 25

[1] Each value represents the average of 8 to 10 determinations ± SE of mean.

[2] The composition of the diets is represented by the following symbols: F = high fat; P = high protein; S = sucrose; f = low fat; p = low protein; and C = cornstarch.

TABLE 3

Cholesterol content of the serum on grouped regimens [1]

Diet	Cholesterol content of serum			
	Control diet period	Experimental diet period	Experimental diet + 1% cholesterol period	Experimental diet + 1% cholesterol + methyltestosterone period
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Low fat	148 ± 7	167 ± 7 [2]	371 ± 22	264 ± 19 [3]
High fat	146 ± 7	223 ± 6 [3]	442 ± 76	427 ± 43 [2]
Low protein	151 ± 6	206 ± 8 [3]	417 ± 22	358 ± 37 [2]
High protein	138 ± 8	176 ± 7 [4]	386 ± 27	323 ± 33 [2]
Sucrose	137 ± 6	195 ± 7 [3]	424 ± 29	421 ± 42 [2]
Cornstarch	153 ± 7	188 ± 9 [4]	379 ± 19	260 ± 15 [3]

[1] Each group of 5 dogs was maintained with 4 different diets and the cholesterol pattern of each dog's serum was determined twice during each dietary period. Each value represents the mean of 38 to 40 analyses ± SE of mean.
[2] Not significantly different from preceding period.
[3] Significantly different from preceding period, $P < 0.001$.
[4] Significantly different from preceding period, $P < 0.005$.

TABLE 4

Cholesterol content of the α-lipoprotein fraction on grouped regimens [1]

Diet	Cholesterol content of α-lipoprotein fraction			
	Control diet period	Experimental diet period	Experimental diet + 1% cholesterol period	Experimental diet + 1% cholesterol + methyltestosterone period
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Low fat	113 ± 5	123 ± 5 [2]	147 ± 4	84 ± 3 [3]
High fat	114 ± 4	155 ± 4 [3]	154 ± 4	90 ± 4 [3]
Low protein	119 ± 4	147 ± 4 [3]	148 ± 5	83 ± 3 [3]
High protein	107 ± 5	129 ± 5 [3]	152 ± 3	91 ± 3 [3]
Sucrose	108 ± 5	143 ± 4 [3]	146 ± 5	83 ± 3 [3]
Cornstarch	118 ± 5	134 ± 5 [4]	154 ± 4	91 ± 3 [3]

[1] Each group of 5 dogs was maintained with 4 different diets and the cholesterol pattern of each dog's serum was determined twice during each dietary period. Each value represents the mean of 38 to 40 analyses ± SE of mean.
[2] Not significantly different from preceding period.
[3] Significantly different from preceding period, $P < 0.001$.
[4] Significantly different from preceding period, $P < 0.005$.

TABLE 5

Cholesterol content of the β-lipoprotein fraction on grouped regimens [1]

Diet	Cholesterol content of β-lipoprotein fraction			
	Control diet period	Experimental diet period	Experimental diet + 1% cholesterol period	Experimental diet + 1% cholesterol + methyltestosterone period
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Low fat	35 ± 3	44 ± 2 [4]	224 ± 24	180 ± 15 [2]
High fat	32 ± 3	68 ± 4 [3]	288 ± 28	337 ± 45 [2]
Low protein	32 ± 2	59 ± 4 [3]	269 ± 25	272 ± 36 [2]
High protein	31 ± 3	47 ± 3 [3]	233 ± 28	232 ± 33 [2]
Sucrose	29 ± 2	53 ± 3 [3]	278 ± 33	338 ± 44 [2]
Cornstarch	35 ± 3	54 ± 4 [3]	225 ± 19	169 ± 13 [4]

[1] Each group of 5 dogs was maintained with 4 different diets and the cholesterol pattern of each dog's serum was determined twice during each dietary period. Each value represents the mean of 38 to 40 analyses ± SE of mean.
[2] Not significantly different from preceding period.
[3] Significantly different from preceding period, $P < 0.001$.
[4] Significantly different from preceding period, $P < 0.025$.

dogs were shifted from the stock diet to the experimental diets, there was a statistically significant increase in serum total cholesterol concentration ($P < 0.005$) except with the low fat diet. This increase was almost equally divided between the α- and β-fractions and is probably due to the lard content of the diet (5). The addition of 1% cholesterol to the experimental diets produced a large increase in serum total cholesterol concentrations with almost all of the increase occurring in the β-fraction. None of the 3 dietary variables had a significant effect upon the magnitude of this increase.

The oral administration of 200 mg of methyltestosterone to dogs maintained with the cholesterol-containing experimental diets produced a decrease in serum total cholesterol concentrations (table 3). In the α-fraction this decrease was observed on all regimens and was always statistically significant ($P < 0.001$). The cholesterol content of the β-fractions was decreased only with diets low in fat and in those containing cornstarch, resulting in a statistically significant decrease in serum *total* cholesterol concentrations. With diets high in fat and in those containing sucrose as the source of carbohydrate, the cholesterol content of the β-fraction increased. Variation of the protein content of the diet had no influence on the cholesterol content of this fraction.

DISCUSSION

The mechanism of action of methyltestosterone is not known. Many studies have been undertaken to explore various pathways by which this androgen as well as the related compound, testosterone propionate, exert their hypocholesterolemic effect. Furman (15) has studied the incorporation of acetate-1-^{14}C in vivo in dogs treated with methyltestosterone. He reported a depression of specific activity of serum free cholesterol in his treated dogs, whereas the specific activity of the ester cholesterol fraction was variable. It appears likely that the hypocholesterolemic action of methyltestosterone cannot be ascribed solely to its effect on cholesterol biosynthesis. This conclusion is supported by our experiments on methyltestosterone-induced hypocholesterolemia in cholesterol-fed animals (5, 8), where liver sterol biosynthesis is suppressed (16).

Attempts have been made to relate the cholesterol-lowering effect of the androgen to its anabolic action. Wilson (17) investigated the action of testosterone upon protein synthesis in the seminal vesicle of rats and noted that protein biosynthesis from labeled amino acids increased within 12 hours to 2 days after the administration of testosterone to his animals. Recently Wilson [4] further observed that the acceleration of protein synthesis in the preen gland of the duck as a result of the intravenous injection of testosterone-1,2-^{3}H is associated with an increased rate of synthesis of ribonucleic acid within the cell nuclei of this target organ. We have investigated the changes in the synthesis of the protein moiety of serum lipoproteins in methyltestosterone-treated rats in vivo. When ^{14}C-labeled amino acids were injected into control and treated rats, we were able to demonstrate an increased uptake of labeled amino acid only into the low density (β-) lipoprotein-protein under the conditions of our experiments.[5] These experiments do not explain the consistent lowering of the cholesterol content of the α-lipoprotein fraction in intact animals, since the incorporation of the labeled amino acids into the α-lipoprotein protein was not affected by methyltestosterone administration. However, studies by Furman (18) and Fredrickson [6] in man suggest the possibility that the low density (β-) fraction obtained by our fractionation procedure may contain an α_1-lipoprotein. The effect of methyltestosterone upon this fraction is unknown and requires further investigation.

In a search for possible mechanisms it was thought that in view of the large dose of methyltestosterone required, the effect could be indirect and might be mediated via the pituitary gland. However, we have been able to demonstrate in hypophysectomized rats (19) that the hypocholesterol-

---

[4] Wilson, J. D., and P. M. Loeb 1965 Intranuclear localization of testosterone-1,2-^{3}H in the preen gland of the duck. J. Clin. Invest., 44: 1111 (abstract).

[5] Unpublished observations.

[6] Levy, R. I., R. S. Lees and D. S. Fredrickson 1965 A functional role for plasma α_1-lipoprotein. J. Clin. Invest., 44: 1068 (abstract).

TABLE 6

Increments in cholesterol concentrations

Shift in diet	Dietary variable	Change in cholesterol content		
		Serum	α-Lipoprotein	β-Lipoprotein
		mg/100 ml	*mg/100 ml*	*mg/100 ml*
From control to experimental	low fat	19	10	9
	high fat	77	41	36
	low protein	55	28	27
	high protein	38	22	16
	sucrose	59	35	24
	cornstarch	35	16	19
From experimental to experimental + 1% cholesterol	low fat	204	24	180
	high fat	219	−1	220
	low protein	211	1	210
	high protein	209	23	186
	sucrose	228	3	225
	cornstarch	191	20	171
From experimental + 1% cholesterol to methyltestosterone	low fat	−107	−63	−44
	high fat	− 15	−64	+49
	low protein	− 59	−65	+ 6
	high protein	− 62	−61	− 1
	sucrose	− 3	−63	+60
	cornstarch	−119	−63	−56

emic effect of this steroid is not mediated via this gland.

In the present study we have attempted to evaluate the influence of changes in the diet upon the hypocholesterolemic action of methyltestosterone. To present the results obtained more conveniently, we have listed in table 6 the increments (increases or decreases) in cholesterol concentration which occurred in serum and in the 2 lipoprotein fractions when the dogs were shifted through the sequence of diets outlined above. A shift from the stock diet to the experimental diets produced an increase in serum cholesterol concentrations which was most pronounced with high fat diets and with those containing sucrose, and least with low fat diets and those containing cornstarch. This latter diet resembles the control diet in overall composition. The increase in serum cholesterol was not due to the powdered form of the experimental diet. When powdered stock diet was fed, neither the serum total cholesterol concentration nor the distribution of cholesterol between the α- and β-lipoprotein fractions was affected.

Feeding the experimental diets containing 1% cholesterol caused large elevations in serum total cholesterol levels. These changes were largest with high fat and sucrose diets, where all the increase occurred in the β-fraction. The factors which are sometimes implicated in human arteriosclerosis, namely, high intake of fat and refined carbohydrates also appear to be operative in the dog in elevating serum cholesterol levels.

The hypocholesterolemic effect of methyltestosterone upon the α-lipoprotein fraction of dogs receiving high cholesterol diets has been observed previously (5). In the present study, the effect on the α-fraction was independent of the diet. In contrast, the effect of methyltestosterone upon the β-fraction was diet-dependent: those factors, namely sucrose and high fat, which caused the highest serum cholesterol levels in the dogs fed the high cholesterol-experimental diets appeared to counteract the cholesterol-lowering effect of methyltestosterone on this fraction. The serum total cholesterol concentrations decreased significantly ($P < 0.001$) only in the case of the low fat and cornstarch diets.

From these data it must be concluded that the overall hypocholesterolemic effect of methyltestosterone is diet-depend-

ent. However, the effects of fat and carbohydrate cannot be dissociated under the conditions of these experiments; changes in the protein content of our diets had no effect. The fact that the hypocholesterolemia is most pronounced with diets low in fat and in those containing cornstarch, and least evident in diets high in fat and in those containing sucrose, suggests an effect of methyltestosterone upon the intestinal absorption of cholesterol. An alteration of the intestinal bacterial flora in methyltestosterone-treated dogs cannot be excluded, but appears less likely since the androgen exerts a hypocholesterolemic action when injected intramuscularly (5).

It is known that in dogs the serum cholesterol concentration is controlled, at least in part, by changes in the conversion of cholesterol to bile acids (20). In preliminary balance studies (21), we were unable to show, however, that fecal bile acid excretion in dogs fed either low or high cholesterol diets was affected by methyltestosterone, administered in quantities sufficient to lower serum cholesterol levels. This does not exclude the possibility that the changes in serum cholesterol concentrations observed in the present study were associated with changes in fecal bile acid excretion. Balance studies with dogs maintained with diets of different compositions will be needed to establish this point.

ACKNOWLEDGMENT

We wish to thank Minnie B. Myers, Peter O. Milch and Mrs. Susan Samuels for their technical assistance.

LITERATURE CITED

1. Furman, R. H., R. P. Howard, L. N. Norcia and E. C. Keaty 1958 The influence of androgens, estrogens, and related steroids on serum lipids and lipoproteins. Am. J. Med., *24:* 80.
2. Robinson, R. W., R. J. LeBeau and W. D. Cohen 1964 Effects of methyltestosterone upon serum lipids. J. Clin. Endocrin. Metab., *24:* 708.
3. Fillios, L. C., and G. V. Mann 1956 The importance of sex in the variability of the cholesteremic response of rabbits fed cholesterol. Circulation Res., *4:* 406.
4. Furman, R. H., L. N. Norcia, C. W. Robinson and I. E. Gonzalez 1957 Influence of testosterone, methyltestosterone, and DL-ethionine on canine liver lipids, serum lipids and lipoproteins. Am. J. Physiol., *191:* 561.
5. Abell, L. L., E. H. Mosbach and F. E. Kendall 1962 Hypocholesteremic effect of 17α-methyltestosterone in dogs. Circulation Res., *10:* 846.
6. Pick, R., J. Stamler, S. Rodbard and L. N. Katz 1959 Effects of testosterone and castration on cholesteremia and atherogenesis in chicks on high fat, high cholesterol diets. Circulation Res., *7:* 202.
7. Raiford, R. L., and H. Y. C. Wong 1962 In vivo incorporation of acetate-1-^{14}C into cholesterol and fatty acids following testosterone propionate administration. Circulation Res., *11:* 753.
8. Abell, L. L., and E. H. Mosbach 1962 Hypocholesteremic effect of methyltestosterone in rats. J. Lipid Res., *3:* 88.
9. Sperry, W. M., and M. Webb 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
10. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.
11. Shull, K. H., G. V. Mann, S. B. Andrus and F. J. Stare 1954 Response of dogs to cholesterol feeding. Am. J. Physiol., *176:* 475.
12. Steiner, A., F. E. Kendall and M. Bevans 1949 Production of arteriosclerosis in dogs by cholesterol and thiouracil feeding. Am. Heart J., *38:* 34.
13. Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.
14. Havel, R. J., H. A. Eder and J. H. Bragdon 1955 The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest., *34:* 1345.
15. Furman, R. H., C. W. Robinson, Jr., R. H. Bradford, P. Alaupovic and L. N. Norcia 1963 Methyltestosterone effect on serum cholesterol radioactivity in dogs given acetate-^{14}C. Proc. Soc. Exp. Biol. Med., *113:* 789.
16. Gould, R. G. 1951 Lipid metabolism and atherosclerosis. Am. J. Med., *11:* 18.
17. Wilson, J. D. 1962 Localization of the biochemical site of action of testosterone on protein synthesis in the seminal vesicle of the rat. J. Clin. Invest., *41:* 153.
18. Furman, R. H., K. Lakshmi, L. N. Norcia and R. P. Howard 1960 Effect of in vitro sonic oscillation on distribution of serum lipoproteins in idiopathic "essential" hyperlipemia and other hyperlipidemic states. J. Clin. Invest., *39:* 988.
19. Mosbach, E. H., S. Zapp and L. L. Abell 1963 Hypocholesteremic action of 17α-methyltestosterone (MeT) in hypophysectomized rats. Proc. Soc. Exp. Biol. Med., *114:* 595.

20. Abell, L. L., E. H. Mosbach and F. E. Kendall 1956 Cholesterol metabolism in the dog. J. Biol. Chem., 220: 527.
21. Mosbach, E. H., L. L. Abell and F. E. Kendall 1961 Effect of 17α-methyltestosterone on cholesterol metabolism in the dog. In: Drugs Affecting Lipid Metabolism, eds., S. Garattini and R. Paoletti. Elsevier Publishing Company. Amsterdam, Netherlands, p. 121.

Induction of Premature Birth in Rats by a Methionine Antagonist [1]

BACON F. CHOW AND CONRADO E. AGUSTIN
Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

ABSTRACT Pregnant rats injected with ethionine have shown a high incidence of resorption and stillbirth and pups born alive have been smaller than normal. Without precise knowledge of the time of conception it has not been known to what extent the low birth weight could be attributed to prematurity. The present study was designed to shed light on this question. Pregnant rats were injected with graded doses of ethionine up to and including the amount known to cause resorption or stillbirth. The date of conception was inferred from the first appearance of sperm in the vagina. Relatively small doses of ethionine caused premature birth with only a low incidence of resorption and stillbirth. The incidence of prematurity, and its degree, were directly related to the dose of ethionine. The premature pups grew faster than the control pups, thus correcting an initial weight deficit by the age of 3 months. Sub-lethal doses of ethionine given to non-pregnant animals did not adversely affect subsequent conception or gestation.

The nutritional environment of the fetus has been shown to have an important influence on subsequent development. Thus it was reported that general dietary restriction of mother rats (1, 2) results in permanent growth-stunting and abnormal protein metabolism in the progeny;[2] it does not, however, result in shortening of the gestation period. Nonetheless, it appeared possible that more specific nutritional stresses might advance parturition. Certain antimetabolites of amino acids, when given during pregnancy, have been observed to cause a high incidence of resorption or stillbirth (3–5),[3] with low birth weight of surviving pups. We have studied the effect of smaller quantities of one such substance, ethionine, on the course, outcome and especially the duration of pregnancy in rats.

MATERIALS AND METHODS

Animals. Most of the rats were from our colony of McCollum strain. The females were 3 to 4 months old and weighed from 210 to 240 g. The males were the same age and weighed 340 to 380 g. They were raised and maintained during the experiments with a commercial chow stock diet [4] fed ad libitum. Some rats of another strain [5] were used in the later experiments with ethionine; they were 3 months old and had an average weight of 200 g.

Mating. Sets of 3 female rats selected at random were mated with one male from 5 PM to 9 AM the following day. Immediately afterwards vaginal smears were examined for sperm. Mating was continued until conception, defined by the first appearance of sperm in the vagina, following which the females were housed in individual cages. They were randomized for experimental treatment, thus breaking up the mating sets.

Daily injections of ethionine. Two experiments of this type were carried out. In both, injections were given daily from day 12 of pregnancy until delivery. The injections, which were of one milliliter, were given subcutaneously. The resorption incidence, length of gestation, stillbirth incidence, litter size and birth weights were noted. Any rat that had not delivered by day 23 of gestation was killed in order to examine the uterus for

Received for publication June 14, 1965.
[1] Supported in part by the National Vitamin Foundation, New York.
[2] Unpublished data, B. F. Chow and C. J. Lee, 1965.
[3] Marsh, M. E., L. D. Greenberg and J. F. Rinehart 1955 Studies on the pregnant rat injected with ethionine. I. Effect on litter size and lactation. Federation Proc., *14*: 442 (abstract).
[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[5] Dublin Resistant strain, Dublin Rat Laboratories, Dublin, Virginia.

resorption sites. All the pups were weaned 21 days after birth and then fed our stock diet ad libitum. Their growth rate was recorded.

(a) Thirty-eight pregnant rats were divided at random into 3 groups. Group A received 51 mg DL-ethionine [6]/animal/day; group B received 17 mg ethionine/animal/day; group C received saline solution.

(b) Eighty-five pregnant rats were divided at random into 6 groups. Groups A, B, C, D and E received 8.5, 17, 35, 55 and 70 mg ethionine/animal/day, respectively. Group F received saline.

Single injections of large doses of ethionine. Forty non-pregnant rats were divided at random into 3 groups. All animals were given a single one-milliliter subcutaneous injection. Group A received saline solution; group B received 100 mg ethionine and group C received 200 mg ethionine. The number of deaths in each group was noted. Survivors of the ethionine treatment were mated after a 21-day recovery period. The length of gestation, litter size and birth weight were noted.

RESULTS

Effect of daily ethionine. These results are summarized in tables 1 and 2. There is evidence of a direct relationship of dose to incidence of resorption and stillbirth, length of gestation and birth weight. A dose of ethionine of 35 mg/animal/day or more resulted in a high incidence of resorption or stillbirth, whereas 17 mg or less usually caused only a reduction of gestation period with small but viable offspring.

The rates of weight increase for the 2 classes of pups are shown in figures 1 and 2. Pups born to mothers receiving ethionine, i.e., born prematurely, despite their low initial birth weight and weaning weight, actually grew faster, after weaning, than the control pups and average weights were equal by the eleventh week. No congenital malformations were observed.

Effect of a single large dose of ethionine. Of the 16 animals receiving 100 mg

[6] Purchased from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Effect of various doses of ethionine in pregnant rats (exp. 1)

Group	Dose of ethionine	No. of pregnant rats	No. of rats showing resorption	No. of rats with live births	No. of litters with stillbirths	Length of gestation [1]	Birth weight [1]
	mg/rat/day					*days*	*g*
A	51	10	8	2	2	17.5(17–18) [2]	3.2(2.8–3.4)
B	17	12	3	9	4	19.8(19–21)	4.4(3.3–5.2)
C	0	16	0	16	0	21.2(21–22)	5.8(5.4–6.3)

[1] Live births.
[2] Mean and range.

TABLE 2

Effect of various doses of ethionine in pregnant rats (exp. 2)

Group	Dose of ethionine	No. of pregnant rats	No. of rats showing resorption	No. of rats with live births	No. of litters with stillbirths	Length of gestation [1]	Birth weight [1]
	mg/rat/day					*days*	*g*
A	8.5	12	0	12	0	21.5(21–22) [2]	6.1(5.6–6.8)
B	17	30	5	25	3	20.1(19–21)	4.2(3.4–5.0)
C	35	13	6	7	4	19.2(17–20)	3.5(2.0–4.5)
D	55	10	6	4	4	19.4(18–20)	3.2(1.9–3.6)
E	70	10	8	2	2	18.9(17–19)	3.0(1.1–3.4)
F	0	10	0	10	0	21.1(21–22)	6.3(5.9–7.2)

[1] Live births.
[2] Mean and range.

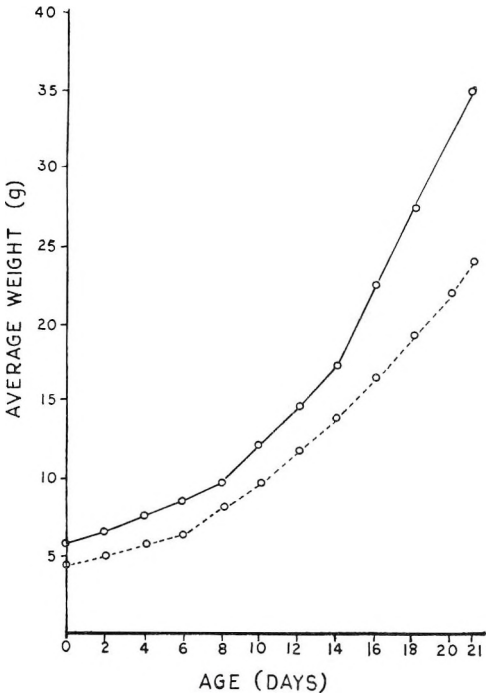

Fig. 1 Weights of the offspring from ethionine-treated (- - - - -) and saline-treated (———) mothers plotted against time from birth.

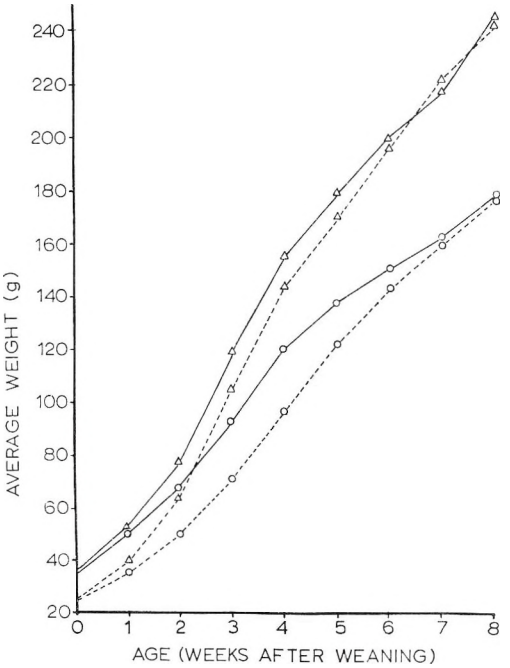

Fig. 2 Weights of the offspring from ethionine-treated (- - - - -) and saline-treated (———) mothers plotted against time after weaning. △, male; ○, female.

ethionine, 5 had died within 2 days; of the 16 animals receiving 200 mg all were dead within 3 days. When 7 of the survivors of group B were mated 21 days later they all became pregnant. Gestation lasted for at least 21 days and the pups had an average birth weight of 5.2 g. The gestation period of the group A control rats was the same and their pups had an average birth weight of 5.4 g, which is not significantly different from the treated group. These results, presented in table 3, suggest that near lethal doses of ethionine have no permanent effect on the reproductive capacity of the rat.

DISCUSSION

We have presented evidence which suggests that 1) ethionine, a known antagonist of methionine, can cause premature birth; 2) the weight deficit of the resulting pups is not permanent; and 3) subsequent pregnancies in the treated animals are of normal duration and result in apparently normal pups.

It remains to be established that pups born prematurely following the injection of ethionine are in all respects similar to pups delivered prematurely by Caesarean section or after injections of oxytocin. Our preliminary evaluation of the pups by

TABLE 3

Effect of a single large dose of ethionine before conception in rats

Group	Dose of ethionine	No. of rats	No. of deaths			No. that became pregnant	Length of gestation	Mean birth wt
			Day 1	Day 2	Day 3			
	mg/rat						*days*	*g*
A	0	8	0	0	0	6	21–22	5.2
B	100	16	0	5	0	10	21–22	5.4
C	200	16	8	4	4	0	—	—

growth rate suggests that they differ from those born to mothers upon whom overall dietary restriction has been imposed during pregnancy. Although both result from a nutritional stress to the mother and in each case the pups are below the normal weight at birth their subsequent performance is quite different. Progeny of restricted mothers grow slowly and never attain the normal weight for their age; they waste nitrogen and show an abnormal pattern of nitrogen excretion. However, the premature pups resulting from ethionine treatment grow rapidly and overcome their early deficit in weight by the age of 3 months. We have not yet confirmed that their pattern of nitrogen excretion is normal but there is no reason to believe otherwise. Because of the implications for humans, it is important to emphasize that low weight at birth may be associated with at least 2 quite different courses of subsequent development.

The study of prematurity in man has been hampered by the criterion adopted to define it. For the apparently sound practical reason that it is often impossible to establish accurately the time of conception, prematurity is most often defined by the weight of the infant. It is recognized in principle, but not always remembered in practice, that a full-term infant may fall well below this arbitrarily chosen borderline. This has caused, in the past, a failure to differentiate the truly premature from the "dysmature" infant. Evidence is accumulating which suggests that the latter type may resemble the stunted offspring of the restricted rat. It is already clear that the risks associated with each type differ considerably. Low birth weight is certainly not a clinical entity; it is merely a manifestation shared by separate syndromes.

Some data relating to the effect of another nutritional stress should also be mentioned.[7] Of 10 rats receiving a single injection containing 50 mg tryptophan and 50 mg lysine in the final week of pregnancy, eight delivered one day earlier than 8 control rats mated at the same time. The remaining two treated rats delivered on the same day as the controls. The mean birthweight was 4.8 g in the treated group and 6.0 g in the controls. Although the difference in birthweight is significant, we are unwilling to conclude, without knowing the exact date of conception, that gestation was in fact shortened.

LITERATURE CITED

1. Chow, B. F., and C. J. Lee 1964 Effect of dietary restriction of pregnant rats on body weight gain of offspring. J. Nutrition, *82:* 10.
2. Chow, B. F. 1964 Growth of rats from normal dams restricted in diet in previous pregnancies. J. Nutrition, *83:* 289.
3. Lee, C. M., J. T. Wiseman, S. A. Kaplan and J. Warkany 1955 Effects of ethionine injections on pregnant rats and their offspring. J. Arch. Pathol., *59:* 232.
4. Schultz, R. L., P. W. Schultz and A. A. Corn 1960 Ethionine-induced resorption of the rat conceptus. Proc. Soc. Exp. Biol. Med., *105:* 88.
5. Proffit, W. R., and L. E. Edwards 1962 Effects of ethionine administration during pregnancy in the rat. J. Exp. Zool., *150:* 135.

[7] Unpublished observations, B. F. Chow, 1964.

Dietary Regulation of Pancreatic Enzyme Synthesis, Secretion and Inactivation in the Rat

JEAN TWOMBLY SNOOK [1]
Department of Animal Husbandry, University of California, Davis, California

ABSTRACT Dietary regulation of the synthesis, secretion and inactivation of trypsin, chymotrypsin and amylase was studied in rats fed a nitrogen-free ration or diets containing casein, whole-egg protein or hydrolysates of these proteins. Measurements of the enzyme content of the pancreas after an 11-hour fast showed that substitution of the nitrogen-free diet for the casein diet depressed synthesis of the 3 enzymes, whereas replacement with whole-egg protein usually increased synthesis of trypsinogen and chymotrypsinogen but not amylase. Relatively more of the amino acid supply of the pancreas was diverted to chymotrypsinogen and less to amylase when whole-egg protein was fed. The reverse was true when casein was given. Rats fed the nitrogen-free diet synthesized relatively more trypsinogen. With respect to trypsinogen and chymotrypsinogen, the secretory response elicited by hydrolyzed casein and hydrolyzed egg protein was similar to the one evoked by casein. However, hydrolyzed egg protein was not equivalent to whole-egg protein. Interpretation of these results is based on the premise that dietary protein regulates pancreatic enzyme synthesis, in part, through amino acid pool(s) in the pancreas. The observation that the nitrogen-free diet retarded the rate of inactivation of amylase more than did the protein or hydrolyzed protein diets may be related to a protective effect of starch which was included in the nitrogen-free diet.

The results of previous studies suggest that dietary proteins influence the tryptic, chymotryptic and amylolytic activities of the intestinal contents by regulating the synthesis, secretion and inactivation of digestive enzymes (1). This suggestion is corroborated by a number of observations. For example, Desnuelle et al. (2) and Howard and Yudkin (3) established that the pancreas "adapts" to a higher level of casein in the diet by increasing the synthesis of protease and decreasing the synthesis of amylase. Wang and Grossman (4) reported that the enzymic output of the pancreas is augmented by the presence of amino acids and peptones within the small intestine. Snook and Meyer (5) observed that the rate of enzyme inactivation within the intestine is retarded when protein diets as opposed to protein-free diets are fed.

The mechanisms regulating the response of digestive enzymes to dietary protein are complex and must be clarified. They were studied in the experiments reported herein by comparing changes in enzyme levels of pancreas and intestine when rats adapted to casein were changed to a nitrogen-free diet or to diets containing whole-egg protein or acid-hydrolysates of casein or egg protein.

EXPERIMENTAL

Male Sprague-Dawley rats, weighing approximately 180 g and trained to eat, as in previous studies (1), for 1-hour intervals spaced 12 hours apart, were used in all experiments. The rats were fed one of the following diets: nitrogen-free (essentially), casein, hydrolyzed casein, whole-egg protein,[2] and hydrolyzed egg protein. The proteins or hydrolyzed proteins usually made up 15% of the ration (a ration containing 30% hydrolyzed casein was also fed) and were added at the expense of sucrose in the diet. The remainder of each diet was composed of 5% cellulose powder, 5% cottonseed oil, 4% salts [3] and 1% vitamin mix.[4] The nitrogen-free diet con-

Received for publication February 1, 1965.

[1] This investigation was carried out during the tenure of a Postdoctoral Fellowship from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service. Present address: Department of Food and Nutrition, New York State College of Home Economics, Cornell University, Ithaca, New York.

[2] Whole-egg powder, hexane-extracted, was fed at a level of 18.6% to attain a 15% level of protein.

[3] The detailed composition of the vitamin and mineral mixes is presented elsewhere (1).

[4] See footnote 3.

tained 20% starch added at the expense of sucrose to improve palatability. The food intake of rats fed casein or whole-egg protein was restricted to that of rats fed hydrolyzed protein or nitrogen-free diets.

The whole-egg protein was hydrolyzed in 4 N H_2SO_4 by autoclaving for 24 hours at 120°. The acid solution was neutralized with $Ba(OH)_2$, filtered, clarified, and then dried by vacuum distillation followed by vacuum evaporation at 50°. Cystine and tryptophan (2.4 and 1.5 g/g of nitrogen, respectively) were added to the final dry product to compensate for losses sustained during preparation. Egg white trypsin inhibitor (EWTI) was added to one batch of ration in an amount approximating that in the original whole-egg protein, which was found to contain 4% EWTI. Tryptophan (1.2 g/g of nitrogen) was added to the acid-hydrolyzed casein, which was obtained from commercial sources as a salt-free powder.

The rats were killed by chloroform anesthesia 12 (zero time) or 2.5 hours after the initiation of the last feeding period, and the intestinal tracts and pancreatic glands were excised immediately. The small and large intestines were washed out separately with cold distilled water. The contents were maintained thereafter at 4°. The pancreatic glands were placed in 0.25 M sucrose solution and were homogenized for 2 minutes at 31,000 rev/min in a Virtis "45." Each homogenate was made up to a final volume of 10 ml with 0.2 M tris buffer, pH 8.1, containing 0.05 M $CaCl_2$; bovine trypsin (0.25 mg) was added to activate the pancreatic zymogens. The activation step was carried out at 4° for 24 hours. This mode of activation resembled that described in an earlier paper (5). The chromatographic data obtained from the earlier experiment indicated that little autodigestion of enzymes occurs during activation. Presumably, the enzymes are stabilized, to some extent, by the presence of calcium ion. The activated pancreatic solutions and the contents of the intestines were centrifuged for 15 minutes at 14,000 × *g* to remove particulate matter before aliquots of each were assayed for enzyme activity.

Details of all enzyme assays were presented in a previous paper (1). Chymotryptic and tryptic activities were estimated using N-acetyl-tyrosine ethyl ester (ATEE) and *p*-toluene sulfonyl-L-arginine methyl ester (TAME), respectively. Starch was used to test for amylase. The enzyme values presented in table 1–6 are averages of results from 4 rats and represent the total activity of intestinal contents or activated pancreas. The total rather than the specific activity of the pancreas probably more accurately depicts the ability of the pancreas to respond to a given amount of diet especially in an experiment of short duration.

RESULTS

Dietary change and intestinal and pancreatic enzyme levels. The effect of variations in dietary protein on enzyme synthesis secretion and inactivation was assessed by studying changes in the intestinal and pancreatic enzyme content of rats subjected to the dietary treatments summarized below and in tables 1–4. Rats fed casein for at least 1 week were changed to a nitrogen-free or whole-egg protein diet. Intestinal contents and activated pancreatic glands were assayed immediately prior to (zero time) or 2.5 hours after the initiation of the first or seventh feeding of the new diet. (Previous studies (1) indicated that under conditions of restricted feeding maximal adaptation of pancreatic enzymes to dietary protein is achieved by the seventh feeding period, whereas the enzyme activity of the intestinal contents appears to be greatest about 2.5 hours after feeding.) Then, groups of rats that had been given the nitrogen-free diet for 7 feedings were refed casein or were given the whole-egg protein ration. The digesta and pancreas of each of these rats were also assayed prior to or 2.5 hours after the first or seventh feeding of the new diet. According to the design of the experiment, all rats killed at zero time (12 hours after the initiation of the previous 1-hour feeding period) had been fasted for 11 hours.

It was hypothesized that differences in pancreatic enzyme levels, as assessed after an 11-hour fast when enzyme secretion was probably at a minimum and zymogen accumulation at a maximum, would reflect

the synthetic adaptation of the pancreas to diet. Intestinal enzyme levels 2.5 hours after one feeding of a different diet would be determined by the relative ability of the diet to act as a secretagogue and an inhibitor of enzyme inactivation. The difference in intestinal enzyme levels after 1 and 7 feedings would indirectly reflect the adaptation of the pancreas to the new diet.

The effect of the dietary regimen described above on intestinal protease activity is shown in table 1. Substitution of the nitrogen-free and whole-egg protein diets for the casein diet caused roughly a 100% increase and a 50% decrease, respectively, in intestinal chymotryptic and tryptic activities. After 7 feedings, differences between treatments were even more pronounced; statistically, the tryptic and chymotryptic activities increased significantly ($P < 0.01$) when rats were fed whole-egg protein instead of casein and decreased significantly ($P < 0.01$) when rats were fed the nitrogen-free diet instead of casein.

Intestinal tryptic activity increased significantly ($P < 0.01$) as soon as casein or whole-egg protein was given to rats fed the nitrogen-free diet for 7 feedings. The increase in chymotryptic activity was not as significant. The difference in the intestinal response of the 2 enzymes must have reflected, somewhat, the enzyme content of the pancreas of these rats during the early portion of the digestive period.

The enzyme activities of the large intestine, which are also listed in table 1, demonstrate that the tryptic and chymotryptic activities of the large intestine 2.5 hours after feeding were generally less than 10% of those of the small intestine despite an increase in the concentration of undigested material. These results are in agreement with the view (1, 5) that enzymes are largely inactivated and digested within the intestinal tract. The disappearance of activity in the large intestine also indicated that the substrate specificity of bacterial proteases does not correspond to that of trypsin and chymotrypsin. In line with this observation was the demonstration of Pelot and Grossman (6) that the intestinal contents of the rat lose virtually all ability to hydrolyze TAME and ATEE after a 16-hour diversion of pancreatic juice. Activities measured with less specific substrates (casein, starch) could represent a sum of the activities of bacterial, intestinal as well as pancreatic enzymes. The significant accumulation of tryptic activity which occurred in the large intestine when egg protein was fed may be related to the superior protection afforded by egg protein (1).

The pancreatic adaptation induced by the dietary regimen described above is shown in table 2. Measurements made after an 11-hour fast demonstrated that pancreatic synthesis of chymotrypsinogen and trypsinogen decreased significantly ($P < 0.01$) when rats were given the nitro-

TABLE 1

Adaptation of intestinal protease activity to dietary change

Dietary change		Duration of change	Trypsin [1]		Chymotrypsin [1]	
From	To	No. of feedings	Small intestine [2]	Large intestine [2]	Small intestine	Large intestine
			μmole TAME/min		*μmole ATEE/min*	
Casein	Casein	> 15	192 ± 14 [3]	19 ± 6	180 ± 26	13 ± 1
	Egg	1	366 ± 30	55 ± 12	384 ± 116	13 ± 5
	N-free	1	103 ± 5	4 ± 0	87 ± 12	5 ± 1
	Egg	7	483 ± 37	107 ± 25	621 ± 84	5 ± 1
	N-free	7	59 ± 10	7 ± 4	61 ± 9	3 ± 1
N-free	Casein	1	290 ± 14	5 ± 3	117 ± 19	1 ± 0
	Egg	1	338 ± 28	26 ± 10	202 ± 44	4 ± 2
	Casein	7	269 ± 27	11 ± 4	216 ± 15	2 ± 1
	Egg	7	356 ± 97	29 ± 5	529 ± 44	5 ± 1

[1] TAME and ATEE indicate p-toluene sulfonyl-L-arginine methyl ester and N-acetyl-L-tyrosine ethyl ester, respectively.
[2] Enzyme activities of contents of small and large intestines were determined 2.5 hours after the initiation of feeding.
[3] SE of mean.

gen-free diet for 6 feedings in place of casein. Chymotrypsinogen increased significantly ($P < 0.05$) when whole-egg protein was substituted for casein. Trypsinogen, apparently, was unaffected by this treatment. An analysis of combined data of this as well as similar experiments, one of which is summarized in table 6, indicated that synthesis of trypsinogen does increase when whole-egg protein replaces casein in the diet.

When rats that had been given the nitrogen-free diet for 7 feedings were changed to casein or whole-egg protein, the pancreatic content of trysinogen and chymotrypsinogen increased siginificantly ($P < 0.02$) within 6 feedings. Prior treatment with the nitrogen-free diet even appeared to augment the magnitude of the enzymic response to casein and whole-egg protein.

Pancreatic enzymes did not always adapt in a parallel fashion even when identical amounts of one adequate protein were substituted for another. For example, according to the data shown in table 2, the ratio of chymotrypsinogen to trypsinogen increased significantly when whole-egg protein was given in place of casein. This phenomenon was also demonstrated in the analysis of combined data.

The data presented in table 3 show that intestinal amylase activity usually varied with dietary change in the same direction as tryptic and chymotryptic activities. However, the adaptation of pancreatic amylase to dietary protein did not always parallel that of the proteolytic enzymes. Pancreatic amylase did decrease significantly ($P < 0.01$) when rats were fed the nitrogen-free diet. In this and subsequent experiments the amylolytic activity of the pancreatic glands of rats fed the casein and whole-egg protein rations never differed significantly. However, the ratio of amylase to trysinogen was significantly decreased for the pancreas of rats fed

TABLE 2

Effect of dietary change on pancreatic protease content

Dietary change		Duration of change	Trypsinogen [1]	Chymotrypsinogen [1]	Chymotrypsinogen / Trypsinogen
From	To	No. of feedings	*μmole TAME/min*	*μmole ATEE/min*	
Casein	Casein	> 15	732 ± 79 [2]	1415 ± 211	1.93 ± 0.14
	Egg	6	724 ± 52	2068 ± 132	2.86 ± 0.03
	N-free	6	328 ± 20	539 ± 81	1.64 ± 0.19
N-free	Casein	6	939 ± 57	1915 ± 385	2.04 ± 0.22
	Egg	6	1183 ± 89	3260 ± 384	2.74 ± 0.13

[1] Enzyme activities of activated pancreas were determined 12 hours after initiation of last feeding period.
[2] SE of mean.

TABLE 3

Influence of dietary protein on pancreatic and intestinal amylolytic activity

Dietary change		Duration of change	Intestinal contents [1]		Pancreas [2]	
From	To	No. of feedings	Amylase [3] units	Amylase [4] / Trypsin	Amylase [3] units	Amylase [4] / Trypsinogen
Casein	Casein	> 15	461 ± 113 [5]	2.32 ± 0.47	6325 ± 537	8.76 ± 0.55
	Egg	1	1000 ± 243	2.86 ± 0.79	—	—
	N-free	1	537 ± 76	5.26 ± 0.74	—	—
	Egg	6–7 [6]	826 ± 131	1.71 ± 0.31	4650 ± 505	6.39 ± 0.26
	N-free	6–7	217 ± 52	3.97 ± 1.02	1757 ± 75	5.42 ± 0.40
N-free	Casein	6–7	618 ± 85	2.29 ± 0.57	7435 ± 670	8.20 ± 1.03
	Egg	6–7	765 ± 270	2.98 ± 0.93	7262 ± 961	6.66 ± 0.85

[1] Contents of small intestine were assayed 2.5 hours after initiation of feeding.
[2] Pancreatic glands were assayed 12 hours after initiation of feeding.
[3] Milligrams starch digested per minute.
[4] Ratio of amylase to trypsin or trypsinogen.
[5] SE of mean.
[6] Intestinal contents and pancreatic glands were assayed after 6 and 7 feedings, respectively.

whole-egg protein instead of casein. The observation that whole-egg protein caused amylolytic activity to increase within the intestine of the fed rat but not the pancreas of the fasting rat might be explained again in terms of enzyme protection.

Determination of intestinal ratios of amylase to trypsin 2.5 hours after rats adapted to casein were fed casein, whole-egg protein or the nitrogen-free diet showed that relative to trypsin the nitrogen-free diet followed by the whole egg protein diet most effectively retarded the rate of inactivation of amylase. The stability of trypsin in intestinal contents always exceeded that of amylase. Chymotrypsin was more stable than amylase when protein diets were given; the opposite was true when the nitrogen-free diet was fed.

Effect of feeding on changes in pancreatic and intestinal enzyme levels. The variations of enzyme levels of the digestive system that occurred 2.5 hours after the regimens summarized in tables 1–3 were fed, were a function of the relative effect of each diet on enzyme synthesis, secretion and inactivation.

Intestinal enzyme activity =
enzyme secreted − enzyme inactivated
Pancreatic enzyme content =
enzyme synthesized − enzyme secreted

The net change in the enzyme activity of the intestine during the first 2.5 hours of the digestive period was determined by subtracting the mean intestinal enzyme activity of rats killed 2.5 hours after the initiation of the feeding period from that of rats killed at zero time. The pancreatic change was calculated in a similar manner.

The net change, presented in table 4 as a percentage of the enzyme level at zero time, demonstrated once again that digestive enzymes did not react to diet in a completely parallel fashion. Assuming that enzymes stored in the zymogen granules were secreted into the intestine in parallel, the percentage change in the enzyme level of the pancreas was identical for all enzymes studied if, and only if, these enzymes were synthesized in a parallel fashion. This did not appear to be the case. The data are somewhat difficult to interpret since the feeding of a given diet did not produce a consistent effect on the pancreas. However, in rats depleted of protein for 3.5 days the enzyme levels of the pancreas often increased during the first 2.5 hours of the digestive period. Thus, enzymes were synthesized more rapidly than they were secreted. Moreover, because these rats were somewhat depleted of protein, the pancreatic changes produced by refeeding protein should have reflected the quality of the protein more closely than those produced by feeding the same protein to rats with more sizable endogenous nitrogen stores with which dietary amino acids could mix prior to enzyme synthesis.

As soon as the protein-depleted rats were fed whole-egg protein, proportionally more chymotrypsin and less amylase were syn-

TABLE 4

Percentage change in intestinal and pancreatic enzyme levels during the first 2.5 hours of the digestive period

Dietary change		Duration of change	Trypsin		Chymotrypsin		Amylase	
From	To	No. of feedings	ΔI[1]	ΔP[1]	ΔI	ΔP	ΔI	ΔP
			%	%	%	%	%	%
Casein	Casein	> 15	+214	−24	+ 59	−24	+ 62	−32
	Egg	1	+498	−28	+240	−48	+250	−62
	N-free	1	+ 68	−30	− 23	−47	+ 89	−31
	Egg	7	+299	−32	+224	−27	+293	+ 3
	N-free	7	− 18	+16	− 13	− 7	+ 47	+41
N-free	Casein	1	+303	+39	+ 66	+37	+159	+118
	Casein	7	+271	− 6	+ 86	−27	+118	−28
	Egg	1	+370	+17	+188	+49	+230	−19
	Egg	7	+220	− 8	+237	−26	+1062	−27

[1] Δ I and Δ P represent the change in intestinal and pancreatic enzyme levels, respectively. The changes in enzyme level are presented as a percentage of the zero time (pre-feeding) enzyme level. See text for method of calculation.

thesized. The pancreases of the protein-depleted rats given casein synthesized trypsin and chymotrypsin in the same proportion but synthesized relatively more amylase.

The data of table 4 also show that factors influencing intestinal enzyme levels did not affect all enzymes equally. The inactivation phenomenon is probably of greatest significance in this respect. It must be emphasized that the factors which affect inactivation and the protection against inactivation may not have been the same at 2.5 and at 12 hours (zero time) after the initiation of the 1-hour feeding period. For example, previous work (1) demonstrated that proportionally less chymotrypsin was inactivated during in vitro incubation at 4° of intestinal contents obtained at 8 and 12 hours as opposed to 2.5 hours after the initiation of feeding. Also, the tryptic activity of the large intestine was elevated at 2.5 hours when whole-egg protein was fed (table 1) but not at 12 hours. Thus, the relative changes in intestinal enzymes during the first 2.5 hours of the digestive period did not necessarily reflect the relative stability of the enzyme at 2.5 hours because stability at 12 hours also influenced the calculation.

Effect of feeding hydrolyzed diets. A more complete explanation of differences in the pancreatic and intestinal response to casein and whole-egg protein was sought in the experiments summarized in tables 5 and 6. This objective was carried out by feeding hydrolysates of these proteins to determine whether variations in amino acid composition of the diet were sufficient to evoke the differences in response. The data shown in table 5 suggest that the pancreatic content of trypsinogen and chymotrypsinogen and the tryptic and chymotryptic activities of the intestine were not lowered when rats were fed hydrolyzed casein instead of casein. These results did not concur with those of Grossman et al. (7) and Howard and Yudkin (3) who noted that substitution of casein hydrolysate for casein caused the proteolytic enzyme content of the pancreas to decrease. However, these investigators fed their rats ad libitum, conducted feeding trials of longer duration and did not assay for trypsinogen and chymotrypsinogen but for total protease.

The data of table 5 also show that the level of chymotrypsinogen was raised but not significantly at 2.5 hours when rats were fed 15% casein hydrolysate. Trypsinogen and chymotrypsinogen increased at 2.5 and at 12 hours (after 6 feedings) when 30% hydrolyzed casein instead of 15% casein was fed. The increases in trypsinogen and chymotrypsinogen were significant ($P < 0.05$). The chymotryptic activity of the small intestine also increased significantly ($P < 0.05$) when hydrolyzed casein was fed. Because previous work indicated that dietary protein protects digestive enzymes within the intestine (1), chymotrypsin was probably inactivated more rapidly within the digesta of rats fed hydrolysate instead of protein. Hence, enzyme secretion may have increased considerably when hydrolysate was given, and the increase in pancreatic

TABLE 5

Influence of hydrolyzed casein diets on protease content of intestine and pancreas

Ration	No. of feedings	Time [1]	Small intestine		Pancreas	
			Trypsin	Chymotrypsin	Trypsinogen	Chymotrypsinogen
		hours	*μmole TAME/min*	*μmole ATEE/min*	*μmole TAME/min*	*μmole ATEE/min*
Casein, 15%	> 60	12	55 ± 29 [2]	73 ± 9	700 ± 72	1350 ± 200
Casein, 15%	> 60	2.5	84 ± 32	93 ± 9	624 ± 29	1075 ± 33
Hydrolyzed casein, 15%	1	2.5	99 ± 9	123 ± 2	634 ± 40	1469 ± 166
Hydrolyzed casein, 30%	1	2.5	103 ± 26	129 ± 10	915 ± 85	2105 ± 223
Hydrolyzed casein, 15%	6	12			832 ± 68	1581 ± 152
Hydrolyzed casein, 30%	6	12			1180 ± 197	3000 ± 605

[1] Hours after the initiation of the 1-hour feeding period.
[2] SE of mean.

TABLE 6

Effect of hydrolyzed egg protein and egg-white trypsin inhibitor (EWTI) on pancreatic and intestinal enzymes

Ration	No. of feedings	Time [1]	Small intestine			Pancreas		
			Trypsin	Chymotrypsin	Amylase	Trypsinogen	Chymotrypsinogen	Amylase
		hours	*μmole TAME/min*	*μmole ATEE/min*	*mg starch/min*	*μmole TAME/min*	*μmole ATEE/min*	*mg starch/min*
Egg protein	1	2.5	168 ± 31 [2]	302 ± 28	1383 ± 154	488 ± 47	1125 ± 224	5361 ± 1727
Hydrolyzed egg protein	1	2.5	169 ± 37	171 ± 43	677 ± 92	643 ± 44	1205 ± 44	5160 ± 1109
Hydrolyzed egg protein + EWTI	1	2.5	121 ± 26	182 ± 34	966 ± 331	563 ± 44	1340 ± 102	4399 ± 557
Casein	> 15	12				580 ± 65	1350 ± 154	5339 ± 1212
Egg protein	6	12				859 ± 102	1900 ± 308	6110 ± 1434
Hydrolyzed egg protein	6	12				579 ± 38	1215 ± 172	3963 ± 529
Hydrolyzed egg proten + EWTI	6	12				610 ± 67	1227 ± 164	3349 ± 852

[1] Hours after the initiation of the 1-hour feeding period.
[2] SE of mean.

enzymes at 2.5 hours suggested an increase in enzyme synthesis rather than a decrease in enzyme release.

According to the data tabulated in table 6, the hydrolyzed egg protein diet, even when it contained EWTI, was not equivalent to whole-egg protein. Hydrolysis appeared to destroy those properties which enable egg protein to act as an effective secretagogue or inhibitor of enzyme inactivation, or both, as is evidenced by the fact the chymotryptic and amylolytic activities of the intestinal contents were lower when hydrolysate was given instead of whole-egg protein.

Pancreatic adaptation to egg protein was also affected by hydrolysis. After 6 feedings of hydrolyzed egg protein, the pancreatic content of trypsinogen and chymotrypsinogen remained at the level established on the casein diet, whereas it increased when whole-egg protein was fed. The amylolytic content of the pancreas was also reduced. The effect of hydrolyzed egg protein on pancreatic enzymes at 2.5 hours is debatable. In all instances, the addition of EWTI to the hydrolyzed diet appeared to have little effect on the pancreatic and intestinal enzymes.

DISCUSSION

Results of the experiments reported herein establish more firmly that the protein components of the diet alter the enzyme levels of the intestine by regulating pancreatic enzyme synthesis, secretion, and inactivation. Comparison of the enzyme activities of small and large intestine and pancreas provided additional information about the inactivation phenomenon. Probably more than 90% of trypsin and chymotrypsin are inactivated within the digestive tract. Twombly and Meyer (8) previously calculated, using chromic oxide as an indicator, that about 90% of the endogenous protein secreted or sloughed into the digestive tract is digested and absorbed. It should be stressed that digestion of endogenous protein is probably not the complete responsibility of the proteolytic enzymes normally associated with the digestive process. Borgström et al. (9) have suggested that microbial intestinal inhabitants are at least partially responsible for the normal inactivation of digestive en-

zymes. Hsu and Tappel (10), upon demonstrating the presence of lysosomal enzymes in rat intestinal mucosa, postulated that these enzymes could digest mucous and sloughed mucosal cells.

Relative to trypsin, the nitrogen-free diet more effectively than whole-egg protein retarded the rate of inactivation of amylase. The egg diet, itself, definitely provides a great deal of protection because the amylase content of the rat intestine was lowered more than 50%, whereas trypsin remained constant when hydrolyzed egg rather than whole-egg protein was fed (table 6). Perhaps, the starch in the nitrogen-free diet inhibited the digestion of amylase. The binding of starch and amylase might protect amylase from attack by the proteolytic enzymes. This observation merits further investigation.

A comparison of the relative effects of feeding hydrolyzed casein and the nitrogen-free diet (tables 1, 4, 5), neither of which retarded the inactivation of proteolytic enzymes, shows that the presence of dietary amino acids in the small intestine increased the enzymic output of the pancreas. Therefore, it appears that under normal feeding conditions the major stimuli of enzyme secretion are the dietary proteins and their breakdown products. The role of other foodstuffs, of breakdown products of endogenous proteins, and of neural mechanisms not related to the presence of dietary amino acids in the digestive tract appears to be much less important.

One of the more interesting aspects of these investigations was the observation that the enzymic complement of the pancrease can be altered without changing the level of nitrogen in the diet. Several investigators have shown that the enzyme levels of the pancreas are related to the amino acid content of the diet. Magee and Hong (11) reported that the addition of methionine, phenylalanine or isoleucine to a low casein (7%) diet increased the protease content of the rat pancreas. Lyman and Wilcox (12) observed that essential amino acid deficiencies lowered the enzyme content of the rat pancreas with certain enzymes being affected more by one deficiency than another. Véghelyi and Kemény (13) showed that the pancreatic glands of rats fed methionine-deficient diets for 4 weeks contained normal amounts of amylase, reduced amounts of chymotrypsin and no trypsin. The data shown in table 2 demonstrated that pancreatic enzyme levels were altered even when one adequate protein (whole-egg) was substituted for another (casein).

Interpretation of these observations is somewhat difficult considering the myriad of factors that influence protein synthesis. However, Munro (14) has indicated that the endoplasmic reticulum of the pancreatic acinar cell is not particularly sensitive to changes in diet. He observed that protein depletion did not affect RNA metabolism in the pancreas or alter the amount of RNA per cell. Thus, the synthesis of chymotrypsinogen, trypsinogen, and amylase may be regulated in part by the size and composition of the amino acid pool(s) existing in the pancreas at the time of synthesis. A change in the size of the amino acid pool(s) could explain the decrease and increase, respectively, in protease synthesis when the nitrogen-free diet and 30% hydrolyzed casein diet were substituted for the 15% hydrolyzed casein diet.

Also, results with hydrolyzed protein diets suggest that dietary protein does not necessarily have some unique quality which induces greater synthesis of pancreatic protease. After 6 feedings and an 11-hour fast, the pancreatic enzyme levels produced with the 15% hydrolyzed casein diet were comparable to those observed when casein was fed. The levels were not similar 2.5 hours after the first feeding of the hydrolyzed diet. The difference in response to the 2 diets must reflect the known difference in their respective rates of assimilation. Although hydrolyzed egg was not equivalent to whole-egg protein, its influence on the synthesis of chymotrypsinogen and trypsinogen was similar to that of casein. Perhaps, the difference between the hydrolyzed egg and whole-egg diets can be ascribed to variations in assimilation of the 2 diets or to an uncompensated hydrolytic loss of amino acids. In line with the latter hypothesis was the observation that the growth-promoting properties of casein which are lost during acid hydrolysis can be restored by making

the proper additions to the hydrolysate (15).

A comparison of ratios of pancreatic enzymes (tables 2 and 3) demonstrates that when whole-egg protein was substituted for casein, proportionally more of the amino acid supply of the pancreas was used to synthesize chymotrypsin and less was diverted to amylase. This effect was comparable to the one produced by feeding whole-egg protein to protein-depleted rats (table 4). The observation that rats fed casein synthesize relatively more amylase might be related to the methionine content of casein. Methionine is not required for maximal synthesis in vitro of amylase (16).

Variations during the digestive period (such as at 2.5 and 12 hours) in the relative enzymic composition of pancreas of rats adapted to their diet for at least 3 days may have been related to changes in the composition of the pancreatic amino acid pools caused by differences in the mixing, interaction, and compartmentalization of dietary and endogenous amino acids. At the same time, the possibility cannot be excluded that dietary protein also regulates pancreatic enzyme synthesis by mechanisms not directly involving an amino acid pool.

LITERATURE CITED

1. Snook, J. T., and J. H. Meyer 1964 Response of digestive enzymes to dietary protein. J. Nutrition, *82:* 409.
2. Desnuelle, P., J. P. Reboud and A. Ben Abdeljlil 1961 Influence of the composition of the diet on the enzyme content of rat pancreas. Ciba Foundation Symposium on the Exocrine Pancreas. J. and A. Churchill Ltd., London, p. 90.
3. Howard, F., and J. Yudkin 1963 Effect of dietary change upon the amylase and trypsin activities of the rat pancreas. Brit. J. Nutrition, *17:* 281.
4. Wang, C. C., and M. I. Grossman 1951 Physiological determination of release of secretin and pancreozymin from intestines of dogs with transplanted pancreas. Am. J. Physiol., *164:* 527.
5. Snook, J. T., and J. H. Meyer 1964 Effect of diet and digestive processes on proteolytic enzymes. J. Nutrition, *83:* 94.
6. Pelot, D., and M. I. Grossman 1962 Distribution and fate of pancreatic enzymes in small intestine of the rat. Am. J. Physiol., *202:* 285.
7. Grossman, M. I., H. Greengard and A. C. Ivy 1944 On the mechanism of the adaptation of pancreatic enzymes to diet. Am. J. Physiol., *141:* 38.
8. Twombly, J., and J. H. Meyer 1961 Endogenous nitrogen secretions into the digestive tract. J. Nutrition, *74:* 453.
9. Borgström, B., A. Dahlquist, B. E. Gustafsson, G. Lundh and J. Malmquist 1959 Trypsin, invertase, and amylase content of feces of germfree rats. Proc. Soc. Exp. Biol. Med., *102:* 154.
10. Hsu, L., and A. L. Tappel 1965 Lysosomal enzymes and mucopolysaccharides in the gastrointestinal tract of the rat and pig. Biochem. Biophys. Acta, *101:* 83.
11. Magee, D. F., and S. S. Hong 1956 Changes in pancreatic enzymes brought about by amino acid additions to the diet. Am. J. Physiol., *184:* 449.
12. Lyman, R. L., and S. S. Wilcox 1963 Effect of acute amino acid deficiencies on carcass composition and pancreatic function in the force-fed rat. I. Deficiencies of histidine, methionine, phenylalanine, and threonine. J. Nutrition, *79:* 28.
13. Véghelyi, P. V., and T. T. Kemény 1961 Protein metabolism and pancreatic function. Ciba Foundation Symposium on the Exocrine Pancreas. J. and A. Churchill Ltd., London, p. 329.
14. Munro, H. N. 1964 General aspects of the regulation of protein metabolism by diet and by hormones. In: Mammalian Protein Metabolism, eds., H. N. Munro and J. B. Allison. Academic Press, New York.
15. Breuer, Jr., L. H., W. G. Pond, R. G. Warner and J. K. Loosli 1964 The role of dispensable amino acids in the nutrition of the rat. J. Nutrition, *82:* 499.
16. Hokin, L. E. 1951 Amino acid requirements of amylase synthesis by pigeon pancreas slices. Biochem. J., *50:* 216.

Amino Acid Balance and Nitrogen Retention in Man as Related to Prior Protein Nutriture [1]

HANS FISHER, M. K. BRUSH, P. GRIMINGER AND E. R. SOSTMAN
Nutrition Laboratories, Departments of Animal Sciences and Home Economics, Rutgers-The State University, New Brunswick, New Jersey

ABSTRACT A series of experiments was carried out with male college students to determine the influence of prior protein nutriture on the utilization of high N-low tryptophan diets. There was an inverse relationship between prior protein nutriture and the subsequent utilization of the high N-low tryptophan diet. When subjects consumed a balanced, high protein diet they were in strong positive N balance, which, when followed by the high N-low tryptophan diet, led to little N retention. When the initial protein intake consisted of a low level of hydrolyzed casein, supplemented with adequate tryptophan, the initial N balance was essentially zero; during the intake of the high N-low tryptophan diet, the subjects were in greater positive N balance than previously noted following intake of the balanced, high protein diet. When, during a third experiment, the initial period consisted of a low protein intake coupled with an inadequate intake of tryptophan, the subjects were in strong positive balance during the subsequent ingestion of the high N-low tryptophan diet. When, during a fourth experiment, the subjects were immediately given the high N-low tryptophan diet, they were in severe negative N balance. Significant differences in urinary creatinine excretion were observed in some experiments, with no consistent pattern relative to N intake. The variations in urinary creatinine as well as the changes from normal observed in plasma urea N and amino acid N suggest that in young adults there are marked differences in the ultilization of high N diets inadequate in one or more amino acid which, in immature animals, would bring about depressed growth. Changing the N source used to supplement hydrolyzed casein from gelatin to an isonitrogenous mixture of nonessential amino acids did not change the results obtained with the imbalanced diet.

The importance of amino acid balance in the nutrition of the growing rat and chicken has been well documented (1). In short-term studies with male college students (2), however, we did not observe any impairment in nitrogen utilization with diets which would lead to growth depression in young animals. Swendseid and associates (3) have reported impaired N utilization in men over 60 years old, when the N intake was increased while the level of essential amino acid N remained constant. With college-age men and women these workers observed no evidence of inferior N utilization under similar experimental conditions (3). The present studies were undertaken to extend our earlier work with specific reference to the role of prior protein nutriture on subsequent N retention with diets high in N but inadequate with respect to an essential amino acid.

EXPERIMENTAL

Five experiments were carried out. The essential details of the experimental procedures, including the composition of the basic low and high N-low tryptophan diets, have been described previously (2). The experiments varied in length from two to four weeks and were subdivided into periods of 7 days' duration (periods were lettered A to D). Four or five male college students made up the experimental subjects. Two subjects that served in experiment 1 served again in experiment 2 and again a different pair of subjects that served in experiment 4 served again in experiment 5. All analyses except that for creatinine were made by procedures described previously (2). Creatinine was de-

Received for publication May 21, 1965.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick. Supported in part by grants-in-aid from the U. S. Public Health Service A-4904 and the National Science Foundation.

termined by the method of Owen et al. (4), which we had found satisfactory in other studies (5).

Table 1 outlines the experimental design, and details the source and quantity of dietary N. The protein and amino acid intake during the initial dietary period (period A) preceding the intake of the high N-low tryptophan diet was the variable in experiments 1 to 4. In experiment 1, a high level of good quality protein that provided a large excess of tryptophan was given during the initial period; in experiment 2 a barely adequate level of protein from salt-free hydrolyzed casein with just sufficient tryptophan to meet this amino acid requirement (200 mg) was provided; in experiment 3 the same barely adequate level of salt-free hydrolyzed casein as used in experiment 2 was initially supplemented with an inadequate amount of tryptophan (100 mg); in experiment 4 the subjects were immediately given the imbalanced, high N-low tryptophan diet.

Experiment 5 was essentially a repetition of experiment 3 except that a mixture of nonessential amino acids [2] was substituted for gelatin during the imbalance period (period B) with the high N, inadequate tryptophan diet.

RESULTS

Experiments 1 to 3. Table 2 shows that during period A with the balanced, high protein-high tryptophan intake (exp. 1) the subjects were in strong positive balance. They were essentially in N equilibrium with the minimal N intake provided by salt-free hydrolyzed casein supplemented with an adequate amount of tryptophan during period A of experiment 2. All subjects were in negative N balance when the level of tryptophan provided in conjunction with a minimal intake of hydrolyzed casein protein was sub-optimal (period A, experiment 3). The N balance observed for all 3 experiments during period B was inversely related to the response during period A. Thus, the subjects just maintained N equilibrium following the strong N retention with the balanced, high protein-high tryptophan diet; they were in good positive N balance during

[2] The nonessential amino acid mixture was composed of the following in g N: glycine, 5.6; DL-alanine, 2.5; and L-aspartic acid, 1.9.

TABLE 1

Experimental design and details of dietary nitrogen supplied

Exp. no.	Period [1]	Source and quantity of dietary nitrogen supplied							Dietary L-tryptophan
		Mixed foods [2]	Salt-free hydrolyzed casein	Gelatin	Miscellaneous foods	Glycine	DL-Alanine	L-Aspartic acid	
		g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*mg/day*
1	A	18.13							1100 [3]
	B		9.0	10.0	0.59				113
	C		9.0	10.0	0.59				213
2	A		9.0	0	0.47				213
	B		9.0	10.0	0.47				113
	C		9.0	10.0	0.47				213
3	A		9.0	0	0.47				113
	B		9.0	10.0	0.47				113
	C		9.0	10.0	0.47				213
4	A		9.0	10.0	0.47				113
	B		9.0	10.0	0.47				113
	C		9.0	10.0	0.47				213
	D		9.0	10.0	0.47				213
5	A		9.0	0	0.47				113
	B		9.0	0	0.47	5.6	2.5	1.9	113

[1] Each period was of 7 days' duration.
[2] Mixed foods included eggs, orange juice, biscuits, margarine, heavy cream, low fat bacon, Cheddar cheese, whole wheat bread, sliced peaches, skim milk, cookies, low fat beef, carrots, potatoes, white bread, lettuce, and vanilla ice cream.
[3] Calculated from the tables of Watt and Merrill (7).

TABLE 2

Nitrogen balance and plasma and urinary N constituents of male college students receiving different amounts of dietary N and tryptophan

Period [1]	Daily intake		N balance	Urine		Plasma	
	Nitrogen [2]	Tryptophan		Creatinine	Volume	Urea N	Amino acid N
	g	*mg*	*g/day*	*g/day*	*ml/day*	*mg/100 ml*	*mg/100 ml*
			Experiment 1				
A	18.13	1100 [3]	2.58 ± 0.29 [4]	1.82 ± 0.03	1725 ± 165	11.8 ± 0.5	5.36 ± 0.15
B	19.59	113	0.30 ± 0.10	2.38 ± 0.06	2346 ± 102	21.5 ± 2.7	7.27 ± 0.25
C	19.59	213	−0.35 ± 0.53	2.21 ± 0.09	1730 ± 93	21.4 ± 2.1	7.44 ± 0.39
			Experiment 2				
A	9.47	213	−0.59 ± 0.32	1.23 ± 0.07	1328 ± 94	13.4 ± 0.9	6.86 ± 0.32
B	19.47	113	1.78 ± 1.00	1.66 ± 0.09	1739 ± 139	19.2 ± 1.8	8.26 ± 0.24
C	19.47	213	1.38 ± 0.82	1.71 ± 0.04	1620 ± 147	18.9 ± 1.2	7.50 ± 0.68
			Experiment 3				
A	9.47	113	−1.62 ± 0.31	1.67 ± 0.11	1158 ± 294	12.7 ± 0.3	6.61 ± 0.42
B	19.47	113	3.78 ± 1.22	1.61 ± 0.05	1461 ± 344	21.3 ± 2.0	9.00 ± 0.44
C	19.47	213	1.92 ± 0.52	1.72 ± 0.08	1439 ± 294	17.7 ± 1.3	8.42 ± 0.59
			Experiment 4				
A	19.47	113	−1.19 ± 0.49	1.88 ± 0.16	1267 ± 231	17.4 ± 0.7	8.18 ± 0.34
B	19.47	113	−0.42 ± 0.10	1.94 ± 0.13	1314 ± 185	18.8 ± 0.7	8.51 ± 0.34
C	19.47	213	1.17 ± 0.38	1.88 ± 0.07	1280 ± 229	16.4 ± 0.8	8.30 ± 0.84
D	19.47	213	2.35 ± 0.33	1.97 ± 0.02	1106 ± 186	18.0 ± 0.6	8.25 ± 0.32
			Experiment 5				
A	9.47	113	0.10 ± 0.19	1.70 ± 0.07	836 ± 83	11.3 ± 0.6	6.20 ± 0.26
B	19.47	113	1.66 ± 0.44	1.62 ± 0.06	1018 ± 79	22.7 ± 3.3	7.58 ± 0.41

[1] Each period was of 7 days' duration.
[2] See table 1 for details of dietary N.
[3] Calculated, using the table of Watt and Merrill (7).
[4] Standard errors were calculated from the average values for 4 to 5 male college students; the average value for each student represents four 24-hour collections in the case of urinary constituents and N balance, and a single weekly determination for the plasma analyses.

period B following a minimal but adequate intake of protein and tryptophan (exp. 2); and they were in very strong positive N balance during period B of experiment 3 when the prior tryptophan intake had been inadequate and the subjects had been in severe negative N balance.

During experiment 1 there was a highly significant increase ($P < 0.001$) in creatinine excretion from period A to B. In experiment 2 creatinine excretion also increased significantly from period A to B, whereas essentially no change occurred during experiment 3. The increases in urinary creatinine cannot be ascribed to dietary creatine or creatinine since, during period B in all of these experiments, the diet was devoid of these constituents. In all 3 experiments there were increases in urine volume during period B in comparison with period A. Increases were also noted in plasma urea N and in plasma amino acid N. There appeared to be a direct relationship between the extent of N retention during period B and the level of plasma amino acid N.

Experiment 4. The essential inadequacy of the high N-low tryptophan diet used during periods B and C of experiments 1 to 3 is illustrated by the negative N balance of all subjects given this diet as the starting diet (period A, exp. 4, table 2). Continuing with the same N and tryptophan intake the subjects during period B were in less negative N balance. During periods C and D with the tryptophan intake doubled the subjects were in good positive N balance. There were no changes in creatinine excretion among periods. The improved N retention during periods C and D was not reflected in any decrease in plasma urea or amino acid nitrogen.

Experiment 5. The results for experiment 5 were essentially similar to those observed during periods A and B of experiment 3 (table 2). The subjects were in N equilibrium during period A and were in strong positive N balance during period B when 10 g of nonessential amino acid N was added replacing 10 g N from gelatin as used for experiments 1 to 4. As in the earlier experiments, during period B there was an increase in urine volume, in plasma urea N and plasma amino acid N. No change in creatinine excretion between periods was noted.

DISCUSSION

These experiments reveal an inverse relationship between N retention and protein nutriture prior to the ingestion of a diet high in N and inadequate with respect to at least one essential amino acid. The important question that arises concerns the significance of the considerable N retention that occurred when the subjects had been in negative N balance prior to eating the imbalanced diet. When the plasma urea N and amino acid N concentrations during period A of experiment 1 (good quality, high N diet) are compared with those of period A for experiments 2 and 3, the results suggest that a portion of the retained N during period B of experiments 2, 3 and 5 (poor quality, high N diet) is present as non-protein N.

The results observed in experiments 1 to 3 during period B with the high N-low tryptophan diet are all the more striking in view of the poor results obtained with the same diet during periods A and B of experiment 4. A dietary history recall showed that the subjects used for experiment 4 had a range of N intake between 13.3 and 29.8 g during the week prior to the start of the experiment. The quality of the protein was generally good. It is difficult to decide whether the improved N balance during periods C and D of experiment 4 resulted from the higher tryptophan intake or reflects N lost the previous weeks. There was certainly no reduction in plasma urea or amino acid N attributable to the higher intake or changed N balance. The essentially unchanged plasma urea and amino acid N levels during periods A to D of experiment 4 when the subjects changed from severe negative to strong positive N balance provides no clue as to the nature of the retained N and even casts doubt on the suggestion made earlier that the high plasma N levels represent a portion of the retained N. These studies corroborate our earlier one (2) and indicate no overt, adverse effects in young adults from the short-term ingestion of a diet which, if given to growing animals, would produce a depression in food intake and in growth rate. Nonetheless, the increase in non-protein nitrogenous components of blood and urine suggest a different pattern of metabolism from that observed when large amounts of balanced protein are ingested (compare, for example, the values for period A, experiment 1 with those of period B for all other experiments).

Of considerable importance was the observation that creatinine excretion could vary significantly with dietary manipulation. The observed increased excretions corresponded in both instances with a change from a balanced to an imbalanced diet; in experiment 1, also, the increase occurred with the essentially creatine-creatinine-free diet (during period B), whereas the earlier diet (consumed during period A) contributed small amounts of creatine in the bacon and beef. These results support conclusions reached in a series of studies with rats (5) to the effect that considerable caution must be exercised in using creatinine excretion results as constants for the calculation of other biological values.

The observations of experiment 5 (table 2) suggest that the results obtained during period B of experiment 3 are not due to the unique amino acid pattern of gelatin. Since a mixture of nonessential amino acids gave a response similar to that of gelatin, it is not unexpected that the addition of an adequate level of tryptophan to the casein-gelatin diet during period C of experiments 1 to 4 did not bring about a "normalization" in blood-urea and amino acid N values. This suggests that the elevated plasma levels did not necessarily result from the inadequate tryptophan supplied during period B.

The observation of considerable N retention from sources such as gelatin or nonessential amino acids in young adults

is in line with observations that a portion of the protein reserves of adult cocks could be replenished, following depletion, with an N source of nonessential amino acids (6).

ACKNOWLEDGMENT

We would like to thank Mrs. Carol Berdanier, Mrs. Eileen Robinson, Mrs. Mildred Prieto, Hans Lutz, and John Ashley for their help and cooperation throughout these studies.

LITERATURE CITED

1. Harper, A. E. 1964 Amino acid toxicities and imbalances. In Mammalian Protein Metabolism, eds., H. N. Munro and J. B. Allison. Academic Press, New York.
2. Fisher, H., M. K. Brush, R. Shapiro, J. P. H. Wessels, C. D. Berdanier, P. Griminger and E. R. Sostman 1963 Amino acid balance in the adult: High nitrogen — low tryptophan diets. J. Nutrition, *81:* 230.
3 Swendseid, M. E. 1963 The protein needs of adult man. Proceedings of a Symposium on Protein Nutrition and Metabolism, The University of Illinois. Special Publication no. 4.
4. Owen, J. A., B. Iggo, F. J. Scandrett and C. P. Stewart 1954 The determination of creatinine in plasma or serum, and in urine: A critical examination. Biochem. J., *58:* 426.
5. Fisher, H. 1965 Variations in the urinary creatinine excretion of rats fed diets with different protein and amino acid content. J. Nutrition, *85:* 181.
6. Wessels, J. P. H., and H. Fisher 1965 Estimation of protein reserves and the nitrogen content of organs in protein depleted and repleted cocks. Brit. J. Nutrition, *19:* 57.
7. Watt, B. K., and A. L. Merrill 1963 Composition of Foods. Handbook no. 8. U. S. Department of Agriculture, Washington, D. C.

Aortic Acid Mucopolysaccharides and Collagen in Scorbutic Guinea Pigs [1,2]

IRA GORE, YUKIO TANAKA, TAKAO FUJINAMI AND MAX L. GOODMAN
Department of Pathology, University Hospital, Boston University Medical Center, Boston, Massachusetts

ABSTRACT After 4 weeks of receiving an ascorbic acid-deficient diet, otherwise healthy young guinea pigs displayed: an increase of total aortic acid mucopolysaccharides, essentially the result of increased hyaluronic acid content and a decrease of aortic collagen measured as hydroxyproline. These changes are similar to those observed in wound repair during scurvy, although they are quantitatively less. Collagen in the aortic wall was metabolically more active than it was in other body structures.

Morphologically, ascorbic acid deficiency is characterized by inadequate formation of collagen (1). The defect is most readily observed in reparative and proliferative mesenchymal reactions, or both, but can be demonstrated in uninjured tissue as well (2). In the deficiency state, hydroxyproline, an essential constituent of collagen, is not formed from its precursor, proline (3–7). Concurrently, the fibroblast displays ultrastructural changes indicative of impaired protein synthesis (8). Since acid mucopolysaccharides are also produced by the fibroblast, and bear a close histologic relationship to collagen fibers, there has been substantial interest in ascertaining how they may be affected by the scorbutic state. Penney and Balfour (9) reported diminished metachromasia in scorbutic wounds, whereas Bunting and White (10), as well as Persson (11) observed an increase; Persson (11), moreover, demonstrated an increased content of hexosamine. However, neither Ludwig (12) nor Kodicek and Loew (13) observed an influence of scurvy upon the hexosamine content of proliferating mesenchymal tissue. Dunphy and Udupa (14), Robertson and Hinds (15), and Kimoto and associates (16, 17), contrarily, determined that the mucopolysaccharide content of scorbutic granulation tissue was increased. With respect to its composition, there is general agreement that the hyaluronic acid predominates (15, 18, 19). A decrease of sulfated acid mucopolysaccharides has been shown with radioisotopic sulfur (9, 13, 18) and confirmed by histochemistry (20).

Studies of injury and repair in scorbutic animals have been so revealing that investigations of uninjured tissues have been neglected. Of these, it seemed most promising to explore the changes produced by scurvy in the aorta, since it is a structure exposed to continuous physiologic stress. Willis (21) has reported that scurvy in guinea pigs caused superficial lipid deposits in the aorta, and Myasnikov (22) observed that the administration of ascorbic acid to cholesterol-fed rabbits reduced the severity of intimal lesions. Previous studies (23) have indicated that variations in the quantity and composition of aortic acid mucopolysaccharides in man could be an important factor in the development and progression of atherosclerosis in man. The question of whether similar changes might explain the reported occurrence of intimal lesions in scorbutic guinea pigs, merited investigation.

METHODS

Seventy young adult female guinea pigs weighing 350 to 400 g each were fed a commercial scorbutogenic diet [3] formulated as recommended by Woodruff et al. (24), to which a vitamin fortification mix-

Received for publication June 15, 1965.

[1] Presented in part at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1965, Atlantic City, New Jersey.

[2] Supported in part by Public Health Service Research Grant no. HE 0-7327 from the National Institutes of Health, and a grant-in-aid from the Tobacco Research Council.

[3] Nutritional Biochemicals Corporation, Cleveland.

ture,[4] but devoid of ascorbic acid, was added. Sixty control animals were maintained with a similar diet, supplemented with the complete vitamin diet fortification mixture[5] which provided 45.0 g of ascorbic acid for each 45.4 kg of diet. All animals were killed at the end of 4 weeks. By this time, guinea pigs fed the deficient diet displayed weight loss, weakness, periarticular hemorrhages, and greatly reduced plasma ascorbic acid levels (less than 0.2 mg/100 ml). By contrast, the control animals gained weight, appeared healthy, and had plasma ascorbic acid levels in excess of 0.6 mg/100 ml. After gross examination, the aortas of all animals were stripped of adventitia, dehydrated in several changes of acetone, and air-dried. Because of their small size, 10 aortas were pooled for the subsequent assay of hydroxyproline and acid mucopolysaccharide after grinding in a Wiley mill and further delipidization in chloroform-methanol (1:1).

Hydroxyproline was measured by the method of Neuman and Logan (25) on the hydrolysate of 50 to 100 mg of defatted aortic powder in 6 N HCl in sealed glass tubes at 100° for 12 hours.

The procedure for extracting acid mucopolysaccharides (AMPS) as described previously (25) consisted essentially of enzymatic deproteinization, removal of peptides, and precipitation as the potassium salt in an ethanolic solution of potassium acetate and acetic acid. The uronic acid content of the final product was measured by both the carbazole (27) and orcinol techniques (28, 29). Fractionation was conducted after the technique of Antonopoulos and associates (30, 31) by elution of a cetylpyridinium chloride — AMPS complex from a cellulose column. Using successively 0.35 M NaCl (fraction 1), 0.6 M NaCl (fraction 2), and 1.5 M $MgCl_2$ solutions for elution at room temperature, 3 fractions were obtained. A calcium salt was formed from the 1.5 M $MgCl_2$ eluate and eluted at 4° from a column of Hyflo-supercel with ethanol at concentrations of 30% (fraction 4) and 10% (fraction 3).

The identity of the separated fractions was established by electrophoresis on acetate paper[6] using acetic acid-pyridine-water (100:5:895 v/v) pH 3.6 as a buffer, and a current of 0.5 ma/cm for 120 minutes (26); 0.1% alcian blue in acetic ethanol (5% acetic acid in 20% ETOH) was used as a stain. For verification, the component sugars were identified in the four fractions by descending paper partition chromatography[7] after hydrolysis in 4 N HCl at 100° for 12 hours in sealed tubes. Butanol-pyridin-water (5:3:2 v/v) was used as a solvent; ninhydrin and phthalic acid-aniline solution were the stains. Finally, infrared spectroscopy was performed between 1,330 and 400 cm^{-1} using a Perkin-Elmer, model 337 spectrophotometer, and a cell plate composed of thallium iodide, 56%, thallium, bromide, 4%, and sodium chloride, 40%.[8]

RESULTS

A significant decrease of hydroxyproline ($P < 0.05$) was observed in the aortas of scorbutic guinea pigs (fig. 1, table 1). Total aortic acid mucopolysaccharides were slightly increased in the deficient

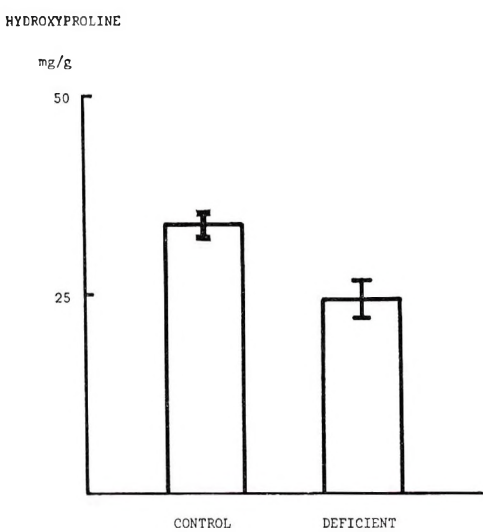

Fig. 1 Hydroxyproline content of normal and scorbutic guinea pig aortas.

[4] Contained per 45.4 kg of diet: (in grams) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35 (Vitamin Fortification Mixture, obtained from Nutritional Biochemicals Corporation).

[5] See footnote 4.

[6] Sepraphore III, Gelman Company, Chelsea, Michigan.

[7] Unpublished data, Y. Tanaka and I. Gore.

[8] Hitachi Limited, Tokyo, Japan.

TABLE 1

Aortic hydroxyproline in scorbutic guinea pigs (8/group)

	mg/g
Control	33.94 ± 2.09 [1,2]
Scorbutic	24.69 ± 2.60

[1] Mean ± SE.
[2] Significant difference, $P < 0.05$.

animals ($0.1 < P < 0.2$) (fig. 2, tables 2 and 3). The successive AMPS fractions 1, 2, 3 and 4, were shown by the identifying procedures to be hyaluronic acid, heparitin monosulfate, chondroitin sulfate B and chondroitin sulfate C, respectively. In the chromatographic profile (fig. 3) a sharp separation of the individual substances is shown. Chondroitin sulfate A, keratosulfate, and heparin were not observed in guinea pig aortas. In scurvy, hyaluronic acid was increased ($P < 0.05$) both absolutely and relatively; chondroitin sulfate B was sharply reduced ($P < 0.05$), but the slight decrease of the total sulfated fraction was not significant. Grossly, the aortas of scorbutic and control guinea pigs alike, were characterized by a smooth unmarred intima. Fatty deposits, such as those described by Willis (21) were absent in this, as well as in a parallel study (32) of the endothelium in scurvy, involving a total of 105 animals exclusive of controls.

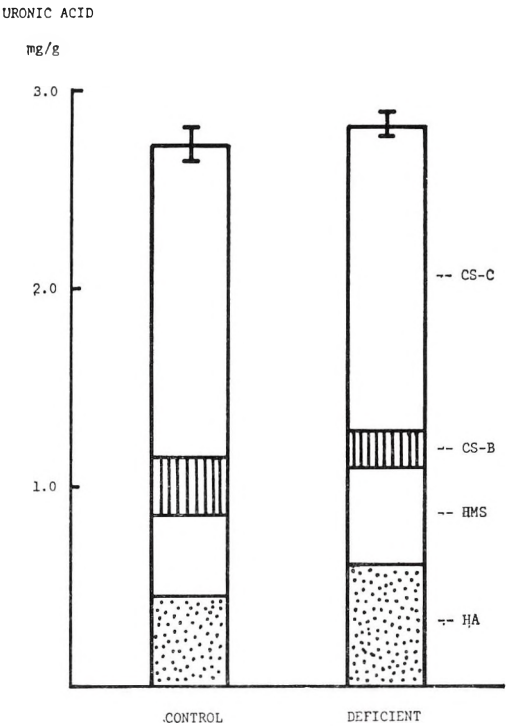

Fig. 2 Acid mucopolysaccharides of normal and scorbutic guinea pig aortas indicating the relative proportions of hyaluronic acid (HA), heparitin monosulfate (HMS), chondroitin sulfate C (CS-C).

TABLE 2

Aortic acid mucopolysaccharides (AMPS) in scorbutic guinea pigs; cellulose column chromatography

AMPS [1]	Quantity applied	Quantity recovered		0.35 M NaCl Hyaluronic A		0.6 M NaCl Heparitin S		1.5 M $MgCl_2$ Chondroitin S	
mg/g	*μg*	*μg*	*%*	*μg*	*%*	*μg*	*%*	*μg*	*%*
				Control					
2.73 ± 0.16 [2]	839	820	97.9	134	16.0	127	15.1	559	66.6
	984	957	97.3	158	16.1	144	14.6	655	66.6
	1034	1031	99.7	167	16.2	165	16.0	699	67.6
Mean ± SE			98.2 ± 0.8		16.1 ± 0.0		15.2 ± 0.4		66.9X0.5
				Scorbutic					
2.83 ± 0.10	1265	1225	96.8	258	20.4	184	14.5	783	61.9
	1102	1074	97.5	231	21.0	187	17.0	656	59.5
	1143	1136	99.4	249	21.8	209	18.3	678	59.3
Mean ± SE			97.9 ± 0.8		21.1 ± 0.4 [3]		16.6 ± 1.1		60.2 ± 0.9

[1] AMPS measured as uronic acid. Fractions determined in triplicate from a pool of 30 samples.
[2] Mean ± SE.
[3] Significant difference, $P < 0.01$.

TABLE 3

Ethanolic partition of chondroitin sulfates (CS)
(1.5 M $MgCl_2$ chromatographic fraction)

Quantity applied	Quantity recovered		CS–C 30% ETOH		CS–B 10% ETOH	
μg	μg	%	μg	%	μg	%
			Control			
447	413	92.3	365.5	55.1	47.5	11.5
524	465	88.7	419.9	56.9	45.1	9.7
583	501	85.9	415.0	56.6	86.0	10.9
	Mean ± SE			56.2 ± 0.6		10.7 ± 0.5
			Scorbutic			
626	585	93.5	542.9	54.7	42.1	7.2
525	468	98.11	437.6	53.0	30.4	6.5
542	494	91.2	467.3	53.9	26.7	5.4
	Mean ± SE			53.9 ± 0.5		6.4 ± 0.5

[1] Significant difference, $P < 0.05$.

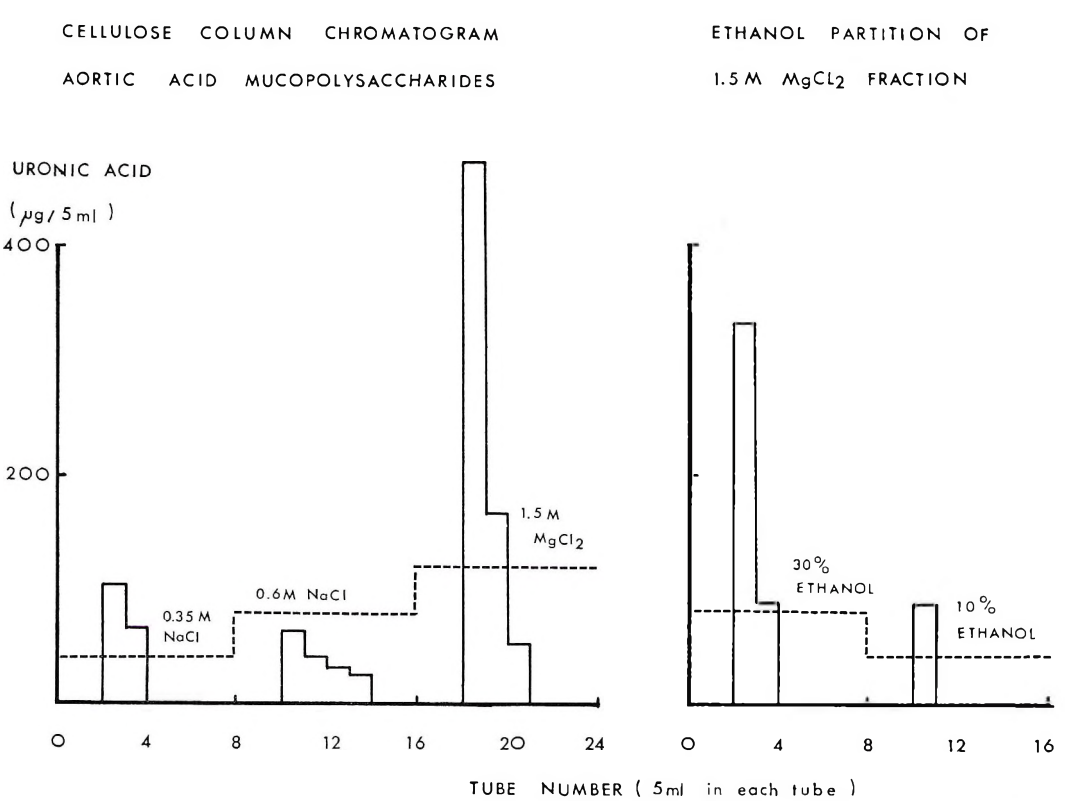

Fig. 3 *Left:* Chromatographic profile of fractional elution of acid mucopolysaccharides from cellulose column with 0.35 M NaCl, 0.6 M NaCl, and 1.5 M $MgCl_2$. *Right:* Ethanolic partition of 1.5 M $MgCl_2$ fraction (chondroitin sulfate) from a column Hyflo-supercel.

DISCUSSION

Scurvy in guinea pigs produced changes in the aortic acid mucopolysaccharides (AMPS) which are qualitatively identical to, but quantitatively less than those observed in carrageenan granulomas (15–17). The reduction in the sulfated fraction, except for chondroitin sulfate B is only relative, although other studies of wound repair suggest an absolute decrease (9, 18, 20). Possibly, there is a true difference between tissue repair after an injury, and physiological maintenance of an anatomically intact structure, but it is also possible that the only difference is methodologic. The observations of Friberg (33) recently confirmed by Kofoed and Robertson [9] indicate that when the effects of inanition are considered, ascorbic acid deficiency does not affect the synthesis of sulfated acid mucopolysaccharides. The isotope technique utilized in their studies, however, does not discriminate between the sulfated fractions, and is completely insensitive to changes in the hyaluronic acid fraction. Chondroitin sulfate B has been identified as the specific fraction of arterial AMPS most likely to be implicated in atherogenesis (23). It contributes to the normal anticoagulant character of the vessel wall, and by virtue of its stimulatory effect on lipid clearing, participates in the transport of lipid through the vessel wall. A reduction in these functions is conducive to the development of lipid, or other atheromatous change in the intima, such as Willis (21) has described. We can only suggest quantitative differences to account for our failure to observe similar lesions, despite a significant reduction of chondroitin sulfate B.

The hydroxyproline content of scorbutic guinea pig aorta, too, is decreased, but not to the same extent as the collagen content of wound repair. Nevertheless, a substantial reduction of aortic hydroxyproline after 4 weeks of dietary ascorbic acid deficiency (and less than that interval of tissue deficiency) indicates that the turnover of collagen in this structure normally is appreciably greater than whole body studies of collagen degradation would indicate (34). No attempt was made to determine soluble and insoluble collagen fractions; however, the magnitude of the observed changes necessitates major involvement of the insoluble component.

LITERATURE CITED

1. Wolbach, S. B. 1933 Intercellular substances in experimental scorbutus. Am. J. Pathol., 9: 689 (suppl.).
2. Gross, J. J. 1959 Studies on the formation of collagen. IV. Effect of vitamin C deficiency on the neutral salt-extractible collagen of skin. J. Exp. Med., *109*: 559.
3. Gould, B. S., and F. J. Woessner 1957 Biosynthesis of collagen: The influence of ascorbic acid on the proline, hydroxyproline, glycine, and collagen content of regenerating guinea pig skin. J. Biol. Chem., 226: 289.
4. Robertson, W. V. B., J. Hewitt and C. Herman 1959 The relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carrageenan granuloma. J. Biol. Chem., 234: 105.
5. Gerber, G. B. 1960 Some interrelated asaspects of proline and hydroxyproline metabolism. Nature, *185*: 767.
6. Mitoma, C., and T. E. Smith 1960 Studies on the role of ascorbic acid in collagen synthesis. J. Biol. Chem., 235: 426.
7. Williams, G. 1959 Some histological aspects of connective tissue metabolism in acute scorbutus. Brit. J. Exp. Pathol., 40: 176.
8. Ross, R., and E. P. Benditt 1964 Wound healing and collagen formation. IV. Distortion of ribosomal patterns of fibroblasts in scurvy. J. Cell. Biol., 22: 365.
9. Penney, J. R., and B. M. Balfour 1949 The effect of vitamin C on mucopolysaccharide production in wound healing. J. Pathol., Bact., *61*: 171.
10. Bunting, H., and R. F. White 1950 Histochemical studies of skin wounds in normal and in scorbutic guinea pigs. Arch. Pathol., *49*: 590.
11. Persson, B. H. 1953 Studies on connective tissue ground substance. II. Organization of the ground substance in ascorbic acid deficiency and its modification by the action of cortisone. Acta Soc. Med. Upsala, 58: 2.
12. Ludwig, A. W. 1954 Development of experimental exophthalmos in scorbutic guinea pigs. Proc. Soc. Exp. Biol. Med., 85: 424.
13. Kodicek, E., and G. Loew 1955 Uptake of sulfur-35 by mucopolysaccharides of granulation tissue. Proc. Roy. Soc. London (Series B), *144*: 100.
14. Dunphy, J. E., and K. N. Udupa 1955 Chemical and histochemical sequences in the normal healing of wounds. New England J. Med., 253: 847.
15. Robertson, W. V. B., and H. Hinds 1956 Polysaccharide formation in repair tissue during ascorbic acid deficiency. J. Biol. Chem., 221: 791.

[9] Kofoed, J. A., and W. V. Robertson 1965 Ascorbic acid and the synthesis of sulfated mucopolysaccharides. Federation Proc., 24: 354 (abstract).

16. Kimoto, E., Y. Tanaka and H. Ueno 1959 Influence of ascorbic acid upon mucopolysaccharide formation in alginic acid granuloma of guinea pigs. Kurume Med. J., 6: 146.
17. Kimoto, E., Y. Tanaka and Y. Imoto 1960 Connective tissue growth in alginic acid granuloma of rats. J. Biochem. (Tokyo), 47: 97.
19. Stein, O., and M. Wolman 1958 A histogrowth stimulated by corrageenin. 3. The nature and amount of polysaccharide produced in normal and scorbutic guinea pigs and the metabolism of a chondroitin sulphuric acid fraction. Biochem. J., 69: 125.
19. Stein, O., and M. Wolman 1958 A histochemical study of wound healing in scorbutic guinea pigs. Brit. J. Exp. Pathol., 39: 418.
20. Fuks, B. B., V. V. Vinogradov, G. S. Shishkin and L. F. Maksimovskii 1965 Proteins, mucopolysaccharides, enzymes and DNA in skin wounds in alimentary and combined alimentary and chemical avitaminosis C. Federation Proc., (Trans. Suppl.), 24: 213.
21. Willis, G. C. 1958 The reversibility of atherosclerosis. Canad. M. A. J., 77: 106.
22. Myasnikov, A. L. 1958 Influence of some factors on development of experimental cholesterol atherosclerosis. Circulation, 17: 99.
23. Gore, I., and B. J. Larkey 1960 Functional activity of aortic mucopolysaccharides. J. Lab. Clin. Med., 56: 839.
24. Woodruff, C. W., and W. J. Darby 1948 In vivo effect of pteroylglutamic acid upon tyrosine metabolism of scorbutic guinea pig. J. Biol. Chem., 172: 851.
25. Neuman, R. E., and M. A. Logan 1950 The determination of hydroxyproline. J. Biol. Chem., 184: 299.
26. Gore, I., Y. Tanaka and H. A. White 1964 Arterial acid mucopolysaccharide changes produced by protamine. Arch. Pathol., 78: 181.
27. Dische, Z. 1947 New specific color reaction of hexuronic acids. J. Biol. Chem., 167: 189.
28. Bollet, A. J., D. V. Anderson and W. F. Simpson 1958 Measurement of tissue acid mucopolysaccharides. J. Clin. Invest., 35: 858.
29. Tanaka, Y., Y. Matsusita, K. Kayanagi, M. Takeuchi and Y. Koyanagi 1961 Determination method of mucopolysaccharide by orcinol. J. Kurume Med. Assoc., 24: 1671.
30. Antonopoulos, C. A., E. Boreliu, D. Gardell, B. Hammstrom and J. E. Scott 1961 The precipitation of polyanions by long chain aliphatic ammonium compounds. IV. Elution salt solutions of mucopolysaccharide-quarternary ammonium complexes absorbed on a support. Biochem. Biophys. Acta, 54: 213.
31. Antonopoulos, C. A., S. Gardell, J. A. Szirmai and E. R. DeTyssonski 1964 Determination of glycosaminoglycans (mucopolysaccharides) from tissues on the microgram scale. Biochem. Biophys. Acta, 83: 1.
32. Gore, I., T. Fujinami and T. Shirahama 1965 Endothelial changes produced by vitamin C deficiency in guinea pigs. Arch. Pathol., 80: 371.
33. Friberg, V. 1958 Role of inanition in the reduced synthesis of chondroitin sulfuric acid in guinea pig rib cartilage in vitamin C deficiency. Arkiv. J. Kemi., 12: 501.
34. Prockop, D. J. 1964 Isotopic studies on collagen degradation and the urine excretion of hydroxyproline. J. Clin. Invest., 43: 453.

Effect of Diet on Accumulation of Gossypol in the Organs of Swine [1,2]

F. H. SMITH AND A. J. CLAWSON
Department of Animal Science, North Carolina State University at Raleigh, Raleigh, North Carolina

ABSTRACT A study was made to determine the effect of diet on the accumulation of gossypol in the organs of growing pigs. The dietary variables tested were: 1) soybean meal; 2) high quality cottonseed meal alone; 3) with added lysine; 4) with injected iron dextran; 5) with dietary iron; and 6) with an exhaustively extracted cottonseed meal. All diets were fed at a 15% protein level and contained 0.06% free gossypol except the diet containing the extracted cottonseed meal. After 15 to 17 days the pigs were killed and the organs were analyzed for free and bound gossypol. No differences in the gossypol content of the organs resulted from diets 1 to 3. The free gossypol was lower and the bound was higher in the livers and spleens of the pigs injected with iron. The greatest effects on organ gossypol resulted from the dietary iron in diet 5. The gossypol values for organs of pigs consuming the exhaustively extracted cottonseed meal were lower than corresponding values from the organs of pigs fed diets 1 through 4.

Cottonseed meal contains gossypol ([2, 2′-binaphthalene] -8,8′ - dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy, 5,5′ -diisopropyl -3,3′-dimethyl), a substance which is toxic to nonruminant animals (1–7) when present in the free state in excessive amounts. Consequently, the use of some cottonseed meals as protein concentrate has been limited in the diets of pigs because of their sensitivity to gossypol (4–8). Diets containing 0.02 to 0.03% free gossypol cause a high incidence of death in growing pigs (4, 7, 8); however, diets containing levels of 0.01% or less appear to be nontoxic (4). Development of methods for the determination of the gossypol content of tissues (9, 10) opened new avenues for investigating factors affecting gossypol toxicity in swine. The performance of pigs fed various cottonseed meals was improved by additions of lysine (11–13), of fish meal (11) and of iron salts (14, 15). These observations indicate that the toxicity associated with feeding high gossypol-containing cottonseed meal to swine can be reduced. Thus, a study was made to determine the effect of various dietary constituents on the accumulation of gossypol in the organs of swine fed diets containing supplemental gossypol.

MATERIALS AND METHODS

The primary differences in the experimental diets (table 1) were as follows: 1) Soybean meal (SOM), 2) high quality cottonseed meal (CSM), 3) CSM + lysine, 4) CSM + iron injected into the pigs as iron dextran in 500-mg doses on the day before initiation and on the eleventh day of the experiment (CSM + Fe-inj), and 5) CSM with 0.1% iron as ferrous sulfate heptahydrate mixed with the diet (CSM + $FeSO_4$). Diets 1 through 5 were designed to contain 0.06% free gossypol, of which either all or a part was supplied by gassypol acetic acid.[3] This compound was ground thoroughly in a portion of the starch by means of a large mechanical mortar. The gossypol-starch mixture was blended with the other dietary ingredients in a mechanical mixer in 18.2-kg batches. Diet 6 was included to determine the effect of exhaustive extraction of a highly toxic meal on the accumulation of gossypol in the organs. The diets were mixed fresh every 6 days and stored under refrigeration until fed. The diets were analyzed for free gossypol. The free gossypol was extracted with 50 ml of an ethanol-water-ether-glacial acetic acid (715:285:200:0.2 ml, re-

Received for publication May 20, 1965.

[1] Contribution from the Animal Science Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper no. 1997 of the Journal Series.

[2] This study was partially supported by the National Cottonseed Products Association, Memphis, Tennessee.

[3] The gossypol-acetic acid was supplied by V. L. Frampton of the Southern Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture.

TABLE 1

Diets fed to pigs in gossypol study

	Diets					
	1	2	3	4 [1]	5	6
	%	%	%	%	%	%
Soybean meal	29.16					
Cottonseed meal		35.33	35.16	35.33	35.13	
Extracted cottonseed meal						32.94
Cornstarch	34.70	31.60	31.49	31.60	31.47	32.71
Corn sugar	28.82	25.73	25.65	25.73	25.63	26.82
Peanut oil	4.44	4.47	4.45	4.47	4.44	4.71
Defluorinated phosphate	1.88	0.94	0.94	0.94	0.94	0.94
Trace mineral salt [2]	0.47	0.47	0.47	0.47	0.47	0.47
Vitamin supplement [3]	0.47	0.47	0.47	0.47	0.47	0.47
Limestone		0.94	0.94	0.94	0.94	0.94
L-Lysine (monohydrochloride)			0.39			
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)					0.46	
Added gossypol	0.06	0.047	0.047	0.047	0.047	
Total	100.00	100.00	100.01	100.00	100.00	100.00
Total protein	15.09	15.05	14.97	15.05	14.96	15.04

[1] 500 mg of iron dextran injected into pigs one day before initiation of dietary treatment and at 11 days afterward.

[2] Supplied per kg of diet: sodium chloride, 4.8 g; zinc, 40 mg; manganese, 30 mg; iron, 10 mg; copper, 3 mg; iodine, 0.8 mg; and cobalt, 0.75 mg.

[3] Supplied per kg of diet: vitamin A, 2200 USP units; vitamin D, 1100 USP units; vitamin E, 1.1 IU; riboflavin, 4.4 mg; pantothenic acid, 10.1 mg; niacin, 22 mg; choline chloride, 55 mg; vitamin B_{12}, 22 μg; and oxytetracycline, 22 mg.

spectively) + 1 ml of pyridine by homogenizing in a Servall Omni-Mixer having the chamber surrounded by ice water. The remainder of the procedure was as described previously (16).

Pigs ranging in weight from 26.4 to 39.5 kg from 2 litters of purebred Durocs were placed in individual pens one week before receiving the experimental diets. During this preliminary period the rate of feed consumption was determined. Three pigs were assigned at random to each diet, which was fed at the rate of 5% of body weight.

Although this was not a balanced study, near the end of the trial, samples of feces were collected from each pig and composited according to dietary treatment and dried. The fecal samples were analyzed for free gossypol by the procedure described for the diets. They were analyzed for total gossypol by the method of Pons and Hoffpauir (17). The bound gossypol was obtained by difference between total and free gossypol.

Free gossypol is defined as that gossypol which can be extracted with ethyl ether, acetone, or a mixed solvent such as that used in this study. Bound gossypol is that remaining in the sample after the extraction of the free gossypol and is in combination with some component of the sample, probably the free ε-amino groups of lysine (18).

One replication, one pig from each diet, was killed at daily intervals on days 15, 16, and 17. The organs were removed, weighed, and stored in plastic bags at −18°. The tissues were thawed partially, ground in a food chopper, mixed and stored in glass bottles at −18° after sampling for dry matter and gossypol determinations. Free and bound gossypol were determined by methods described previously (10), except that chloroform instead of hexane was used for the extraction of bound gossypol.

RESULTS AND DISCUSSION

The results from the analysis of the diets for free gossypol are shown in table 2. The recovery of free gossypol from diets 2 to 4 were in good agreement with that added, whereas that from diets 1 and 5 was approximately 81 and 53%, respectively, of that added. The feces from all treatments contained some free gossypol (table 2). However, the lowest amounts were from diets 1 and 5 and the highest from diet 6. The dietary iron decreased the

TABLE 2

Gossypol content of diets and feces

Diet no.	Diets [1]			Feces	
	Free		Bound	Observed	
	Calculated	Observed	Calculated	Free	Bound
	%	%	%	%	%
1	0.060	0.049	0.000	0.015	0.200
2	0.060	0.057	0.311	0.058	0.695
3	0.060	0.057	0.308	0.080	0.760
4	0.060	0.056	0.308	0.071	0.703
5	0.060	0.032	0.308	0.026	0.435
6	0.008	0.018	0.367	0.137	0.939

[1] Diets 2–5, cottonseed meal contained, 0.053% free and 0.868% bound gossypol; diet 6, cottonseed meal contained, 0.024% free and 1.325% bound gossypol.

TABLE 3

Gossypol content of organs and body fluid of pigs fed diets containing gossypol [1,2]

Tissue	Gossypol form	Dietary treatment (3 pigs/treatment)						L.S.D.	
		1 SOM [3]	2 CSM	3 CSM + LYS	4 CSM + FE INJ	5 CSM + $FeSO_4$	6 CSM–EXT	0.05	0.01
		μg gossypol/g dry matter							
Liver	F [4]	465	410	453	370	138	237	65.8	92.5
	B	424	477	455	597	163	226	124.4	174.4
Kidney	F	208	204	214	173	46	61	46.5	65.2
	B	233	204	204	183	66	75	42.7	59.8
Spleen	F	67	52	43	49	27	35	19.4	—
	B	327	308	331	469	205	186	112.1	157.2
Lymph nodes	F	69	73	73	49	27	27	15.2	21.8
	B	228	171	207	227	55	56	63.6	91.5
Heart	F	79	81	65	78	29	40	18.1	20.3
	B	110	108	120	123	75	83	25.3	28.5
Lungs	F	43	50	42	41	20	22	14.0	16.7
	B	194	178	192	185	151	167	ns	—
Diaphragm muscle	F	14	19	23	26	18	16	ns	—
	B	58	56	52	60	56	50	ns	—
Pancreas	F	27	40	31	31	27	21	7.3	10.2
	B	47	47	47	48	27	28	8.3	11.6
Brain	F	13	10	3	6	7	10	ns	—
	B	38	47	48	48	43	44	ns	—
Blood serum, μg/ml	F	1.6	1.3	1.7	1.8	0.3	0.7	ns	—
	B	21.0	17.2	16.5	13.7	3.6	10.9	9.8	11.7
Bile, μg/g	F	52	28	85	178	25	27	—	—
	B	6	3	3	77	2	4	—	—

[1] Diets 1 to 5 contained 0.06% and diet 6 contained 0.008% free gossypol.

[2] Expressed as mean for 3 pigs/treatment.

[3] SOM indicates soybean meal; CSM, cottonseed meal; CSM + LYS, cottonseed meal + lysine; CSM + FE INJ, cottonseed meal + Fe injection; CSM + $FeSO_4$, cottonseed meal with 0.1% iron as ferrous sulfate heptahydrate mixed with diet; and CSM–EXT, extracted cottonseed meal.

[4] F indicates free gossypol; B, bound gossypol.

amount of both free and bound gossypol in the feces, treatment 5 versus 2, 3, and 4. Much of the free gossypol in the SOM diet was converted to bound gossypol during passage through the alimentary canal. The high level of free gossypol in feces from diet 6 (CSM-EXT) suggests that some of the bound gossypol was hydrolyzed to the free state. The high mortality, 7 pigs out of 8,[4] observed when this meal was fed prior to extraction for protein evaluation may have resulted from hydrolysis of bound gossypol.

The results, expressed as mean values from the tissue analysis (table 3), indicate that the sequence of decreasing concentrations of free gossypol in organs is liver, kidney, heart, lymph nodes, spleen, lungs, pancreas, diaphragm muscle, and brain; whereas the sequence for bound gossypol is liver, spleen, lungs, kidney, lymph nodes, heart, diaphragm muscle, brain and pancreas. Although the pigs in this study had not shown any signs of acute toxicity before they were killed, the level of gossypol in the organs was in general agreement with pathological observations of organs from pigs that had died of gossypol toxicity (19). The pathological study of the pigs that died of gossypol toxicity showed severe damage to the liver, heart, kidney, lungs, spleen, and lymph nodes.

A comparison of the effects of diets 1, 2, and 3 on the accumulation of gossypol in the organs reveals a significant difference only for free gossypol in the pancreas and the spleen.

The free gossypol accumulation in the livers of pigs fed diet 4 and receiving injected iron was lower ($P < 0.05$) than that of pigs receiving diets 1 and 3. This effect was even more marked in the lymph nodes where significantly higher values ($P < 0.01$) were observed for those pigs consuming diets 2 and 3. In contrast, the bound gossypol in the liver and spleens of pigs fed diet 4 were significantly higher ($P < 0.05$) than those receiving either diet 1 or 3; spleen-bound gossypol was higher ($P < 0.05$) for pigs fed diet 4 than was that from either diets 1, 2, or 3. This indicates that the injected iron had an effect on the formation of bound gossypol in these organs. Tissue staining techniques showed that those pigs injected with iron dextran had more iron in the livers and spleens than did those receiving the other diets.

The most striking observation of this study was the highly significant reductions in the levels of both free and of bound gossypol in certain organ tissues from the pigs receiving the dietary iron (diet 5). Free gossypol in liver, kidney, lymph nodes, lungs, and bound gossypol in liver, kidney, lymph nodes, heart, and pancreas was lower ($P < 0.01$) from diet 5 than from diets 1 to 4. The spleen-free gossypol from diet 5 was lower than that from diets 1, 2, and 4, and the spleen-bound gossypol was lower than that from diets 1, 3, and 4 ($P < 0.05$). Likewise, bound-serum gossypol was lower than that from diet 4 ($P < 0.05$) and from diets 1, 2, and 3 ($P < 0.01$). The dietary iron presumably reacts with gossypol upon ingestion and reduces its absorption; thus, much of it never reached the organs analyzed. This hypothesis is supported by the analysis of the diets for free gossypol and the feces for both free and for bound gossypol. When iron was mixed with the diet, the amount of free gossypol recovered from the diet and of both free and bound gossypol recovered from the feces was lower than corresponding amounts from diets 2, 3, and 4. These lower recoveries indicate that some detoxification takes place and that the gossypol molecule is so altered that it no longer responds to the reagents of the analytical procedure.

The gossypol content, free and bound, of the livers and the kidneys of pigs fed the exhaustively extracted cottonseed meal, diet 6, was lower than corresponding values for treatments 1 to 4 ($P < 0.01$) and the liver-free gossypol was higher for treatment 6 than for treatment 5 ($P < 0.01$). The free gossypol of the lymph nodes and heart and the bound gossypol of the spleen, lymph nodes and heart were lower than corresponding values for treatments 1 to 4 ($P < 0.01$).

The ratio of bound to free gossypol, irrespective of treatment, was variable among the different tissues. The bound to free ratio was lowest in liver and kidney tissues, approximately 1:1, and was greatest for spleens, being approximately 6:1. The

[4] Unpublished data.

ratio was greater for blood serum than for tissues, ranging from 7.6 to 15.6:1. The high ratio of bound to free gossypol in the brain may be related to the level of phospholipids in that organ. It has been reported that gossypol has been observed in combination with phospholipids after the processing of cottonseed (20–22). A similar reaction could bind some of the gossypol to the phospholipids in the animal tissues; however, it is possible that much of the gossypol was bound through a combination with free amino groups of protein (18).

The greatest amount of free gossypol per organ was observed in the liver. The average amount of free gossypol per liver ranged from a high of 122.3 mg for pigs fed diet 1 to a low of 28.5 for pigs fed diet 5 containing 0.1% iron as ferrous sulfate. The next highest value for free gossypol per organ was a 3-mg average observed in the kidney of pigs fed diet 1. The corresponding value for pigs fed diet 5 was 0.6 mg.

A high level, although variable, of free gossypol was observed in the bile. This may be one of the pathways of excretion of absorbed gossypol. The gossypol may form a water-soluble salt in the basic bile and then be excreted with the feces. This may be the explanation for the high level of free gossypol noted in some fecal samples from pigs that have consumed diets containing gossypol.

ACKNOWLEDGMENT

The authors wish to express their appreciation to J. C. Osborne for performing the autopsies and for the histological examination of the tissues.

LITERATURE CITED

1. Withers, W. A., and F. E. Carruth 1915 Gossypol the toxic substance in cottonseed meal. J. Agr. Res., *5:* 281.
2. Schwartze, E. W., and C. L. Alsberg 1924 Pharmacological action of gossypol. J. Agr. Res., *28:* 191.
3. Cabell, C. A., and T. P. Earle 1956 Relation of amount and quality of protein in the diet to free gossypol tolerance by the rat. J. Am. Oil Chemists' Soc., *33:* 416.
4. Hale, F., C. M. Lyman and H. A. Smith 1958 Use of cottonseed meal in swine rations, Texas Agricultural Experiment Station Bull. 898. College Station, Texas.
5. Sewell, W. E. 1943 Detoxification of cottonseed meal for hogs. Alabama Polytech. Inst. Agr. Exp. Bull. 259. Auburn, Alabama.
6. Hale, F., and C. M. Lyman 1957 Effect of protein level in the rations on gossypol tolerance in growing-fattening pigs. J. Anim. Sci., *16:* 364.
7. Kornegay, E. T., A. J. Clawson, F. H. Smith and E. R. Barrick 1961 Influence of protein source on toxicity of gossypol in swine rations. J. Anim. Sci., *20:* 597.
8. Clawson, A. J., F. H. Smith and E. R. Barrick 1962 Accumulation of gossypol in livers and factors influencing the toxicity of injected gossypol. J. Anim. Sci., *21:* 911.
9. Smith, F. H. 1963 The isolation of gossypol from porcine liver tissue. J. Am. Oil Chemists' Soc., *40:* 60.
10. Smith, F. H. 1965 The determination of free and bound gossypol in swine tissue. J. Am. Oil Chemists' Soc., *42:* 145.
11. Stephenson, E. L., P. R. Noland and A. A. Camp 1952 The use of cottonseed meal in swine production. Arkansas Agricultural Experiment Station Bull. 523. Fayetteville, Arkansas.
12. Clawson, A. J., F. H. Smith, J. C. Osborne and E. R. Barrick 1961 Effect of protein source, autoclaving, and lysine supplementation on gossypol toxicity. J. Anim. Sci., *20:* 547.
13. Hale, F., and C. M. Lyman 1961 Lysine supplementation of sorghum grain-cottonseed meal rations for growing and fattening pigs. J. Anim. Sci., *20:* 734.
14. Withers, W. A., and F. E. Carruth 1917 Iron as an antidote to cottonseed meal injury. J. Biol. Chem., *32:* 245.
15. Robinson, W. L. 1936 Making cottonseed meal safe and effective when fed as the only source of protein concentrate to pigs in dry-lot. Proc. Am. Soc. of Animal Prod., p. 87.
16. Smith, F. H. 1946 Estimation of gossypol in cottonseed meal and cottonseed meats. Ind. Eng. Chem. (Anal. ed.), *18:* 43.
17. Pons, W. A., Jr., and C. L. Hoffpauir 1957 Determination of free and total gossypol in mixed feeds containing cottonseed meal. J. Ass. Offic. Agr. Chem., *40:* 1068.
18. Lyman, C. M., B. P. Baliga and M. W. Slay 1959 Reaction of protein with gossypol. Arch. Biochem. Biophys., *84:* 486.
19. Smith, H. A. 1947 The pathology of gossypol poisoning. Am. J. Pathol., *33:* 353.
20. Martin, J. B. 1959 Chemistry of gossypol complexes. Proc. of the Conference on the Structure and Reactions of Gossypol and Non-gossypol Pigments of Cottonseeds, Nat. Cottonseed Products Ass., Dallas, Texas, p 71.
21. Pons, W. A., Jr., J. Pominski, W. H. King, J. A. Harris and T. H. Hopper 1959 Recovery of gossypol from cottonseed gums. J. Am. Oil Chemists' Soc., *36:* 328.
22. Umarov, A. Y., and A. L. Markman 1960 Chromatographic studies of cottonseed oil. Uzbek. Khim. Zhur., no. 5, 66.

Interrelationship between the Biological Oxidation Mechanism, Serum Lipids and the Serum Iron Transport System in the Rat [1]

RICHARD R. ROEHM AND HELEN L. MAYFIELD
Department of Home Economics Research, Montana State University Agricultural Experiment Station, Bozeman, Montana

ABSTRACT This study was made to investigate the interrelationships in hemoglobin formation, the iron-transport system, serum protein, lipid metabolism and succinic dehydrogenase activity (SDH) of heart, liver and kidney of rats fed diets known to be hypercholesterolemia-inducing. These diets were fed with and without 1% added cholesterol and with and without sufficient iron. Control rats were fed a chow diet. Hemoglobin, hematocrits and serum iron of rats fed the 3 experimental diets were lower and serum protein, higher, than those for control rats. Serum cholesterol was higher in all 3 groups and serum triglycerides markedly increased in the iron-deficient rats. Heart weights of iron-deficient rats increased and kidney weights of the 3 experimental groups were lower than those of controls. The SDH activity of the heart was not affected by feeding the experimental diets. Liver SDH per gram of rat decreased in rats fed the 3 experimental diets and appeared related to the hypercholesterolemic effect of the diet and not to its effect of lowering the hemoglobin level. The kidney SDH was lower in rats fed the experimental diets and appeared related to the lowered hemoglobin level, for rats fed the iron-deficient diet had significantly lower SDH than those with somewhat higher hemoglobin levels.

Work in this laboratory and elsewhere has shown that lowered hemoglobin levels may be observed in laboratory animals fed diets high in fat or high in cholesterol (1–5). These hypercholesterolemia-inducing diets, containing adequate amounts of iron for the hematopoietic processes under normal dietary conditions, induce a suboptimal blood picture and a condition of abnormal lipid metabolism in rats, guinea pigs and rabbits. A recent report (6) indicates that man also shows a decrease in hemoglobin and hematocrit values when given multiple infusions of a fat emulsion. The factors involved in the lowered hemoglobin levels are unexplained at present.

There is evidence that the activity of some of the enzymes functioning in the biological oxidation mechanism, in particular that of succinic dehydrogenase (SDH), is proportional to the iron content of the enzyme (7). Beutler and Blaisdell (8) have reported on the SDH activity in the liver, kidney and heart of iron-deficient rats. After moderately severe iron deficiency, they observed no decrease in the SDH activity in the liver but some loss of activity in the heart and kidneys of these iron-deficient rats. However, it is not known whether lowered hemoglobin levels induced by diets high in fat (or cholesterol), rather than low in iron, would influence the activity of this enzyme in a similar manner.

This study was planned to obtain such information concerning the interrelationships between a hypercholesterolemia-inducing diet (with and without added cholesterol), changes in hemoglobin level, certain abnormalities in lipid metabolism and the activity of the succinic dehydrogenase enzyme system in the heart, liver and kidney of the rat. For comparative purposes rats were also fed a similar hypercholesterolemia-inducing diet with cholesterol but iron-deficient, and a normal diet of laboratory chow.

METHODS

The design of this experiment was influenced by the limitations imposed by the methods, equipment and time available for the enzyme determinations in the heart, liver and kidney of the rats. Wean-

Received for publication June 5, 1965.
[1] This study was supported in part by funds from the Regional Project W-44 of the Western Agricultural Experiment Stations. Contribution from Montana State University, Montana Agricultural Experiment Station, Paper no. 695, Journal Series.

TABLE 1

Composition of experimental diets

	Group		
	1	2	3
	%	%	%
Casein [1]	5.0	5.0	5.0
Albumin, egg [2]	10.0	10.0	10.0
Cottonseed oil [3]	10.0	10.0	10.0
Sucrose	68.8	67.8	67.8
Salt mixture [4]	4.0	4.0	4.0 [5]
Vitamin mixture [6]	2.2	2.2	2.2
Cholesterol [7]	0.0	1.0	1.0

[1] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.

[2] Nutritional Biochemicals Corporation.

[3] Wesson oil, Wesson Oil Sales Company, Fullerton, California.

[4] Salt mixture, USP XIV, obtained from Nutritional Biochemicals Corporation. This salt mixture contains the following: (in g/kg of mix) cupric sulfate, 0.08; ferric ammonium citrate, 15.28; manganese sulfate, 0.20; ammonium alum, 0.09; potassium iodide, 0.04; sodium fluoride, 0.51; calcium carbonate, 68.6; calcium citrate, 308.3; calcium biphosphate, 112.8; magnesium carbonate, 35.2; magnesium sulfate, 38.3; potassium chloride, 124.7; dibasic potassium phosphate, 218.8, and sodium chloride, 77.1.

[5] Salt mixture, USP XIV, as above but with all iron salts omitted, prepared by Nutritional Biochemicals Corporation.

[6] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, contains the following: (in g/kg of mix) vitamin A concentrate, 4.5 (200,000 units/g); vitamin D concentrate, 0.25 (400,000 units/g); α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and the following: (in mg/kg of mix) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35.

[7] Cholesterol USP, Nutritional Biochemicals Corporation.

ling rats of the Holtzman strain [2] were obtained at 6 different times during a 4-month period, in lots of 16 rats each. Each lot of 16 rats was used in a complete replication of the experimental design, 4 rats (2 females, 2 males) per each of 4 groups. Each group, when completed, had 24 rats (12 females, 12 males). Rats were housed individually in screen-bottom cages and maintained in a temperature-controlled room of approximately 24° at an elevation of 1.46 km (4800 ft). After a 2-week preliminary period during which they were fed, ad libitum, a laboratory chow diet,[3] female rats weighed an average of 112 g and males 126 g. Groups 1, 2 and 3 were then fed, ad libitum, one of the 3 experimental diets shown in table 1 and the fourth group continued to receive the laboratory chow. Rats in group 1 were fed a hypercholesterolemia-inducing diet similar to that used by Okey and Lyman (9). The diet fed to group 2 was similar, but with 1% added cholesterol, and that fed to group 3 differed from diet 2 in that all iron salts were omitted from the salt mixture. Weekly food consumption and rat weights were recorded over the 7-week experimental period.

Hemoglobin and hematocrit determinations were made using tail blood samples from non-fasted rats after they had been fed the experimental diets for 3, 5 and 7 weeks. These measurements were made during the morning or early afternoon. Hemoglobin was measured by the cyanmethemoglobin method using a hemoglobin standard.[4] Readings were made using a Beckman B spectrophotometer. Standard heparinized capillary tubes were used for the micro-hematocrits. After centrifuging they were read in a micro-capillary reader.

At the end of the 7-week experimental period and from 1 to 8 days after hemoglobin and hematocrit determinations were made, food was removed from the cages for approximately 10 hours prior to decapitation. Blood was collected from these fasted rats and serum prepared and frozen for later analyses. Because of the enzyme determinations only 2 rats were killed on one day. Liver, heart and kidneys were removed as rapidly as possible, wiped free of blood by pressing lightly with filter paper and placed in covered containers, chilled in crushed ice. Each of the organs was weighed as rapidly as possible and replaced in the chilled container until needed for preparing the homogenates. The whole heart, either one or two of the kidneys, depending upon their weight, and a representative liver sample of known weight (1 to 1.5 g) were used in preparing the respective homogenates of these organs for enzyme activity measurements. Tissue homogenates were prepared [5] and succinic dehydrogenase activity was measured by the method of Schneider and Potter (10) as given by Umbreit et al. (11). A 14-station Warburg apparatus [6]

[2] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[4] Hemoglobin standard. Acuglobin, supplied by Ortho Pharmaceutical Corporation, Raritan, New Jersey.

[5] Homogenates were prepared using a Virtis "45" Homogenizer. The Virtis Company, Yonkers, New York.

[6] Lardy Warburg apparatus, Gilson Medical Electronics, Middleton, Wisconsin.

was used in measuring the SDH activity over a 30-minute interval. Determinations were made in duplicate on each of the 3 organs of 2 rats at one time. This permitted 2 manometers for use as thermobarometers. SDH activity was first calculated in the usual manner as microliters of oxygen (μliter O_2) per hour per milligram dry tissue, and was later expressed on a per milligram of protein basis as well as on a relative basis per gram rat weight which was calculated as follows:

$$\frac{\mu\text{liters } O_2/\text{mg moist tissue} \times \text{organ wt in mg}}{\text{rat weight in g}}$$

For purposes of this calculation, heart tissue had 22% solids, liver 30 and kidney 22.

Determinations of serum iron and total iron-binding capacity (TIBC) of the serum were made following Mandel's (12) modification of the method of Peters et al. (13). Total cholesterol was measured in the

TABLE 2

Effect of feeding hypercholesterolemia-producing diets, varying in cholesterol and iron content, on hemoglobin, the serum iron-transport system, the succinic dehydrogenase activity of heart, liver and kidney tissue and the respective weights of these rat organs [1]

	Experimental diets			
	1 Basal diet	2 Basal diet + 1% cholesterol	3 Basal diet + 1% cholesterol, iron-deficient	Diet 4 Laboratory chow
Rats/group [2]	24	24	24	24
Food and growth				
Food intake, g/day	13.7	14.6	12.5	18.4
Weight, end of test, g	271 ± 9.3 [3]	276 ± 11.7	251 ± 11.5	265 ± 14.0
Blood				
Hemoglobin, g/100 ml	15.4 ± 0.1 [b,4]	15.2 ± 0.1 [b]	9.1 ± 0.3 [c]	16.1 ± 0.1 [a]
Hematocrit, %	47.7 ± 0.2 [b]	47.5 ± 0.3 [b]	31.7 ± 0.7 [c]	49.4 ± 0.3 [a]
Serum				
Iron, μg/100 ml	250.0 ± 19.1 [a]	201.0 ± 15.8 [b]	56.0 ± 12.2 [c]	254.0 ± 19.5 [a]
TIBC,[5] μg/100 ml	775.0 ± 14.2 [b]	713.0 ± 11.0 [a]	613.0 ± 8.0 [c]	728.0 ± 21.2 [a]
Protein, g/100 ml	6.8 ± 0.07 [b]	7.2 ± 0.07 [c]	7.0 ± 0.07 [c]	6.5 ± 0.09 [a]
Cholesterol, mg/100 ml	98.9 ± 2.8 [b]	149.9 ± 11.3 [c]	116.2 ± 10.5 [d]	76.2 ± 1.9 [a]
Triglycerides, mg/100 ml	118.2 ± 10.0 [b]	88.8 ± 6.6 [a]	140.1 ± 17.3 [c]	90.4 ± 4.2 [a]
Organ weights				
Heart, g/100 g rat	0.36 ± 0.01 [a]	0.37 ± 0.01 [a]	0.44 ± 0.01 [b]	0.36 ± 0.01 [a]
Liver, g/100 g rat	2.91 ± 0.06 [b]	3.49 ± 0.08 [c]	3.33 ± 0.03 [a]	3.31 ± 0.04 [a]
Kidney, g/100 g rat	0.74 ± 0.01 [b]	0.75 ± 0.01 [b]	0.73 ± 0.02 [b]	0.82 ± 0.01 [a]
	Succinic dehydrogenase (SDH) activity [6]			
Heart				
μl O_2/hr/mg dry wt tissue	230.0 ± 2.5 [a]	232.5 ± 2.4 [a]	196.8 ± 4.2 [b]	226.7 ± 1.9 [a]
μl O_2/hr/mg protein	266.4 ± 2.7 [b]	254.8 ± 3.2 [c]	209.7 ± 4.2 [a]	209.1 ± 2.4 [a]
Relative SDH/g rat wt [7]	184.7 ± 3.2 [a]	188.0 ± 2.9 [a]	189.3 ± 5.1 [a]	180.4 ± 3.4 [a]
Liver				
μl O_2/hr/mg dry wt tissue	66.8 ± 1.7 [a]	41.5 ± 1.4 [b]	44.8 ± 1.4 [b]	69.4 ± 1.5 [a]
μl O_2/hr/mg protein	92.2 ± 2.3 [a]	62.0 ± 2.1 [b]	66.3 ± 2.0 [b]	94.3 ± 1.9 [a]
Relative SDH/g rat wt	578.8 ± 16.3 [b]	429.3 ± 11.7 [c]	447.5 ± 14.5 [c]	690.8 ± 17.9 [a]
Kidney				
μl O_2/hr/mg dry wt tissue	144.4 ± 3.4 [b]	140.2 ± 1.7 [b]	108.1 ± 1.9 [c]	159.8 ± 2.2 [a]
μl O_2/hr/mg protein	193.1 ± 4.5 [b]	190.1 ± 2.8 [b]	143.4 ± 2.9 [c]	214.5 ± 2.9 [a]
Relative SDH/g rat wt	234.3 ± 5.7 [b]	230.9 ± 4.5 [b]	174.3 ± 5.0 [c]	286.7 ± 4.8 [a]

[1] Rats were fasted approximately 10 hours prior to all determinations other than hemoglobin and hematocrit.
[2] Each group consisted of 12 male and 12 female rats.
[3] SE of mean.
[4] Duncan's multiple range test (14). Comparisons in table are made horizontally. Means with different superscripts are statistically significant at the 1% level.
[5] TIBC indicates total iron-binding capacity.
[6] Per cent solids in heart, 22; liver, 30; kidney, 22.
[7] Relative SDH/g rat wt calculated as follows: $\frac{\mu\text{l } O_2/\text{hr/mg moist tissue} \times \text{organ wt in mg}}{\text{rat wt in g}}$.

serum samples by a micro adaptation of the procedure of Abell et al. (14). The method of Van Handel and Zilversmit (15) with later modification (16) was used in determining serum triglycerides. Serum protein was measured using a Goldberg refractometer with temperature compensator.[7]

Statistical treatment of the resulting data[8] consisted of an analysis of variance and comparisons by Duncan's multiple range test (17). Only those differences statistically significant at the 1% level are considered in this report.

RESULTS AND DISCUSSION

Biochemical changes in the blood, serum, heart, liver and kidney of groups of rats fed a hypercholesterolemia-inducing diet, with and without added cholesterol, diets 1 and 2, respectively; diet 3, similar to diet 2 but iron-deficient; and diet 4, a laboratory chow, are shown in table 2. By use of Duncan's multiple range test (17) statistically significant differences between means for each of the biochemical measurements in the 4 groups were determined. Means with dissimilar superscripts are statistically different at the 1% level. For simplifying presentation, the values given in table 2 are averages of the 12 male and 12 female rats per group, since the 2 sexes responded in a similar manner to the dietary variations in diets 1, 2, 3 and 4. However, there was a significant difference between the sexes in the majority of the determinations and the averages for males and females, separately, are shown and discussed in a subsequent section of this report.

Hemoglobin and hematocrits. In agreement with previous work in this laboratory (1) rats in group 1, fed the hypercholesterolemia-inducing diet (table 1) had slightly, but significantly, lower mean hemoglobin and hematocrit levels than rats fed the laboratory chow, group 4. The addition of 1% cholesterol to this diet, group 2, did not lower these values further, but the omission of iron salts from the salt mixture in diet 3, group 3, greatly lowered these values. Although the hemoglobin and hematocrit values for rats in groups 1 and 2 were within the ranges for normal rats given by Albritton (18) they were significantly lower than those of control rats in this study (group 4) and lower than values previously reported from this laboratory for rats fed a chow diet (19). Hemoglobin and hematocrit levels for control rats at this elevation, 1.48 km (4800 ft), may be slightly higher than values reported by others due to the effect of altitude on hemopoiesis (18).

Serum iron and TIBC. Rats in group 1, fed the hypercholesterolemia-inducing diet with no added cholesterol, had a significantly higher TIBC than the control rats in group 4. However, the serum iron level was not changed. The addition of 1% cholesterol to the hypercholesterolemia-inducing diet did not cause further impairment in the hemopoietic process, since rats in groups 1 and 2 had similar hemoglobin and hematocrit levels, but it did cause a significant decrease in the level of serum iron. Thus a somewhat contradictory disturbance in the iron transport system was indicated. Why this decrease in serum iron was not reflected in a higher hemoglobin concentration is without explanation at this time. Since iron analyses were made only on the rat serums and not on any of the body tissues, the deposition of this dietary iron cannot be traced. Priest and Normann (5) observed large quantities of the iron-containing pigment, hemosiderin, deposited in the cortex portion of the kidneys of rats made anemic by the ingestion of certain high-fat diets.

Serum protein. Serum protein levels of rats fed any one of the 3 experimental diets were significantly higher, 6.8 to 7.2 g/100 ml, than those of the control rats fed chow, 6.5. Although this increase is not very large, these levels of serum protein in the rats fed the experimental diets are greater than those given by Albritton (18) for normal rats, average 6.0, range 5.5 to 6.5 g/100 ml. Rats fed diets containing 1% cholesterol, with and without adequate iron, groups 2 and 3, had slightly, but significantly higher, serum protein levels than those fed the hypercholesterolemia diet without added cholesterol.

[7] Made by American Optical Company, Instrument Division, Buffalo, New York.

[8] Statistical treatment of data made by Montana State University Computing Center.

These slightly, but significantly, higher levels of serum protein may be related to the accompanying changes in serum cholesterol and triglycerides. Based on work reported by Rodbell et al. (20) and discussed by Korn (21) in his report on the role of heparin and lipoprotein lipase in the transport of triglycerides, it appears that most of the exogenous triglyceride and cholesterol transported from the intestinal tract to the tissues via the lymph and blood is in the form of chylomicrons, a complex of triglyceride, cholesterol ester, phospholipid and protein. These workers reported that although in some instances the entire chylomicron may leave the circulation intact, there was also evidence that some of the protein may be left behind when the lipid disappears. In the present study, rats fed the 3 hypercholesterolemia-inducing diets had elevated serum cholesterol and triglyceride levels. The accompanying higher serum protein level may be due to the protein left behind in the blood stream after the partial hydrolysis of the cholesterol-triglyceride-protein-containing chylomicrons. These higher levels of serum protein suggest that the lowered hemoglobin and hematocrit values observed in these rats were not the result of a process of dilution of the blood stream. Some workers (6, 22) have discussed the dilutional aspect of this type of anemia since more water than usual may be consumed by test animals fed high fat diets.

Serum cholesterol. Serum cholesterol levels of rats in the 3 experimental groups, 1, 2 and 3 were significantly higher than the level of the rats in the control group 4 (76 mg/100 ml) and are in accord with values previously reported from this laboratory (1). The addition of 1% cholesterol to the hypercholesterolemia-inducing diet caused a significant increase in serum cholesterol, 150 mg/100 ml, over that obtained when no cholesterol was fed, 99. However, this value was significantly lowered to 116 mg/100 ml when the iron salts were omitted from this 1% cholesterol-containing diet. Rats fed the iron-deficient diet, group 3, consumed less food per day (12.5 g) than those in group 2 (14.6 g) and this somewhat lower food intake may have contributed to the lower serum cholesterol level.

Serum triglycerides. The serum triglyceride level of the control rats fed the laboratory chow, 90 mg/100 ml, is slightly higher than that reported by Bizzi et al. (23) for rats fed laboratory rat cubes, 80 mg/100 ml. It is considerably higher than that obtained by Tinoco et al. (24), 52 mg/100 ml, for male rats fed a purified diet somewhat low in methionine and vitamin B_{12}, but otherwise complete. When rats were fed the hypercholesterolemia-inducing diet without added cholesterol, the serum triglyceride level, 118 mg/100 ml, was significantly higher than that observed in the control rats. However, when 1% cholesterol was added to this diet, group 2, the serum triglyceride level was lowered to 89 mg/100 ml and was similar to that noted in the control rats. At the same time that the serum triglyceride level was lowered in these rats, the accompanying serum cholesterol value was greatly increased, from 99 to 150 mg/100 ml. Rats in group 3 fed the 1% cholesterol, iron-deficient diet, had the highest level of serum triglycerides, 140 mg/100 ml, of any of the 4 groups and at the same time a serum cholesterol level of 116 mg/100 ml, significantly higher than that of the control rats, but significantly lower than that of rats fed a similar diet but with normal iron intake, 150 mg/100 ml. This is a recognized phenomenon (25) that levels of serum cholesterol and serum triglyceride do not always respond to dietary variations by increasing or decreasing in a like manner.

This inverse relationship that exists sometimes between the various levels and kinds of dietary fat, the levels of serum cholesterol and the levels of serum triglycerides has been discussed by Albrink (25) and Bizzi et al. (23). The latter workers (23) recognize these variations and reversals in the components of the blood lipids in the rat but have not fully established all factors within the diet which may bring them about. Their work showed that high levels of serum triglycerides accompanied a condition of thrombosis in the cardiac chambers of the rat and that elevated serum cholesterol, together with normal triglyceride level, was associated with atherosclerosis of the aorta. From the results of the present study, it appears that

the degree of impairment in the iron-transport system, as well as in the hemopoietic process, might be an influencing factor in the thrombogenic system. Rats in group 3, with the highest level of serum triglycerides of any of the groups also had the lowest hemoglobin and hematocrit levels together with the reversed phenomenon of low serum iron. These results were obtained with the feeding of a 10% fat diet rather than a 40% fat diet fed by Bizzi et al. (23) and without the feeding of thiouracil or cholic acid as used by these workers (23) to obtain high levels of serum triglycerides. Since the whole heart was used in the enzyme determination in the present study, no pathological examinations were made of this organ.

Weight of organs. Weights of the heart, liver and kidneys of the rats in the experimental groups, when expressed as grams per 100 g of rat (table 2) show some significant variations from the control group. Only the rats fed the cholesterol-containing, iron-deficient diet had hearts that were significantly heavier than those of the control group, 0.44 g as compared with 0.36 g/100 g rat for the control rats. Again, since no pathological examinations were made of this organ it cannot be said whether this greater weight was due to normal tissue development or to some abnormality, such as a thrombogenic condition within the heart chambers as noted by Bizzi et al. (23). This same diet, group 3, did not affect the weight of the liver, but feeding the 1% cholesterol diet with adequate iron significantly increased the liver weight over that of the controls, whereas the rats in group 1, fed the hypercholesterolemia-inducing diet without cholesterol had smaller livers than the controls. The kidney weights of rats in the 3 experimental groups with varying degrees of lowered hemoglobin and disturbances in the iron-transport system were significantly less than those of the control rats. Priest and Normann (5) did not report on the weights of the kidneys of their rats with renal hemosiderosis and no staining work was carried out in the present study to determine the presence of the iron-containing pigment, hemosiderin; hence it cannot be said whether these smaller kidney weights are related to such an abnormality in the kidney. Beutler and Blaisdell (8) reported that their iron-deficient diet induced similar changes in the weight of these organs, an increase in the weight of the heart and a decrease in the weight of the kidneys.

Succinic dehydrogenase activity (SDH), heart. The iron-deficient, cholesterol-containing diet fed to group 3 brought about a significant lowering in the SDH activity of the heart when expressed as microliters of oxygen per hour, per milligram dry weight of tissue. However, when this was calculated on either a per milligram of protein basis, or on a relative SDH per gram of rat weight basis (see footnote 7, table 2, for this calculation) this lowering was no longer evident and the values were then similar to those of the controls. This may be due in part to the fact that the heart weights of the iron-deficient rats were increased and hence, when the activity was expressed on a relative SDH basis, the total amount per 100 g of rat weight for the control and iron-deficient animals was approximately equal. These results are in general agreement with those reported by Beutler and Blaisdell (8). When the hypercholesterolemia-inducing diet was fed, with or without added cholesterol, and the SDH activity of the heart was expressed on a per milligram of protein basis, the SDH activity was significantly increased. This increased activity, however, was not evident when expressed as activity per milligram dry weight of tissue or as relative SDH activity per gram of rat weight. Thus, although all three of the experimental diets affected the hemopoietic processes and the iron-transport system, these changes were not reflected in the overall activity of the iron-bearing enzyme, SDH, of the heart.

SDH, liver. Rats fed the 3 experimental diets showed a number of significant changes in the SDH activity of the liver. Those fed the hypercholesterolemia-inducing diet, without added cholesterol, group 1, showed a lowered SDH activity of the liver, only when expressed as the relative SDH per gram of rat weight. However, when this diet included 1% cholesterol, group 2, the SDH activity became significantly lower than when no cholesterol was fed and this reduced SDH activity was evident regardless of how it was expressed.

The removal of the iron salts from this cholesterol-containing diet, group 3, did not cause further changes in these values. Hence, these significantly lower SDH values for the livers of these rats fed the 3 experimental diets are probably the result of the hypercholesterolemic effects of the diet and not to its effect of lowering the hemoglobin level. If interpreted in this manner, this is in agreement with the re-

TABLE 3

Sex differences in hemoglobin, the iron-transport system, the succinic dehydrogenase activity of heart, liver and kidney tissue and the respective weights of the organs of rats fed varying hypercholesterolemic diets and laboratory chow [1]

	Significance [2]	Experimental diets: 1 Basal diet		2 Basal diet + 1% cholesterol		3 Basal diet + 1% cholesterol, iron-deficient		Diet 4 Laboratory chow	
Rats/group		12 ♂	12 ♀	12 ♂	12 ♀	12 ♂	12 ♀	12 ♂	12 ♀
Food and growth									
Food intake, g/day		15.7	11.6	16.4	12.8	13.9	11.0	21.1	15.7
Weight, end of test, g		331	211	330	221	303	200	329	200
Blood									
Hemoglobin, g/100 ml	S–DS	15.1	15.7	15.0	15.4	8.5	9.7	16.1	16.2
Hematocrit, %	S–DS	47.4	47.9	47.6	47.4	30.1	33.3	49.8	49.0
Serum									
Iron, μg/100 ml	S–DS	198	303	154	247	68	45	219	289
TIBC,[3] μg/100 ml	—	762	787	707	720	615	610	699	758
Protein, g/100 ml	—	6.9	6.7	7.2	7.3	6.9	7.2	6.6	6.4
Cholesterol, mg/100 ml	S–DS	91	107	110	190	91	142	71	82
Triglycerides, mg/100 ml	S	144	93	112	65	149	131	95	86
Organ weights									
Heart, g/100 g rat	S	0.34	0.39	0.36	0.38	0.40	0.47	0.34	0.38
Liver, g/100 g rat	S–DS	3.03	2.79	3.73	3.24	3.33	3.32	3.34	3.28
Kidneys, g/100 g rat	S	0.70	0.77	0.71	0.78	0.69	0.77	0.80	0.83
Succinic dehydrogenase (SDH) activity [4]									
Heart									
μl O_2/hr/mg dry wt tissue	—	235	226	232	234	196	197	229	223
μl O_2/hr/mg protein	S–DS	263	270	246	263	213	207	201	217
Relative SDH/g rat wt [5]	S	176	194	178	198	173	206	171	190
Liver									
μl O_2/hr/mg dry wt tissue	DS	66	65	39	44	41	48	73	66
μl O_2/hr/mg protein	DS	95	90	59	65	61	72	97	92
Relative SDH/g rat wt	DS	614	543	433	425	413	482	728	653
Kidney									
μl O_2/hr/mg dry wt tissue	—	144	145	140	140	103	113	161	159
μl O_2/hr/mg protein	S	191	195	183	198	134	153	213	216
Relative SDH/g rat wt	S	224	245	221	241	157	192	285	289

[1] Rats were fasted approximately 10 hours prior to all determinations other than hemoglobin and hematocrit.
[2] Based on an analysis of variance:
S denotes significant difference in sex only ($P < 0.01$).
DS denotes significant difference in interaction of diet and sex only ($P < 0.01$).
S–DS denotes significant difference in both sex and in interaction of diet and sex ($P < 0.01$).
[3] TIBC indicates total iron-binding capacity.
[4] Per cent solids in heart, 22; liver, 30; kidney, 22.
[5] Relative SDH/g rat wt calculated as follows: $\frac{\mu l\ O_2/hr/mg\ moist\ tissue \times organ\ wt\ in\ mg}{rat\ wt\ in\ g}$.

port of Beutler and Blaisdell (8) that moderately severe iron deficiency caused no decrease in SDH activity of the liver.

SDH, kidney. The SDH activity of the kidneys in all 3 experimental groups is significantly lower than that of the controls, regardless of the basis for calculation. This decreased activity appears to be related to the degree the hemoglobin was lowered, since rats in group 3, fed the iron-deficient, cholesterol-containing diet, had significantly less SDH activity than rats fed a similar cholesterol-containing diet, but with normal iron intake. Other workers (8) have also reported a significant diminution of SDH activity in the kidneys of iron-deficient rats.

Differences due to sex. It has been observed previously that male and female rats fed hypercholesterolemic diets have shown certain sex differences in the level of hemoglobin, serum cholesterol and serum triglycerides (1, 24). However, there have not been reports on differences due to sex in some of the other biochemical determinations, shown in table 3.

Serum iron levels of female rats fed diets 1, 2 and 4 are considerably, and significantly, higher than those for the males (table 3). When they were fed diet 3, the iron-deficient, cholesterol-containing diet, this was reversed and the female rats had lower serum iron than the males. The sex reversal in the levels of serum cholesterol and serum triglycerides is evident in all 4 groups. Male rats consistently had lower levels of serum cholesterol and higher levels of serum triglycerides than the females. When organ weights were expressed on the basis of grams per 100 g of rat, female rats had slightly, but significantly, heavier heart and kidney weights and somewhat smaller liver weight than the males.

The succinic dehydrogenase activity, expressed as relative SDH per gram of rat weight, was greater in the heart and kidney tissue of the females than in the similar organ of the males. However, the females had slightly less relative SDH activity in the liver than the males when they were fed diets 1, 2 and 4. When fed diet 3, the iron-deficient diet, females had greater SDH activity than the males.

Although many of the biochemical determinations for males and females were significantly different, it has been noted previously that the 2 sexes responded in a similar manner to the dietary variations used in this study.

LITERATURE CITED

1. Roehm, R. R., and H. L. Mayfield 1962 Effect of dietary fat on cholesterol metabolism. J. Am. Dietet. A., *40:* 417.
2. Pinter, G. G., and R. E. Bailey 1961 Anemia of rabbits fed a cholesterol-containing diet. Am. J. Physiol., *200:* 292.
3. Ostwald, R., and A. Shannon 1964 Composition of tissue lipids and anemia of guinea pigs in response to dietary cholesterol. Biochem. J., *91:* 146.
4. Wohl, H., and C. Merskey 1964 Anemia in rats on atherogenic diets. Am. J. Physiol., *206:* 765.
5. Priest, R. E., and S. J. Normann 1962 Diet-induced hemolytic anemia in the rat. Arch. Pathol., *74:* 423.
6. Mueller, J. F., and F. Viteri 1965 Hematologic studies in patients receiving multiple infusions of Lipomul I.V. Am. J. Clin. Nutrition, *16:* 151.
7. Velick, S. F. 1956 Biological oxidations. Ann. Rev. Biochem., *25:* 257.
8. Beutler, E., and R. K. Blaisdell 1960 Iron enzymes in iron deficiency. V. Succinic dehydrogenase in rat liver, kidney and heart. Blood, *15:* 30.
9. Okey, R., and M. Lyman 1957 Dietary fat and cholesterol metabolism. I. Comparative effects of coconut and cottonseed oils at three levels of intake. J. Nutrition, *61:* 523.
10. Schneider, W. C., and V. R. Potter 1943 The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem., *149:* 217.
11. Umbreit, W. W., R. H. Burris and J. F. Stauffer 1959 Manometric Techniques. Burgess Publishing Company, Minneapolis.
12. Mandel, E. E. 1959 Serum iron and iron-binding capacity in clinical diagnosis. Clin. Chem., *5:* 1.
13. Peters, T., T. J. Giovanniello, L. Apt and J. F. Ross 1956 A new method for the determination of serum iron-binding capacity. 1. J. Lab. Clin. Med., *48:* 274.
14. Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.
15. Van Handel, E., and D. B. Zilversmit 1957 Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med., *50:* 152.
16. Van Handel, E. 1961 Modification of the micro determination of triglycerides. Clin. Chem., *7:* 249.
17. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.

18. Albritton, E. C. 1952 Standard Values in Blood. W. B. Saunders Company, Philadelphia.
19. Roehm, R. R., and H. L. Mayfield 1963 Effects of several low protein diets on the serum iron, total iron-binding capacity and serum cholesterol of rats. J. Nutrition, *81:* 131.
20. Rodbell, M., D. S. Fredrickson and K. Ono 1959 Metabolism of chylomicron proteins in the dog. J. Biol. Chem., 234*:* 567.
21. Korn, E. D. 1961 Are heparin and lipoprotein lipase involved in the transport of triglycerides. Colloq. Intern. Centre Natl. Rech. Sci. (Paris), no. 99. (U. S. Public Health Service, Bethesda, Maryland), p. 139.
22. Lynn, W. S. Jr., R. H. Brown, N. C. Perryman and C. Weber 1960 Biochemistry of Lipids, ed. G. Popjak. Pergammon Press, New York.
23. Bizzi, L., A. N. Howard and G. A. Gresham 1963 Plasma triglycerides and thrombosis in rats. Nature, *197:* 195.
24. Tinoco, J., A. Shannon and R. L. Lyman 1964 Serum lipids in choline-deficient male and female rats. J. Lipid Res., *5:* 57.
25. Albrink, M. J. 1962 Triglycerides, lipoproteins, and coronary artery disease. Arch. Intern. Med., *109:* 345.

Level of Readily Fermentable Carbohydrates and Adaptation of Lambs to All-Urea Supplemented Rations [1]

G. A. McLAREN, G. C. ANDERSON, L. I. TSAI AND K. M. BARTH [2]
Department of Animal Industry, West Virginia University, Morgantown

ABSTRACT Digestion and nitrogen metabolism data obtained with 83 lambs fed semipurified rations containing urea as the sole source of supplemental nitrogen were used to estimate the influence of length of time of urea feeding and level of readily fermentable carbohydrates on the retention of absorbed nitrogen. Regression analysis indicated that the retention of absorbed nitrogen by lambs was significantly improved by 3 percentage units with each 10-day period of urea feeding. The retention of absorbed nitrogen was significantly improved by approximately 2 percentage units for each 100 kcal of readily fermentable carbohydrates in the rations of lambs. Improvement in the retention of absorbed nitrogen due to the level of readily fermentable carbohydrates was observed in lambs regardless of the degree of their adaptation to urea feeding. The digestion of crude fiber was significantly decreased by 8 percentage units for each 1000 kcal of readily fermentable carbohydrates in the ration.

Smith et al. (1) found, through the use of regression analysis, that the retention of absorbed nitrogen by lambs was increased by 2 percentage units for each 10-day feeding period up to 50 days. The lambs used by these workers had been fed semi-purified rations in which approximately 63 and 12% of the total nitrogen was furnished by urea and purified soybean protein, respectively.

Although the importance of the type and adequacy of carbohydrates on non-protein nitrogen (NPN) utilization by ruminants has been recognized for many years, the literature contains limited information on the quantitative relationship between readily fermentable carbohydrates and NPN utilization.

The present work was conducted to determine the influence of the length of time (days) of urea feeding and the level of readily fermentable carbohydrates on the retention of absorbed nitrogen by lambs fed semipurified rations in which urea was the sole source of supplemental nitrogen.

EXPERIMENTAL

The influence of the length of time of urea feeding and the level of readily fermentable carbohydrates on the retention of absorbed nitrogen was estimated through regression analysis of data obtained with 83 lambs in 10 metabolism trials. An estimate of the extent of the depression in crude fiber digestibility as a function of the level of readily fermentable carbohydrates in the rations was made through regression analysis of digestibility data obtained with 33 lambs in 6 digestion trials.

The average total nitrogen content of these rations was 1.76%, of which urea and total NPN accounted for approximately 75 and 80%, respectively. Variations in the level of readily fermentable carbohydrates in the ration were accomplished by varying the amounts of cornstarch, dextrose and cane molasses. The average daily intake of the constituents of the rations and the caloric content of the readily fermentable carbohydrates is shown in table 1. The caloric value of the readily fermentable carbohydrates of each ration was obtained by multiplying the grams of total sugars (dextrose plus total reducing sugars in the molasses) and of cornstarch by the factors 3.74 and 4.20 kcal/g, respectively. The total reducing sugar content of the molasses, after clarification and acid inversion, was determined according

Received for publication June 7, 1965.

[1] Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper no. 833 from the Department of Animal Industry and Veterinary Science.

[2] Present address: Department of Animal Science, Purdue University, Lafayette, Indiana.

TABLE 1

Constituents and average daily intake of rations fed to lambs

Trial	Ration constituents							Total readily fermentable carbohydrates [2]
	Chopped wheat straw	Cane molasses 78% solids	Reducing sugars	Concentrate mixture				
				Constant portion [1]	Urea	Cornstarch	Dextrose	
	g	*g*	*g*	*g*	*g*	*g*	*g*	*kcal*
1	400	226	103	59.8	22.7	82.2	35.3	862
2	400	226	103	59.8	22.7	82.2	35.3	862
3	400	226	103	59.8	22.7	82.2	35.3	862
4	400	142	64	59.8	22.7	82.2	35.3	716
5	490	175	78	59.8	22.7	47.5	20.0	565
5	340	175	78	59.8	22.7	152.5	65.0	1173
6	440	378	167	65.8 [3]	25.0	90.4	38.8	1148
7	400	360	159	65.8	25.0	90.4	38.8	1119
8	400	170	75	59.8	22.7	82.2	35.3	758
9	400	170	75	59.8	22.7	82.2	35.3	758
9	400	170	75	59.8	22.7	152.5	65.0	1164
10	400	179	79	59.8	22.7	152.5	65.0	1178

[1] Comprised of corn oil, 29.4 g; mineral mixture, as described by Thomas et al. (17), 27.2 g; fish liver oil, containing not less than 2250 USP units of vitamin A and 300 ICU of vitamin D/g, 2.6 g; choline chloride, 0.6 g and α-tocopherol, 8 mg.

[2] Calculated on the basis of 3.74 kcal/g of dextrose and reducing sugars and 4.20 kcal/g of cornstarch.

[3] The constant portion of this concentrate was comprised of the same ingredients but at a level of 110% of those indicated in footnote 1.

to the procedure of Lane and Eynon as described in AOAC (2) methods.

The digestion and metabolism trials consisted of a 5-day adjustment period and a 10-day preliminary period followed by one or more consecutive 10-day collection periods. The procedures used in conducting the trials, and in the collection, storage and chemical analysis of samples have been described by Anderson et al. (3). Grade wether lambs, approximately 7 months of age at the start of urea feeding, were assigned at random to the indicated treatments within each trial.

The utilization of nitrogen was calculated according to the Thomas-Mitchell equation (4) but expressed as the retention of absorbed nitrogen rather than as biological value as explained by Anderson et al. (3). Values for endogenous urinary nitrogen and metabolic fecal nitrogen used in calculating nitrogen utilization were 0.034 g/kg body weight and 0.435 g/100 g dry matter intake, respectively (5).

The influence of the length of time of urea feeding (X_1), and the level of readily fermentable carbohydrates (X_2), on the retention of absorbed nitrogen (Ya) was estimated by means of multiple regression analysis. The nitrogen retention data used in this regression analysis were obtained with lambs which had been fed urea for periods varying from 15 to 55 days prior to the start of the collection periods. The level of readily fermentable carbohydrates (X_2) fed ranged from 716 to 1148 kcal/day. The influence of the level of readily fermentable carbohydrates (X_2), on the retention of absorbed nitrogen (Ya) by lambs adapted to high-level urea feeding was estimated by means of regression analysis. The nitrogen retention data were obtained with lambs which had been fed urea for at least 50 days prior to the start of the collection periods. Since these lambs were considered to be adapted to urea utilization, the single variable in this regression analysis was the level of readily fermentable carbohydrates (X_2) which was fed in amounts ranging from 564 to 1178 kcal/day. The influence of the level of readily fermentable carbohydrates (X_2) on the digestion of crude fiber (Yc) was also estimated by means of regression analysis. The crude fiber digestion data were obtained with lambs fed readily fermentable carbohydrates at levels ranging from 564 to 1173 kcal/day. Since Smith et al. (1) have shown that the digestion of crude fiber by lambs was not influenced by the length of urea feeding, this was not considered in this regression analysis. Statistical analyses were conducted according to the procedures of Snedecor (6).

RESULTS AND DISCUSSION

Nitrogen metabolism data used to estimate the influence of the length of time of urea feeding (X_1), and the level of readily fermentable carbohydrates (X_2) on the retention of absorbed nitrogen are presented in table 2, and the statistical treatment is presented in analysis number 1 of table 3. Approximately 55% of the total variance was due to the variables (X_1) and (X_2). The influence of the variables (X_1) and (X_2) on the retention of absorbed nitrogen (Ya) was highly significant. The influence of these variables on the retention of absorbed nitrogen is as follows:

$$(1)\ \hat{Y}a = 17.77 + 0.300X_1 + 0.21X_2.$$

This equation indicates that the improvement in the retention of absorbed nitrogen amounts to 3 percentage units for each 10-day period of urea feeding and 2.1% for each 100 kcal of readily fermentable carbohydrates in the ration. When the mean, which was 982 kcal in this regression analysis, is substituted for (X_2) in equation 1, it is simplified to the following equation:

$$(2)\ \hat{Y}a = 48.49 + 0.30X_1.$$

Although the rate of improvement in the retention of absorbed nitrogen was greater than the value of 2 percentage units per 10 days of urea feeding reported by Smith et al. (1), these results tend to substantiate the occurrence of an "adaptation response" to urea feeding (1). The greater rate of change in nitrogen utilization observed in the present work may have been due to differences in the rations fed. Smith et al. (1) fed rations containing nitrogen supplements which contained 84% urea and 16% purified soybean protein, but in the present work urea was the sole source of supplemental nitrogen. This might account for the higher initial retention of absorbed nitrogen obtained by Smith et al. (1). The higher urea content of the rations fed in the present work would have caused the release of more ammonia to the tissues, which might have brought about the greater adaptation of the tissues to ammonia utilization.

Improvement in the retention of absorbed nitrogen as the level of readily fermentable carbohydrates in the ration is increased could involve either increased utilization of nitrogen by the microbial population or better utilization of ammonia nitrogen by the ruminant tissues. The in vitro studies reported by Wegner et al. (7), Pearson and Smith (8), Arias et al. (9) Belasco (10), and Reis and Reid (11) have demonstrated the need for sufficient readily

TABLE 2

Influence of length of time of urea feeding and level of readily fermentable carbohydrates on the retention of absorbed nitrogen by lambs

Trial and period	No. of lambs	Avg lamb weight	Avg daily dry matter intake	Retention of absorbed nitrogen (Ya)	Length of time of urea feeding (X_1)	Readily fermentable carbohydrates (X_2)
		kg	*g*	*%*	*days*	*kcal/day*
1–1	4	29	713	42.5	15	862
1–2	4	29	714	45.3	25	862
2–1	4	30	735	39.4	15	862
2–2	4	30	734	41.8	25	862
3–1	3	34	736	37.7	15	862
3–2	3	34	736	39.8	25	862
4–1	2	35	664	36.1	15	716
4–2	2	35	664	40.5	25	716
4–3	2	35	664	40.9	35	716
4–4	2	35	664	48.0	45	716
4–5	2	35	664	51.0	55	716
6–1	5	36	907	49.5	15	1148
6–2	5	36	908	49.6	25	1148
6–3	5	36	908	52.1	35	1148
7–1	5	37	887	43.8	11	1119
7–2	5	37	899	47.8	21	1119
7–3	4	37	892	45.8	31	1119

TABLE 3

Summary of statistical evaluations

Analysis no.	Statistical item	Retention of absorbed nitrogen (Ya)	Retention of absorbed nitrogen (Ya)	Crude fiber digestibility (Yc)
	Variables	X_1, X_2	X_2	X_2
1	Determination coefficient, $R^2 =$	0.552		
1	Mean square due to regression =	517.59 [1]		
1	Mean square due to deviation from regression =	14.50		
1	Standard partial regression coefficients:	b'y 1·2 = 0.012 [1] b'y 2·1 = 0.00002 [1]		
1	Standard error of regression coefficient:	sby 1·2 = 0.157 sby 2·1 = 0.003		
1	Regression equation:	$\hat{Y}a = 17.77 + 0.300X_1 + 0.021X_2$		
2	Linear correlation coefficient, $R^2 =$		0.454	
2	Mean square due to regression =		565.96 [1]	
2	Mean square due to deviation from regression =		33.90	
2	Linear regression equation:		$Ya = 30.11 + 0.019X_2$	
3	Linear correlation coefficient, R^2			0.146
3	Mean square due to regression			127.19 [2]
3	Mean square due to deviation from regression:			21.13
3	Linear regression equation:			$\hat{Y}c = 53.0 - 0.008X_2$

[1] $P < 0.005$.
[2] $P < 0.025$.

TABLE 4

Influence of level of readily fermentable carbohydrates on the retention of absorbed nitrogen by lambs adapted to urea feeding [1]

Trial	No. of lambs	Avg lamb wt	Avg daily dry matter intake	Retention of absorbed nitrogen (Ya)	Readily fermentable carbohydrates (X_2)
		kg	*g*	%	*kcal/day*
5	5	38	725	55.5	1173
5	5	37	720	40.2	564
8	3	38	699	43.8	758
9	3	36	681	42.5	758
9	3	38	773	50.9	1165
10	3	38	775	49.0	1178

[1] These lambs had been fed urea for at least 50 days prior to the start of the collection period.

available carbohydrates for the utilization of ammonia nitrogen by rumen microorganisms. The reports of Mitchell et al. (12), Harris and Mitchell (13), and Fontenot et al. (14) have shown that the retention of nitrogen is improved as the result of added readily fermentable carbohydrates.

Although neither the site nor the nature of the "adaptation response" to urea feeding is known, it was felt that the level of readily fermentable carbohydrates in the ration might influence it. Metabolism data relating to the influence of readily fermentable carbohydrates on the retention of absorbed nitrogen by lambs adapted to urea feeding are presented in table 4. A summary of the statistical data of the influence of the level of readily fermentable carbohydrates (X_2) on the retention of absorbed nitrogen (Ya) is presented in analysis 2 of table 3. The level of readily fermentable carbohydrates significantly influenced the retention of absorbed nitrogen. This may be expressed in the following regression equation:

$$(3)\ \hat{Y}a = 30.11 + 0.019X_2.$$

These results indicate that the retention of absorbed nitrogen was significantly increased as the level of readily fermentable carbohydrates in the ration was increased. However, a comparison of the value of (X_2) in equations 1 and 2 indicates that the influence of readily fermentable carbohydrates was approximately the same whether or not the lambs were adapted to urea feeding. This suggests that over the range of 565 to 1173 kcal, the level of readily fermentable carbohydrates did not influence the adaptation of the lambs to the utilization of urea nitrogen.

Although Mitchell et al. (12), Hamilton (15), Swift et al. (16) and Fontenot et al. (14) observed that the digestibility of crude fiber was decreased as the result of adding readily fermentable carbohydrates to the ration, it seemed worthwhile to estimate the extent of this depression in lambs fed semipurified rations containing urea as the sole source of supplemental nitrogen.

Data relating to the influence of the level of readily fermentable carbohydrates on crude fiber digestibility are presented in table 5. The statistical data in analysis 3 of table 3 indicate that the digestibility of crude fiber was significantly depressed as the level of readily fermentable carbohydrates in the ration was increased. The relationship of level of readily fermentable carbohydrates on crude fiber digestibility may be expressed in the following regression equation:

$$(4)\ \hat{Y}c = 53.0 - 0.008X_2.$$

TABLE 5

Influence of level of readily fermentable carbohydrates on crude fiber digestibility

Trial	No. of lambs	Avg crude fiber digestibility (Yc)	Readily fermentable carbohydrates (X_2)
		%	*kcal/day*
3	3	43.9	862
4	2	47.5	716
5	5	43.2	1173
5	5	45.6	564
8	9	48.3	758
9	3	52.1	758
9	3	48.5	1165
10	3	49.0	1165

Regression equation 4 indicates that crude fiber digestibility was decreased 8 percentage units for each 1000 kcal of readily fermentable carbohydrates added to the ration.

LITERATURE CITED

1. Smith, G. S., R. S. Dunbar, G. A. McLaren, G. C. Anderson and J. A. Welch 1960 Measurement of the adaptation response to urea-nitrogen utilization in the ruminant. J. Nutrition, *71:* 20.
2. Association of Official Agricultural Chemists 1950 Official Methods of Analysis, ed. 7. Washington, D. C.
3. Anderson, G. C., G. A. McLaren, J. A. Welch, C. D. Campbell and G. S. Smith 1959 Comparative effects of urea, uramite, biuret, soybean protein and creatine on digestion and nitrogen metabolism in lambs. J. Animal Sci., *18:* 134.
4. Mitchell, H. H. 1923–24 A method of determining the biological value of proteins. J. Biol. Chem., 58: 873.
5. McLaren, G. A., G. C. Anderson, J. A. Welch, C. D. Campbell and G. S. Smith 1960 Diethylstilbestrol and length of preliminary period on the utilization of crude biuret and urea by lambs. II. Various aspects of nitrogen metabolism. J. Animal Sci., *19:* 44.
6. Snedecor, G. W. 1956 Statistical Methods, ed. 5. The Iowa State College Press, Ames.
7. Wegner, M. L., A. N. Booth, G. Bohstedt and E. B. Hart 1940 The in vitro conversion of inorganic nitrogen to protein by microorganisms from the cow's rumen. J. Dairy Sci., *23:* 1123.
8. Pearson, R. M., and J. A. B. Smith 1943 The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of proteins in rumen ingesta. Biochem. J., *37:* 153.
9. Arias, C., W. Burroughs, P. Gerlaugh and R. M. Bethke 1951 The influence of different amounts and sources of energy upon in vitro urea utilization by rumen microorganisms. J. Animal Sci., *10:* 683.
10. Belasco, I. J. 1956 The role of carbohydrates in urea utilization, cellulose digestion and fatty acid formation. J. Animal Sci., *15:* 496.
11. Reis, P. J., and R. L. Reid 1959 In vitro studies on the effect of pH and of glucose on ammonia accumulation in the rumen of sheep. Australian J. Agr. Res., *10:* 71.
12. Mitchell, H. H., T. S. Hamilton and W. T. Haines 1940 The utilization by calves of energy in rations containing different percentages of protein and glucose supplements. J. Agr. Res., *61:* 847.
13. Harris, L. E., and H. H. Mitchell 1941 The value of urea in the synthesis of protein in the paunch of the ruminant. II. On growth. J. Nutrition, 22: 183.
14. Fontenot, J. P., W. D. Gallup and A. B. Nelson 1955 Effect of added carbohydrate on the utilization by steers of nitrogen in wintering rations. J. Animal Sci., *14:* 807.
15. Hamilton, T. S. 1942 The effect of added glucose upon the digestibility of protein and of fiber in rations for sheep. J. Nutrition, 23: 101.
16. Swift, R. W., E. J. Thacker, A. Black, J. W. Bratzler and W. H. James 1947 Digestibility of rations for ruminants as affected by proportions of nutrients. J. Animal Sci., 6: 432.
17. Thomas, W. E., J. K. Loosli, H. H. Williams and L. A. Maynard 1951 The utilization of inorganic sulfates and urea by lambs. J. Nutrition, 43: 515.

Vitamin K Activity in Chickens: Phylloquinone and Menadione in the Presence of Stress Agents [1]

PAUL GRIMINGER
Department of Animal Sciences, Rutgers — The State University, New Brunswick, New Jersey

ABSTRACT At low levels of vitamin K supplementation, high levels of vitamin A and of tetracycline slightly increased plasma prothrombin times of chicks; a sulfonamide, at 0.2% of the diet, increased vitamin K requirements two to three times. In all 3 cases a synthetic menadione analogue reversed hypoprothrombinemia as effectively as did phylloquinone. However, in the presence of 2 coumarin-type anticoagulants, given at the level of 100 mg/kg diet, the menadione analogue was essentially incapable of reversing the hypoprothrombinemia. This level of supplementation increased the vitamin K requirement by factors of approximately 650 and 1,100, respectively, for the 2 anticoagulants. The inactivity of menadione may be due to quantitative limitations of intracellular synthesis of a biologically active form of vitamin K [$K_{2(20)}$] from menadione, whereas phylloquinone (K_1) can apparently act in prothrombin synthesis without prior conversion to $K_{2(20)}$.

In contrast with phylloquinone (vitamin K_1), which reverses the hypoprothrombinemia induced by coumarin-type anticoagulants, menadione (vitamin K_3) and its water-soluble analogues have been shown to have little or no activity in several species tested. Miller et al. (1), working with dicumarol, observed this lack of activity with rats, dogs, and humans, and Griminger and Donis (2) recorded similar observations with chicks. Using warfarin, Lowenthal and Taylor (3) noted the same phenomenon in rats, as did Clark and Halliwell (4) with dogs. Nevertheless, it is common knowledge that menadione and its water-soluble analogues will revert the prothrombin time of animals on uncomplicated vitamin K deficiency to normal, and that they are active against sulfaquinoxaline - induced hypoprothrombinemia (2).

To obtain further information on this subject, we have compared the activity of a menadione analogue with that of phylloquinone in the presence of several agents that were expected to increase the vitamin K requirement of chicks, and that hitherto have not been tested with this species. Since high levels of vitamin A have been shown to increase the vitamin K requirement of the rat (5,6), and certain antibiotics have been noted to slow blood coagulation in dogs (7) and increase the incidence of hemorrhages in chicks (8), this study included vitamin A and an antibiotic as well as a sulfonamide and two coumarin-type anticoagulants not studied previously.

EXPERIMENTAL

All chicks used in these experiments were fed vitamin K-deficient diets during their first week (table 1), and then fed the experimental (supplemented) diets for another 2 weeks. Depending on the nature of the study, either a purified diet (A) or a practical-type diet (B) was fed. The chicks used were female cross-breds grown on raised wire floors in battery-type, electrically heated brooders. Blood was drawn by heart puncture at 3 weeks of age and in some instances also at 2 weeks.

The methods used for the determination and evaluation of the plasma prothrombin times have been described in previous reports (2,9). Additional vitamin A, where used, was given in the form of stabilized beadlets (containing 325,000 IU/g) at the level of 220,000 IU/kg feed. The antibiotic (tetracycline) was added at the level of 500 mg/kg diet. Phylloquinone (2-methyl-3 - phytyl-1, 4 - naphthoquinone)

Received for publication June 17, 1965.
[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick. This research was supported in part by a grant from Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Vitamin K-deficient diets for growing chicks

	%
Diet A	
Starch	20.00
Glucose monohydrate	43.05
Egg albumen, dried	25.00
L-Arginine·HCl	0.50
Mineral mixture [1]	4.90
Vitamin mixture [2]	0.25
Choline chloride (70%)	0.30
Non-nutritive fiber [3]	3.00
Corn oil, refined	3.00
Total	100.00
Diet B	
Yellow corn meal	63.00
Soybean meal (50% protein)	28.00
Dried whey	2.50
Fish solubles (50% solids)	2.50
DL-Methionine	0.05
Calcium carbonate	1.60
Dicalcium phosphate	1.75
Trace-mineral mixture [4]	0.10
Sodium chloride	0.25
Choline chloride	0.05
Vitamin mixture [5]	0.20
Total	100.00

[1] Fisher et al. (17).
[2] Provides the following vitamin levels: (mg/kg diet) thiamine·HCl, 25; riboflavin, 16; Ca pantothenate, 20; pyridoxine·HCl, 6; niacin, 150; folic acid, 4; biotin, 0.6; cyanocobalamin, 0.02; ascorbic acid, 250; vitamin A, 10,000 IU; vitamin D_3, 600 IU; and vitamin E, 5 IU.
[3] Solka Floc, Brown Company, Chicago; consisting of 99.5% pure cellulose.
[4] Delamix, Limestone Corporation of America, Newton, New Jersey.
[5] Providing the following vitamin levels: (mg/kg diet) riboflavin, 1.8; niacin, 13; pantothenic acid, 4; cyanocobalamin, 0.009; vitamin A, 3,500 IU; and vitamin D_3, 400 IU.

was given in the form of stabilized 1% or 5% beadlets.[2] Menadione (2-methyl-1, 4-naphthoquinone) was given in the form of a water-soluble menadione sodium bisulfite complex which contains 33% menadione. The sulfanilamide sulfadimethoxine [N^1-(2,6-dimethoxy-4-pyrimidinyl) sulfanilamide] was added to the diet at the 0.2% level. The anticoagulants warfarin [3- (α-acetonylbenzyl) -4- hydroxycoumarin] and Ro 1-7627 (3-[α-(parachlorophenyl -) propyl -] 4 - hydroxycoumarin) were fed, after some preliminary trials, at the level of 100 mg/kg diet. Ten chicks per group were used for the vitamin A test, and groups of 12 chicks (duplicate lots of six) in all others.

The thromboplastin extract used for the prothrombin determinations was freshly prepared for each test; thus there were minor variations in prothrombin times among the separate tests. Since, however, all tests had their own control groups and were therefore independent of each other, the differences in prothrombin times among tests (up to 2 seconds) were not considered to have any bearing on interpretation of the results (10).

RESULTS AND DISCUSSION

A massive dose of vitamin A prolonged prothrombin times of 3-week-old chicks

[2] Prepared by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 2

Plasma prothrombin times of chicks fed graded levels of vitamin K and a high dose of vitamin A [1]

Vitamin K supplement	Control		Vitamin A added [2]	
	MSBC [3]	Vitamin K_1 [4]	MSBC	Vitamin K_1
mg/kg	*sec*	*sec*	*sec*	*sec*
		Diet A		
0	28.4 [5]		67.1(9)	
0.15	15.8	16.3(8)	17.1	17.6
0.60	13.7	13.2	13.5	13.0
1.20	12.8(9)	12.9	13.2	12.8
2.40	—	—	13.2	13.5(9)
		Diet B		
0	50.3		69.0	
0.15	15.2	15.0	18.9	17.3
0.60	13.8	13.9(9)	13.9	13.9
1.20	—	—	14.0	14.3
2.40	—	—	14.1(9)	13.9

[1] The chicks were fed the basal diets for one week, and the experimental diets for 2 weeks. Prothrombin times, measured at 3 weeks of age, were averaged on the basis of their reciprocals.
[2] 220,000 IU/kg feed.
[3] Menadione sodium bisulfite complex, containing 33% menadione.
[4] Vitamin K_1 given in form of stabilized beadlets.
[5] Ten chicks per group, except where shown differently (in parentheses).

TABLE 3

Plasma prothrombin times of chicks fed graded levels of vitamin K and a high dose of an antibiotic [1]

Vitamin K supplement	Control		Antibiotic added [2]	
	MSBC [3]	Vitamin K_1 [3]	MSBC	Vitamin K_1
mg/kg	*sec*	*sec*	*sec*	*sec*
		Diet B		
0	42.9(11) [4]		48.5	
0.1	15.6	15.2	19.2	19.5
0.2	15.6	15.6	15.2	15.9
0.4	14.2	14.4	15.2	15.7
0.8	14.3	14.8	15.0	14.5(11)
1.6	—	—	14.5	15.1

[1] See footnote 1, table 2.
[2] 500 mg tetracycline/kg diet.
[3] See footnotes 3 and 4, table 2.
[4] Twelve chicks per group, except where shown differently (in parentheses).

fed a low vitamin K diet (0.15 mg/kg diet), but the form of vitamin K had no apparent influence on the results (table 2). In terms of prothrombin times, only a moderately severe deficiency was apparent in this test with the vitamin-K-deficient diets. When prothrombin time is plotted against the prothrombin concentration of human plasma, a hyperbolic curve is obtained. Prothrombin times of 55 to 65 seconds can be shown to be equivalent to a level of 5% of the prothrombin observed in normal plasma (11). More limited data from our laboratory indicate a similar relationship for chicken plasma. Thus, the negative controls in these experiments have, with one exception (28.4 sec), a low level of plasma prothrombin concentration, with a concomitantly severe deficiency of vitamin K.

The high level of tetracycline had essentially the same effect as the high dose of

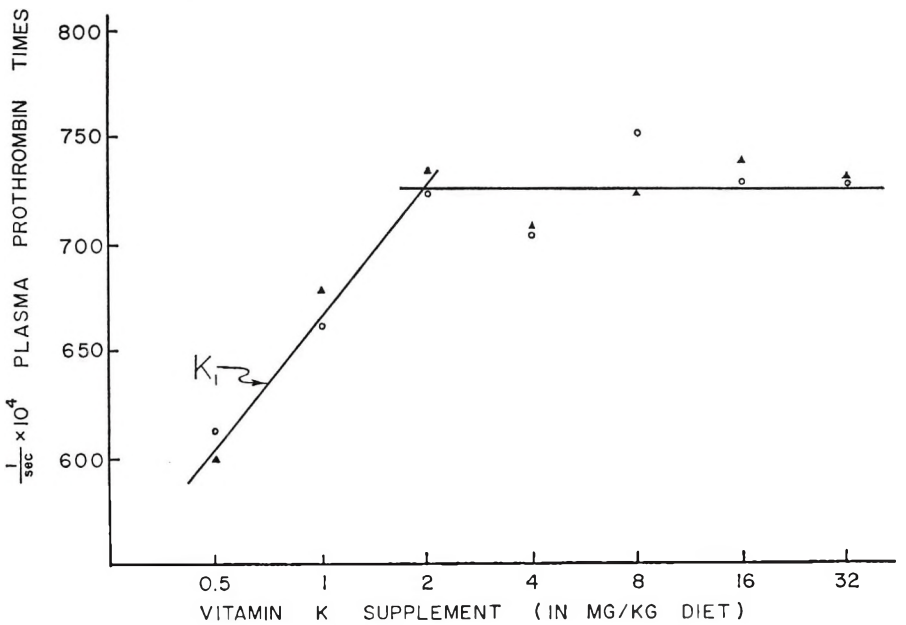

Fig. 1 Effect of continuous feeding of graded levels of vitamin K_1 (○) and MSBC (▲) on plasma prothrombin times of 2- and 3-week-old chicks receiving a low vitamin K diet supplemented with 0.2% of sulfadimethoxine.

vitamin A: it prolonged prothrombin times at the lowest dose of supplemental vitamin K (table 3), but not at higher doses. Again, the form in which vitamin K was given did not influence the results.

The observation that, under certain conditions, both vitamin A and tetracycline influence the utilization of vitamin K recalls earlier observations of an interaction between arsanilic acid and vitamin K (9). Further experimentation would be necessary to estimate the actual increase in the vitamin K requirement imposed by the addition of various levels of vitamin A or antibiotics. This, however, was not within the scope of the present study.

The actual increase in the vitamin K requirement can be more easily estimated when sulfanilamides are included in the ration. When a vitamin K-deficient diet was supplemented with 0.2% sulfadimethoxine (fig. 1), the requirement for vitamin K was increased to 2 mg/kg. (In the absence of sulfadimethoxine, 0.6 to 0.8 mg/kg of vitamin K would satisfy the requirement for optimal prothrombin times with this diet.) Since the results obtained for 2- and 3-week-old chicks were similar, they were pooled. The regression lines calculated by the method of least squares for the 2 vitamin K supplements nearly coincided; the observed differences were judged to be due to chance, and a single regression line calculated. Thus, for this sulfanilamide, which at the 0.2% level of supplementation increased the vitamin K requirement by a factor of two to three, a water-soluble form of menadione (MSBC) was as effective as phylloquinone in reversing the hypoprothrombinemic condition. Sulfaquinoxaline, when added at the same level in an earlier study, increased the vitamin K requirement at least fourfold (2). Although the mode of hypoprothrombinemic action of these 2 sulfa drugs is not understood, it is clear from these and previous reports (2, 12) that they do not increase the vitamin K requirement by reducing synthesis of vitamin K in the intestinal tract.

Because of the lack of information on the effect of warfarin on the prothrombin times of young chicks, several levels of warfarin and phylloquinone were fed to groups of 5 chicks for 2 weeks (table 4). On the strength of this test it was decided to use 100 mg of warfarin/kg of the vitamin K-deficient diet (B) (the somewhat less potent dicumarol had been used in a previous trial at the 400 mg/kg level). Figure 2 shows that phylloquinone given at a level of 400 to 500 mg/kg reversed the anticoagulant action of 100 mg warfarin. Only minimal reductions of plasma prothrombin times were observed with increasing levels of MSBC. Similar results were obtained when Ro-1-7627, another anticoagulant, was studied at the same level of supplementation (100 mg/kg, fig. 3). Since approximately 800 mg vitamin K_1 were required to overcome the hypoprothrombinemia caused by the latter compound, it appears to be an even more potent antivitamin-K than warfarin. Again, the beneficial effects of graded levels of MSBC were minimal.

TABLE 4

Plasma prothrombin times of chicks fed graded levels of warfarin and vitamin K_1 [1]

Vitamin K_1	Warfarin, mg/kg diet			
	0.5	5.0	50	500
mg/kg diet	*sec*	*sec*	*sec*	*sec*
0.5	13.2 [3]	14.4	29.7	151(+) [2]
5.0	13.6	13.6	16.5	180(+)
50	13.7	12.8	14.6	68.5
500	—	12.8	13.9	18.4

[1] The chicks (5/group) were fed basal diet B for 5 days, and the experimental diets (B + supplements) for 14 days. Prothrombin times, measured at 19 days of age, were averaged on the basis of their reciprocals.

[2] Not all samples clotted at 180 seconds, at which time observation was discontinued. Samples not clotted at that time were treated as if they had clotted at 180 seconds. The use of reciprocals for calculations minimizes the error thus incurred.

[3] Five chicks/group, except in the group receiving 500 mg warfarin and 5 mg vitamin K_1, where 2 chicks died during the experiment.

Warfarin was also studied at a lower level of supplementation (50 mg/kg, fig. 4). At this level only 200 mg phylloquinone were needed to obtain normal prothrombin times, and MSBC was again ineffective. For unexplained reasons, the vitamin K deficiency obtained in this test in the absence of phylloquinone was less severe than in other tests.

GENERAL DISCUSSION

These experiments showed that, in the presence of dietary supplements which increase the vitamin K requirement moder-

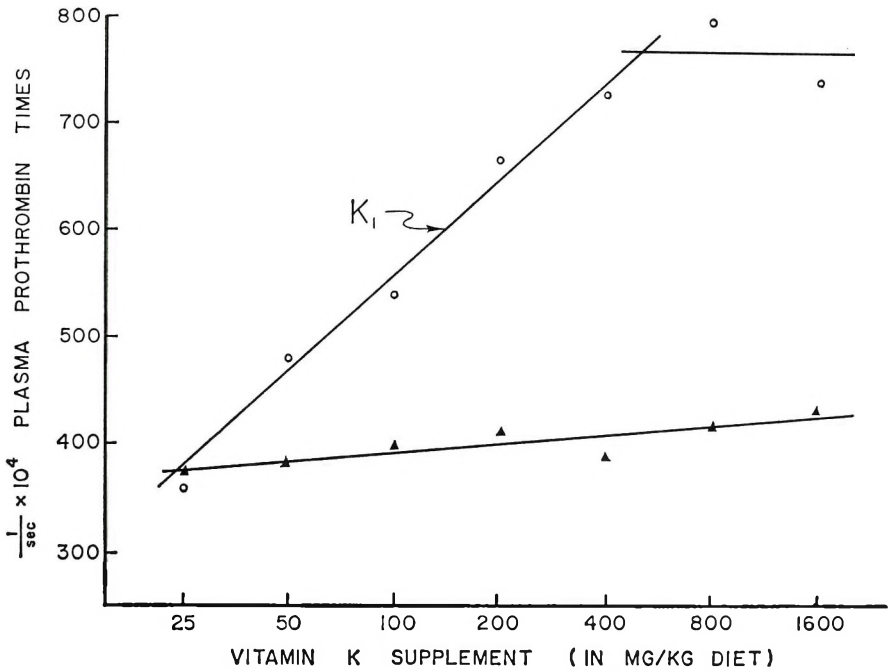

Fig. 2 Effect of continuous feeding of graded levels of vitamin K_1 (○) and MSBC (▲) on plasma prothrombin times of 2- and 3-week-old chicks receiving a low vitamin K diet supplemented with 100 mg of warfarin/kg.

ately, a synthetic form of vitamin K, lacking the side chain, was as effective as the naturally occurring phylloquinone in promoting optimal prothrombin times. When dietary supplements placed a severe stress on the vitamin K requirement, as in the presence of coumarin-type anticoagulants, MSBC appeared to be essentially inactive. Increasing levels of phylloquinone, however, decrease prothrombin times just as they do in the absence of anticoagulants, although at much higher dosage levels of the vitamin. This suggests the possibility that the presence or absence of activity for menadione may not be a qualitative but a quantitative matter that is dependent upon the amount of vitamin required rather than upon the stress agent used.

Billeter and Martius (13) have reported that both menadione and phylloquinone can be converted in the organism to vitamin $K_{2(20)}$ (2-methyl -3- geranyl-geranyl-1, 4-naphthoquinone). Later work from the same laboratory (14) indicated that side chains of vitamin K compounds are split off prior to absorption as a result of the activity of intestinal microorganisms. After absorption the conversion to vitamin $K_{2(20)}$ is continued by intracellular synthesis.

Phylloquinone can also be absorbed without previous removal of the phytyl side chain. This can be explained by assuming either that there are quantitative limitations to the removal of the chain by the microflora, or that the removal of the side chains is incidental to the intact absorption of phylloquinone. Probably there are also limitations to the intracellular synthesis of vitamin $K_{2(20)}$ from menadione. Thus, when relatively small doses of vitamin K are needed (when there is mild or no stress), menadione in its various forms can be readily converted to the biologically active form of vitamin $K_{2(20)}$ to serve in prothrombin synthesis. When, however, menadione is used under conditions of severe hypoprothrombinemia induced by coumarin-type anticoagulants, vitamin $K_{2(20)}$ synthesis is insufficient to provide the necessary amounts of a biologically active form of the vitamin. In

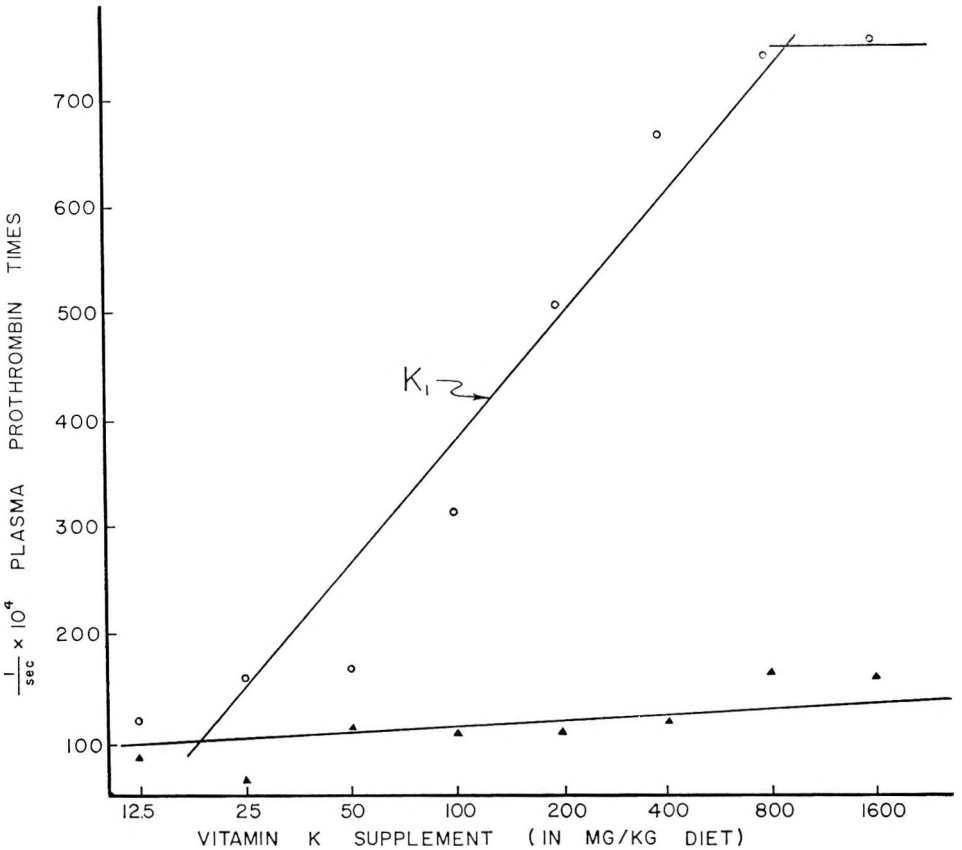

Fig. 3 Effect of continuous feeding of graded levels of vitamin K_1 (○) and MSBC (▲) on plasma prothrombin times of 2- and 3-week-old chicks receiving a low vitamin K diet supplemented with 100 mg of RO-1-7627 anticoagulant/kg.

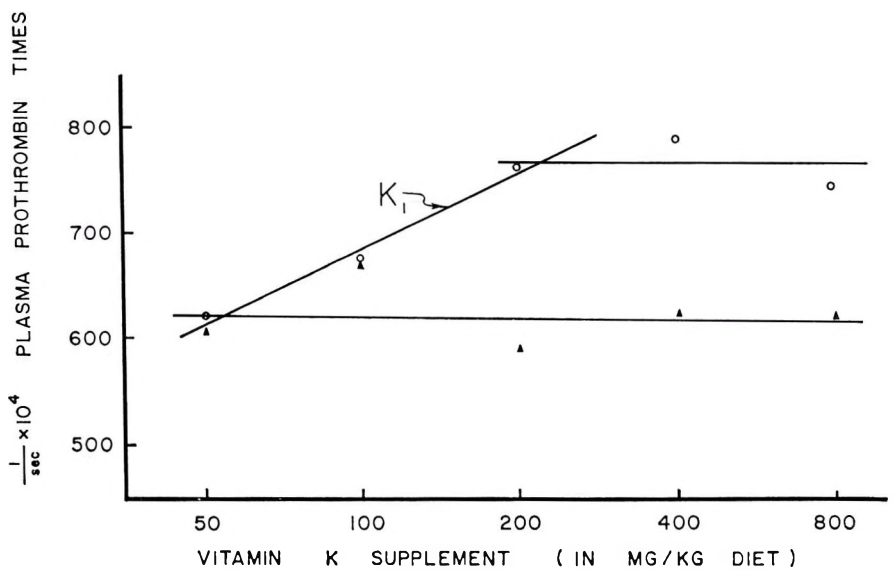

Fig. 4 Effect of continuous feeding of graded levels of vitamin K_1 (○) and MSBC (▲) on plasma prothrombin times of 2-week-old chicks receiving a low vitamin K diet supplemented with 50 mg of warfarin/kg.

this case direct feeding of another biologically active form of the vitamin, such as phylloquinone, is necessary.

Not all available evidence supports this hypothesis of vitamin K absorption. It has, for example, been observed that germfree animals utilize menadione poorly (15). It is possible that certain changes mediated or supported by the microflora (possibly phosphorylation) must take place in the menadione molecule prior to absorption. Other results, relating vitamin K activity with length of the side chain for both vitamins K_1 and K_2 (16), also make it appear doubtful that the removal of the chain is a biological necessity. Perhaps this removal is incidental, and the absorption of intact vitamins K_1 and K_2 is the usual pattern. Whatever the answer, at the present time the observations of the Swiss workers (13,14) appear to offer the best approach to the explanation of the results obtained in the experimentation presented in this report.

LITERATURE CITED

1. Miller, R., W. P. Harvey and C. A. Finch 1950 Antagonism of dicumarol by vitamin K preparations. New England J. Med., *242:* 211.
2. Griminger, P., and O. Donis 1960 Potency of vitamin K_1 and two analogues in counteracting the effects of dicumarol and sulfaquinoxaline in the chick. J. Nutrition, *70:* 361.
3. Lowenthal, J., and J. D. Taylor 1959 A method for measuring the activity of compounds with an activity like vitamin K against indirect anticoagulants in rats. Brit. J. Pharmacol., *14:* 14.
4. Clark, W. T., and R. E. W. Halliwell 1963 The treatment with vitamin K preparations of Warfarin poisoning in dogs. Vet. Rec., *75:* 1210.
5. Matschiner, J. T., and E. A. Doisy, Jr. 1962 Role of vitamin A in induction of vitamin K deficiency in the rat. Proc. Soc. Exp. Biol. Med., *109:* 139.
6. Israels, L. G., J. Foerster and A. Zipursky 1962 The coagulation defect in hypervitaminosis A in rats. Thromb. Diath. Haemorrhag., *7:* 159.
7. Quivy, D. 1952 Antibiotiques et coagulation plasmatique chez le chien. Compt. Rend. Soc. Biol., *146:* 1854.
8. Dempsey, R. J., and P. E. Sanford 1960 Effect of feeding various antibiotics on the hemorrhagic condition in chickens. Poultry Sci., *39:* 691.
9. Griminger, P. 1962 Arsanilic acid and blood coagulation. Poultry Sci., *41:* 982.
10. Griminger, P. 1962 Prothrombin bioassay for vitamin K with different thromboplastin preparations. Internat. Ztsch. Vitaminforsch., *32:* 405.
11. Quick, A. J. 1957 (1959) Hemorrhagic Diseases. Lea and Febiger, Philadelphia.
12. Griminger, P. 1957 On inhibition of vitamin K activity by sulfaquinoxaline in chicks. Proc. Soc. Exp. Biol. Med., *96:* 757.
13. Billeter, M., and C. Martius 1960 Über die Umwandlung von Phyllochinon (Vitamin K_1) in Vitamin $K_{2(20)}$ im Tierkörper. Biochem. Ztschr., *333:* 430.
14. Billeter, M., W. Bolliger and C. Martius 1964 Untersuchungen über die Umwandlung von verfütterten K-Vitaminen durch Austausch der Seitenkette und die Rolle der Darmbakterien hierbei. Biochem. Ztschr., *340:* 290.
15. Gustafsson, B. E., F. S. Daft, E. G. McDaniel, J. C. Smith and R. J. Fitzgerald 1962 Effects of vitamin K-active compounds and intestinal microorganisms in vitamin K-deficient germfree rats. J. Nutrition, *78:* 461.
16. Wiss, O., F. Weber, R. Ruegg and O. Isler 1959 Über die biologische Aktivität der Vitamine K_1 und K_2 und ihrer Isoprenologen. Ztschr. Physiol. Chem., *314:* 245.
17. Fisher, H., P. Griminger, G. A. Leveille and R. Shapiro 1960 Quantitative aspects of lysine deficiency and amino acid imbalance. J. Nutrition, *71:* 213.

Dietary Restriction and Reproduction in the Rat

BENJAMIN N. BERG
Geigy Research, Division of Geigy Chemical Corporation, Ardsley, New York

ABSTRACT The effects of varying levels of food intake on reproduction were observed in Sprague-Dawley rats. The diet consisted of commercial pellets composed of natural foods. Restriction was at 3 levels: 25, 50 and 75%. Maternal and fetal weights varied inversely with degree of restriction. Weight loss of restricted dams bearing young was no greater than that of rats failing to litter. Fetation was an all-or-none phenomenon in that full-sized litters developed or all implants resorbed. Resorptions occurred between days 8 to 11. Number of resorptions and number of viable embryos were nearly identical in 75%-restricted rats examined on day 13. Size of litters of 75%-restricted dams was practically the same on days 13 and 21, and equaled that of unrestricted dams examined at corresponding stages of gestation. Transitory unrestricted feeding during the first 2 or 3 days of pregnancy greatly reduced the incidence of resorptions in 75%-restricted rats, and feeding started on day 8 completely prevented them. Delay of transitory feeding to day 10 was less effective, and on day 12 had no effect. Fetal malformations were never observed. The results obtained with a restricted stock diet were similar to the reported observations in rats receiving a protein-deficient diet.

Purified diets containing graded amounts of protein are usually used in studies on food restriction in relation to reproduction in the rat (1–5). A diet composed of natural foods is used less often (6). A stock diet is unsuited for studying specific deficiencies but is practical for total food consumption. The present investigation deals with observations on the effect of varying levels of intake of a stock diet on reproductive performance, maternal and fetal weights, onset of resorptions, and critical stages of implantation in the rat. The results are similar to those obtained with protein deficiency (2).

METHODS AND MATERIALS

Female rats of the Sprague-Dawley strain, 10 to 11 weeks old and weighing 200 to 220 g were mated, one female with one male during a single estrus cycle. Feeding experiments were started on the day sperm were noted in vaginal washings (day zero). Rats were weighed at weekly intervals, and at time of autopsy after removal of uteri and fetuses. Animals were killed by CO_2 asphyxia and complete autopsies were performed on day 21 of pregnancy. Uteri were inspected for number and size of implants, and for resorptions. Litter size and fetal weights were recorded. Fetuses were inspected for viability and external malformations. Maternal tissues were fixed in 10% formalin, and were prepared for microscopic examination by imbedding in paraffin and staining sections with hematoxylin and eosin. Serial sections of fetuses fixed in Bouin's fluid were examined for internal malformations (7), and cleared specimens were inspected for skeletal development and deformities (8).

The diet consisted of commercially prepared pellets [1] composed of natural foods. According to analysis by the manufacturer, the pellets contained 24.0% of crude protein, 4.0% of crude fat, and 4.5% of crude fiber. Vitamins and minerals were present in amounts adequate for good growth and reproduction. Food intake was at 4 levels: 1) ad libitum (unrestricted); 2) 75% of the ad libitum level, designated as "25% restriction"; 3) one-half of the ad libitum level, designated as "50% restriction"; and 4) 25% of the ad libitum level, designated as "75% restriction." The mean daily food intake of unrestricted rats, calculated from the amount eaten from day zero through day 6 (0–6) of pregnancy was 19 g, from day 7 through day 13 (7–13) 20 g, and from day 14 through day 20 (14–20) 23 g. For ob-

Received for publication June 7, 1965.
[1] Wayne Lab. Blox, Allied Mills, Inc., Chicago.

servations on time of onset of resorptions in relation to level of food intake, varying initial periods of 75% restriction followed by ad libitum feeding were used. The influence of early feeding on implantation was studied by short initial periods of unrestricted food intake and subsequent 75% restriction. One group of 75%-restricted rats was examined for resorptions on day 13 of pregnancy and the results were compared with those on day 21.

RESULTS

Varying levels of dietary restriction, days 0–20. Reproductive performance of 25%-restricted rats was equal to that of unrestricted animals (table 1). With 50% restriction, 77% of rats littered, and at the 75% level of restriction 8 to 36% littered. Fetal weights were approximately the same with unrestricted feeding or 25% restriction. At the 50 and 75% levels, fetal weights were reduced. Litter size ranged from 11 to 14 fetuses, and was unaffected by level of dietary intake. Fetuses of large litters weighed less than fetuses of small litters. Maternal weight varied inversely with degree of dietary restriction. At 50 or 75% levels there was little difference between the weights of rats with or without litters (table 2). At autopsy unrestricted rats showed large deposits of fat in the retroperitoneal and perirenal spaces, and in the mesenteries of the uteri and of the intestines. By contrast, excess fat deposits

TABLE 1

Effect of different levels of food intake on reproduction in the rat

No. of rats	Mean maternal wt [1]		Wt gain or loss	% with resorptions	% with litters	Mean litter size	Mean fetal wt [3]
	Initial	Final [2]					
	g	*g*	%				*g*
			Unrestricted, days 0–20				
10	218	278	+28	10	90	14	4.00 ± 0.09
10	222	276	+24	0	100	13	3.68 ± 0.05
12	206	272	+32	8	92	14	3.97 ± 0.06
			25% Restricted, days 0–20				
12	209	227	+ 9	0	100	13 [4]	3.79 ± 0.16
			50% Restricted, days 0–20				
13	210	193	− 8	23	77	11	3.43 ± 0.04
			75% Restricted, days 0–20				
14	213	144	−32	64	36	12	1.94 ± 0.17
13	220	149	−32	92	8	12	2.07
14	200	148	−26	92	8	14	1.68

[1] Rats with litters.
[2] After removal of uteri and litters.
[3] Mean values ± SE of mean fetal weights/litter.
[4] One litter of 2 fetuses in a single uterine horn omitted.

TABLE 2

Body weights of rats with or without litters

No. of rats	With litters [1]		Wt loss	No. of rats	Without litters [2]		Wt loss
	Mean initial wt	Mean final wt			Mean initial wt	Mean final wt	
	g	*g*	%		*g*	*g*	%
			50% Restricted, days 0–20				
10	210	193	− 8	3	213	206	− 3
			75% Restricted, days 0–20				
5	213	144	−32	9	217	156	−28

[1] After removal of uteri and litters.
[2] All implantations resorbed.

were absent in restricted animals. Edema of the pancreas and the stomach wall was noted in 75%-restricted dams with litters but not in animals with resorptions. Elsewhere there was no evidence of edema or fluid accumulation. Microscopically, no lesions were observed in maternal tissues, nor were any fetal malformations observed. Fetal skeletal development was slightly retarded with 50% restriction, and at the 75% level ossification was delayed by 24 to 36 hours.

Gestation was successful and resulted in full-sized litters or failed completely. This occurred at all levels of dietary intake. Resorption sites were barely visible on day 21 and appeared as brownish spots on the mesometrial side of the uterus close to the terminal branches of the uterine artery. In some instances resorption sites were not discernible. Occasionally, larger ones were noted in uteri carrying litters. Unilateral fetation was observed in a few animals.

Varying initial periods of 75% restriction followed by ad libitum feeding. When ad libitum feeding was instituted on or before day 8, body weight and reproductive performance of 75%-restricted rats equaled that of unrestricted animals (table 3). Delay of ad libitum feeding to day 10 resulted in a 36% decrease in litters, and extension of the interval to days 12 or 14 reduced the number of litters by 64%. Litter size was unaffected, but fetal weights decreased when unrestricted food intake was started on day 8 or later. One restricted rat that was started with ad libitum feeding on day 12 had 4 viable fetuses, 1 fetal resorption, and 12 resorptions of implants.

Varying initial periods of ad libitum feeding followed by 75% restriction. When restricted rats were placed on ad libitum feeding on days 0–1, 14% littered (table 4). Unrestricted food intake on days 0–2 or days 0–3 resulted in an increase in the number of litters to 80%. Maternal weight loss was approximately the same in all 3 groups of animals, whereas fetal weights increased as the interval of ad libitum feeding was extended. Resumption of unrestricted food intake after restriction on days 4 to 12 resulted in higher maternal and fetal weights, but the percentage of litters was unchanged.

TABLE 3

Effect of unrestricted feeding after varying initial periods of 75% dietary restriction on reproduction in the rat

No. of rats	Mean maternal wt		Wt gain	% with resorptions	% with litters	Mean litter size	Mean fetal wt [3]
	Initial	Final [2]					
	g	*g*	%				*g*
		75% Restricted, days 0–3; unrestricted, days 4–20					
14	217	275	+28	0	100	14	3.90 ± 0.07
		75% Restricted, days 0–5; unrestricted, days 6–20					
14	210	267	+27	0	100	13	4.01 ± 0.08
		75% Restricted, days 0–7; unrestricted, days 8–20					
14	213	262	+23	0	100	13	3.75 ± 0.06 [6]
		75% Restricted, days 0–9; unrestricted, days 10–20					
14	209	253	+21	36	64	14 [4]	3.53 ± 0.08 [7]
		75% Restricted, days 0–11; unrestricted, days 12–20					
14	205	243	+19	64	36	14 [5]	3.39 ± 0.18 [8]
		75% Restricted, days 0–13; unrestricted, days 14–20					
14	199	216	+ 9	64	36	12	3.48 ± 0.02

[1] Rats with litters.
[2] After removal of uteri and litters.
[3] Mean values ± SE of mean fetal weights/litter.
[4] One litter of 11 fetuses in a single uterine horn omitted.
[5] One litter of 4 viable fetuses and 12 resorptions omitted.
[6] $P = < 0.05$ for the difference between 4.01 ± 0.08 and 3.75 ± 0.06.
[7] $P = < 0.05$ for the difference between 3.75 ± 0.06 and 3.53 ± 0.08.
[8] No significant difference between 3.53 ± 0.08 and 3.39 ± 0.18.

TABLE 4

Effect of 75% dietary restriction after varying initial periods of unrestricted feeding on reproduction in the rat

No. of rats	Mean maternal wt [1]		Wt loss or gain	% with resorptions	% with litters	Mean litter size	Mean fetal wt [3]
	Initial	Final [2]					
	g	*g*	%				*g*
			Unrestricted, days 0–1; restricted, days 2–20				
14	199	121	−39	86	14	16	1.48
			Unrestricted, days 0–2; restricted, days 3–20				
14	201	138	−31	21	79	13	1.80 ± 0.10
			Unrestricted, days 0–3; restricted, days 4–20				
15	223	158	−29	20	80	14 [4]	2.14 ± 0.12 [6]
			Unrestricted, days 0–3; restricted, days 4–11; unrestricted, days 12–20				
13	225	257	+14	23	77	14 [5]	3.58 ± 0.08

[1] Rats with litters.
[2] After removal of uteri and litters.
[3] Mean values ± SE of mean fetal weights/litter.
[4] One litter of 5 fetuses in a single uterine horn omitted.
[5] One litter of 2 fetuses in a single uterine horn omitted.
[6] $P = < 0.04$ for the difference between 2.14 ± 0.12 and 1.80 ± 0.10.

TABLE 5

Observations in 75%-restricted and unrestricted pregnant rats on day 13

No. of rats	Mean maternal wt [1]		Wt loss or gain	Resorptions of implants	Litters of implants	Mean no. of resorptions	Mean litter size
	Initial	Final					
	g	*g*	%	%	%		
			75% Restricted				
24	207	150	−28	83	17	13 [1]	14
			Unrestricted				
15	209	249	+19	0	100	0	14

[1] Two rats without visible resorptions omitted.

Observations in 75%-restricted and unrestricted pregnant rats on day 13. The percentage of resorptions in 75%-restricted rats on day 13 (table 5) was approximately the same as on day 21. None occurred in the controls. In contrast with the barely visible resorption sites on day 21, they were easily identified on day 13. In 2 instances sites were not discernible. The number of resorptions and the number of viable embryos per litter were nearly identical in the restricted rats. The results on day 13 confirmed the observations on day 21 in that all implants were either resorbed or viable. Litter size was also essentially the same on days 13 and 21, and equaled that of unrestricted rats examined at corresponding stages of gestation.

DISCUSSION

Reproductive performance of rats receiving various levels of a stock diet composed of natural foods was similar to reproduction in rats fed a purified diet containing different amounts of protein (2). With a 50%-restricted stock diet, 77% of rats littered as compared with 70% for animals receiving 5% protein in a purified diet. At the 75% level of restriction, 8 to 36% of stock-fed rats littered as compared with zero to 11% for rats receiving 2.5% protein or a protein-free diet.

Better food utilization or altered tissue metabolism may account for the weight loss being no greater in restricted rats carrying litters than in those that did not. For example, weight reduction of 75%-restricted dams with litters averaging 23 g amounted to 32% as compared with 28% for rats failing to bear young. Edema of the pancreas and the gastric wall in litter-bearing dams was insufficient to explain maintenance of body weight.

Data obtained on day 13 of gestation showed that the number of implants in 75%-restricted rats was the same as in animals fed ad libitum. Fetation was an all-or-none phenomenon in that full-sized litters developed or all implants resorbed. Observations with transitory unrestricted feeding indicated that resorptions took place between days 8 to 11. Feeding started on day 8 was completely preventive, but delay to days 10 or 12 resulted in an increasing number of resportions. Time of onset corresponded with layer differentiation and organogenesis in the embryo (day 9), the stage of greatest susceptibility to the teratologic effects of drugs or other agents.

Kinzey and Srebnick (9) observed that administration of estrone and progesterone from day 5 through day 9 of pregnancy prevented resorptions in rats receiving a protein-free diet. Since a similar result was obtained in 75%-restricted rats by transitory unrestricted feeding on day 8, the beneficial effect on placentation may be attributed to increased production of endogenous hormones and replenishment of depleted circulating estrone and progesterone at a critical stage of placental growth. In contrast, injections of estrone and progesterone from day 1 through day 5 was ineffective against resorptions in protein-deficient rats (9), whereas transitory ad libitum feeding for the first 2 or 3 days of gestation resulted in 80% viable litters. Evidently, exogenous hormones disappeared rapidly from the circulation, but adequate feeding during early pregnancy sustained estrone and progesterone in the circulation at a level necessary for placentation.

Food intake may influence the results obtained in testing drug effects on pregnancy in the rat. The present study shows that maternal nutrition as well as fetal growth are impaired by underfeeding, and resorptions ensue. Moreover, certain deficiencies cause fetal malformations (10). It is necessary, therefore, to distinguish nutritional effects from specific drug action.

ACKNOWLEDGMENTS

The author acknowledges helpful suggestions from Dr. Ernest B. Sigg, and the technical assistance of Mrs. Claire Hickey and Elmer Riesel.

LITERATURE CITED

1. Macomber, D. 1933 Studies of reproduction in the rat. 1. The effect of changes in the protein upon fertility, pregnancy and lactation. New England J. Med., 209: 1105.
2. Nelson, M. M., and H. M. Evans 1953 Relation of dietary protein levels to reproduction in the rat. J. Nutrition, 51: 71.
3. Curtiss, C. 1953 Effect of low protein intake on the pregnant rat. Metabolism, 2: 344.
4. Leathem, J. M. 1959 Extragonadal factors in reproduction. In: Recent Progress in the Endocrinology of Reproduction, ed., C. W. Lloyd. Academic Press, New York, p. 179.
5. Richardson, L. R. 1964 Reproductive performance of rats receiving various levels of dietary protein and fat. J. Nutrition, 82: 257.
6. Chow, B. F., and C. Lee 1964 Effect of dietary restriction of pregnant rats on body weight gain of the offspring. J. Nutrition, 82: 10.
7. Wilson, J. G. 1965 Methods for administering agents and detecting malformations in experimental animals. In: Teratology: Principles and Techniques, eds., J. G. Wilson and J. Warkany. University of Chicago Press, Chicago, p. 262.
8. Staples, R. E., and V. L. Schnell 1964 Refinements in rapid clearing technic in the KOH — alizarin Red S method for fetal bone. Stain Technol., 39: 62.
9. Kinzey, W. G., and H. H. Srebnick 1963 Maintenance of pregnancy in protein-deficient rats with short-term injections of ovarian hormones. Proc. Soc. Exp. Biol. Med., 114: 158.
10. Johnson, E. M. 1965 Nutritional factors in mammalian teratology. In: Teratology: Principles and Techniques, eds., J. G. Wilson and J. Warkany. University of Chicago Press, Chicago, p. 113.

Vitamin B_{12} Content and Binding Capacity of Cow's Milk Proteins [1,2,3]

EVANGELOS GIZIS, Y. P. KIM, J. R. BRUNNER AND B. S. SCHWEIGERT
Department of Food Science, Michigan State University, East Lansing, Michigan

ABSTRACT The vitamin B_{12} content and binding capacity of cow's milk protein fractions after equilibration with excess ^{60}Co-labeled vitamin B_{12} in borate buffer, pH 9.0, were determined by microbiological assay using *Lactobacillus leichmannii* and by assay for radioactivity. The distribution of vitamin B_{12} between the various proteins of milk was ubiquitous but a higher concentration was demonstrated in the whey proteins. The isoelectrically prepared casein fraction contained 98 $\mu\mu$g of vitamin B_{12}/mg of protein; β-lactoglobulin, 870; α-lactalbumin, 90; blood serum albumin, 80; proteose-peptone, 370; fat-globule membrane proteins, 82; and non-protein nitrogen — converted to an equivalent amount of protein, 2,200. The vitamin B_{12} content of the casein complex was distributed between α- and β-caseins: 41 and 20 $\mu\mu$g/mg of protein, respectively. Further fractionation of α-casein into α_s- and k-caseins yielded values for vitamin B_{12} of 5 and 10 $\mu\mu$g/mg of protein, respectively. Apparently, the vitamin was released during the subfractionation of the casein complex. The amount of ^{60}Co-labeled vitamin B_{12} adsorbed by various milk proteins upon equilibration with excess ^{60}Co-labeled vitamin B_{12} was 850 $\mu\mu$g/mg for β-lactoglobulin, 690 for α-lactalbumin, 773 for casein, 1580 for α-casein, 915 for α_s-casein and 2490 for the proteose-peptone fraction. In 8 M urea, β-lactoglobulin bound vitamin B_{12} at a higher level (e.g., $>$ 20%).

A previous report from this laboratory indicated that milk protein fractions contained no unique or predominate quantities of bound vitamin B_{12} and that about 95% of the total vitamin B_{12} in cow's milk was associated with the protein components (1). Variations in the amount of vitamin B_{12} present in different samples of cow's milk indicated that milk proteins, as they exist in milk, do not exhibit their maximum vitamin B_{12} binding capacity.

The present study was planned to investigate the capacity of milk proteins to bind added vitamin B_{12}.

EXPERIMENTAL

Fractionation of milk proteins. The procedures followed in the preparation of casein and whey protein fractions are shown in figures 1 and 2. Proteose-peptone, a minor whey protein fraction, and fat-globule proteins were prepared as described by Brunner and Thompson (7). An enriched immunoglobulin fraction was obtained from a lactoglobulin preparation by collecting the slow-moving peaks from the descending channel of a Tiselius cell following the electrophoretic resolution of the protein components.

Saturation of proteins with ^{60}Co-labeled vitamin B_{12}. An estimated excess of ^{60}Co-labeled vitamin B_{12} (cyanocobalamin), specific activity 1.0 μc/μg, was added to each protein fraction in borate buffer, pH 9.0, $\Gamma/2 = 0.24$ (8). This mixture was allowed to interact for 2 hours at room temperature. The excess ^{60}Co-labeled vitamin B_{12} was removed by passing the reaction mixture through a Sephadex G-25 column (100 ml bed volume and 28 cm high) equilibrated with borate buffer. The effluent was monitored spectrophotometrically at 280 mμ for location of the protein fraction. The radioactivity in the protein effluent was determined in a well-type, NaI(T1) scintillation counter. Measurements were made at the optimal voltage and for sufficient time to obtain statistically significant results.

Received for publication June 28, 1965.

[1] Presented at the 49th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., 1965.

[2] Supported in part by grant no. EF-284 from the Division of Environmental Health and Food Protection, U. S. Public Health Service. We are indebted to Merck and Company, Rahway, New Jersey, for providing the ^{60}Co-labeled vitamin B_{12} used in these studies.

[3] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article no. 3660.

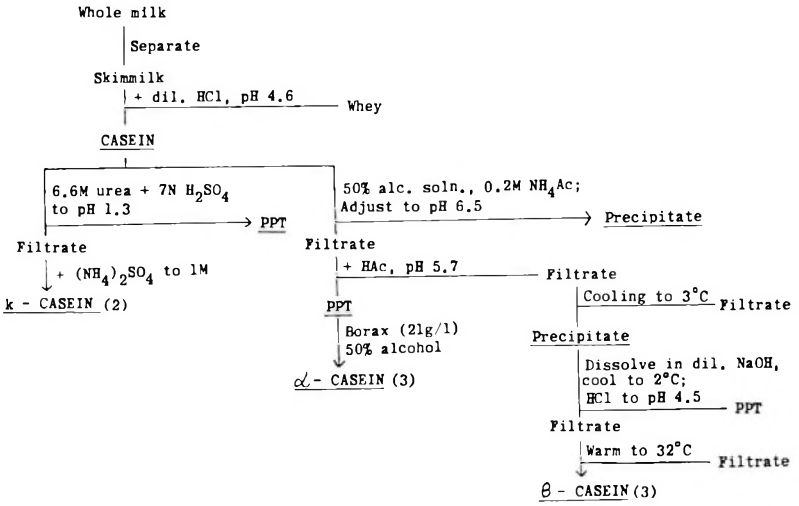

Fig. 1 Fractionation of cow's milk casein into subfractions.

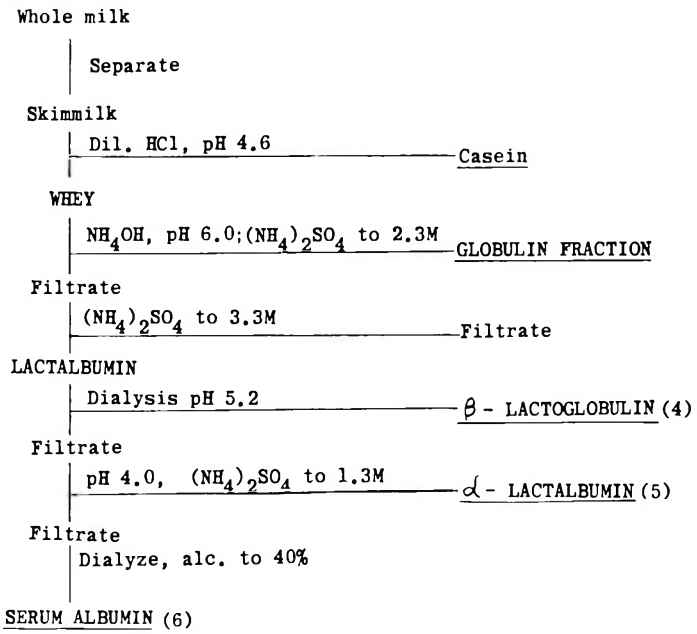

Fig. 2 Fractionation of cow's milk whey proteins.

Analyses. A standard microbiological technique using *Lactobacillus leichmanii* ATCC 7830 and cyanocobalamin as the standard was used to assay for the total bound vitamin B_{12} present with and without ^{60}Co-labeled vitamin B_{12} added to the various protein fractions (1).

The nitrogen content of the protein fraction was estimated by a micro-Kjeldahl method and converted to protein equivalent by the factor 6.38. The relative purity of the protein fractions was assessed by free-boundary electrophoresis in a Perkin-Elmer, model 38-A electrophoresis appara-

TABLE 1

Vitamin B_{12} content and binding capacity of cow's milk protein fractions

	Vitamin B_{12} content of protein fractions [1]	^{60}Co-labeled vitamin B_{12} adsorbed by protein fractions [2]	Total vitamin B_{12} content of protein fractions [1] (native + adsorbed ^{60}Co-labeled vitamin B_{12}
	μμg/mg protein	*μμg/mg protein*	*μμg/mg protein*
Casein [3]	98	773	929
α-casein	41	1580	1690
α_s-casein	5	915	940
k-casein	10	—	—
β-casein	20	810	880
Whey proteins	—	—	—
Lactoglobulin fraction	157		
Immunoglobulins	206		
Lactalbumin fraction	461		
β-Lactoglobulin	870	850	2010
α-Lactalbumin	90	690	734
Blood serum albumin	80	280	280
Proteose-peptone fraction	370	2490	2650
Fat-globule membrane protein	82	—	—
Non-protein nitrogen	2200	—	—

[1] Microbiological assay.
[2] Radiological assay.
[3] Isoelectrically precipitated.

tus. Identification of the protein components was tentatively established by comparing the electrophoretic mobilities of the major boundaries with those reported by Brunner et al. (9).

RESULTS AND DISCUSSION

The descending electrophoretic patterns and corresponding boundary mobilities of the protein preparations used in this study are shown in figure 3. The concentration of vitamin B_{12} in these fractions is reported in table 1, column 1. The values given represent the average of 5 microbiological determinations and the variation between samples was similar to that reported by Kim et al. (1). β-Lactoglobulin contained the highest concentration of vitamin B_{12} of any of the major milk proteins, namely, 870 μμg/mg. However, the second most abundant whey protein — α-lactalbumin — contained only 90 μμ/mg of vitamin B_{12}. Total casein prepared by isoelectric precipitation contained 98 μμg/mg. α-Casein and β-casein fractions contained 41 and 20 μμg of vitamin B_{12}/mg, respectively. α_s-Casein and k-casein, isolated from the α-casein fraction, contained 5 and 10 μμg/mg, respectively. Obviously, the vitamin B_{12} content of these casein subfractions does not agree with the vitamin B_{12} concentrations in the more complex fractions. Presumably, protein-bound vitamin B_{12} was released by the fractionation procedure used to separate the more homogenous components.

The same protein preparations, when exposed to an excess of the ^{60}Co-labeled cyanocobalamin, bound the vitamin in quantities listed in table 1, column 2. These values were determined by radioactivity assay as described previously. To ascertain whether the values represented newly bound vitamin B_{12} or merely a displacement of bound vitamin B_{12} present in the original milk by the ^{60}Co-labeled vitamin B_{12}, the ^{60}Co-labeled vitamin B_{12}-saturated proteins were assayed microbiologically and the results reported in column 3. The values for total vitamin B_{12} should be

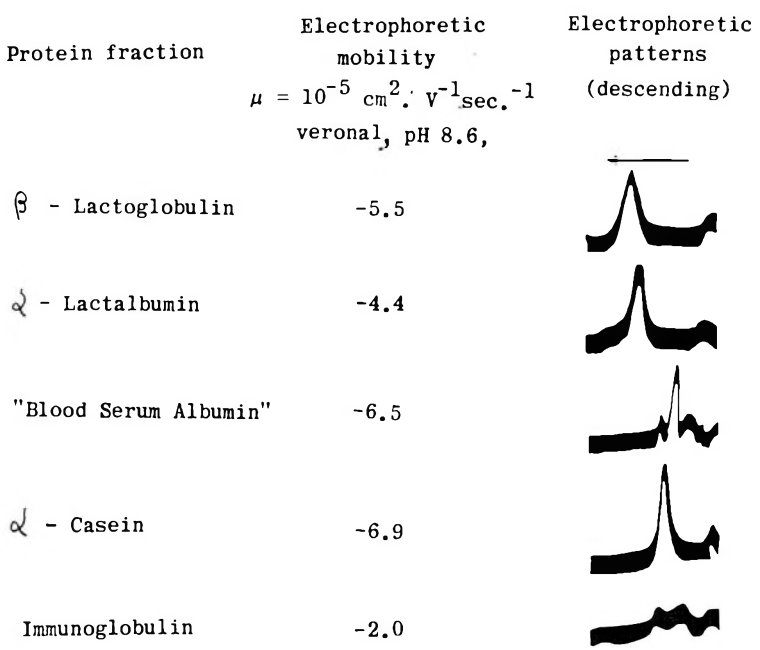

Fig. 3 Descending electrophoretic patterns and boundary mobilities of milk protein fractions run in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$ and in concentration approximating 1.5%.

equal to the combined values presented in columns 1 and 2 for the individual protein fractions which, within experimental error, appeared to be the case. The proteose-peptone and α-casein fractions exhibited the highest binding capacity. These fractions contained a large portion of the glycoproteins present in milk. Casein subfractions bound more ^{60}Co-labeled vitamin B_{12} than total casein, probably as a result of the destruction of the casein micelles during fractionation.

When β-lactoglobulin was dissolved in 8 M urea in the presence of excess ^{60}Co-labeled vitamin B_{12}, about 20% more vitamin B_{12} was bound than in the absence of urea. Presumably this behavior was a ramification of changes in the molecular structure of the protein in the presence of a denaturing agent. The nature of the bonds involved at the protein-vitamin B_{12} binding sites is not known and constitutes the prinicipal objective of our projected studies.

LITERATURE CITED

1. Kim, Y. P., E. Gizis, J. R. Brunner and B. S. Schweigert 1965 Vitamin B_{12} distribution in cow's milk. J. Nutrition, *86:* 394.
2. Zittle, C. A., and J. H. Custer 1963 Purification and some properties of α_s-casein and k-casein. J. Dairy Sci., *46:* 1183.
3. Hipp, N. J., M. L. Groves, J. H. Custer and T. L. McMeekin 1952 Separation of α-, β-, and γ-casein. J. Dairy Sci., *35:* 272.
4. Larson, B. L., and R. Jenness 1955 β-Lactoglobulin. Biochem. Preparations, *4:* 23.
5. Gordon, W. G., and J. Ziegler 1955 α-Lactalbumin. Biochem. Preparations, *4:* 16.
6. Polis, B. D., H. W. Shmukler and J. H. Custer 1950 Isolation of crystalline albumin from milk. J. Biol. Chem., *187:* 349.
7. Brunner, J. R., and M. P. Thompson 1961 Characteristics of several minor-protein fractions isolated from bovine milk. J. Dairy Sci., *44:* 1224.
8. Kakei, M., and G. B. J. Glass 1962 Separation of bound and free vitamin B_{12} on Sephadex G-25 column. Proc. Soc. Exp. Biol. Med., *111:* 270.
9. Brunner, J. R., C. A. Ernstrom, R. A Hollis, B. L. Larson, R. McL. Whitney and C. A. Zittle 1960 Nomenclature of the proteins of bovine milk — first revision. J. Dairy Sci., *43:* 901.

Reproduction and Maternal Response of the Rat when Thiamine Intake is Limited

MYRTLE L. BROWN [1] AND CAROLYN H. SNODGRASS
Human Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

ABSTRACT Reproductive performance, serum proteins and hematological response were studied in female rats of the Wistar strain, maintained from weaning until mating with a diet containing either an adequate or a low level of thiamine. During gestation the rats were fed an adequate diet or the same diet containing one-tenth the level of thiamine. Food intake and weight gain were markedly influenced by diet during pregnancy. In animals fed the low level of thiamine during pregnancy, prepregnancy diet also influenced food intake and maternal weight gain. Heavier young were produced by animals fed adequate thiamine during pregnancy but fetal weight was not affected by prepregnancy diet. Total serum protein was similar for all groups although γ-globulin concentration were lower in animals fed adequate thiamine during pregnancy. Hemoglobin and hematocrit values indicated the expected hemodilution of pregnancy in animals fed adequate thiamine and apparent hemoconcentration in animals fed the low thiamine diet. Thiamine content in the young and livers of mothers were related to thiamine intake during pregnancy and were not significantly influenced by prepregnancy diet.

In a previous report from this laboratory (1) it was shown that female rats reared with a diet partially deficient in thiamine but fed adequate thiamine during pregnancy produced fewer young and had higher percentages of γ-globulin in their serum than animals fed adequate thiamine throughout. Other aspects of reproductive performance, however, were essentially normal. The present report deals with the effect of a limited, but not severely deficient, intake of thiamine fed from weaning followed by a more severe limitation of thiamine intake during the reproductive period. Observations were made upon reproductive performance, maternal serum protein and hematological response.

EXPERIMENTAL

Female rats of the Wistar strain were fed from weaning a basal diet containing 2.5 mg thiamine/kg or the same diet containing 1.0 mg/kg, a borderline level for growth in the rat (2). The diet has been described previously (1) and differs only in level of thiamine used in the low thiamine regimens. At approximately 70 days of age, animals were mated with males that had been reared with laboratory chow and the females were judged to be pregnant if sperm were found in a vaginal smear. Following mating, animals were fed the basal diet in which levels of all vitamins except choline were doubled or the same diet containing one-tenth this level of thiamine. These diets hereafter will be designated as the 5.0 mg/kg diet and the 0.5 mg/kg diet. Details of necropsy carried out on the twenty-second day of gestation and analytical methods have been described (1).

RESULTS

Food intake, weight gain, and reproductive performance. Food intake and weight gain were similar during pregnancy when the level of thiamine was 5.0 mg/kg regardless of whether the diet prior to pregnancy contained 2.5 or 1.0 mg/kg (table 1). Food intake and weight gain were markedly reduced when dietary thiamine was 0.5 mg/kg during pregnancy and the extent of the reduction depended upon the prepregnancy level of thiamine. During the third week of pregnancy, the food intake of rats fed the 0.5 mg/kg diet was 30% of that consumed by rats fed the 5.0 mg/kg diet when prepregnancy level

Received for publication May 1, 1965.

[1] Present address: Department of Home Economics and Department of Public Health, University of Hawaii, Honolulu, Hawaii.

was 2.5 mg/kg and 13% when prepregnancy level was 1.0 mg/kg.

The average weight of fetuses carried by mothers fed the 5.0 mg/kg diet during pregnancy was more than that of animals fed the 0.5 mg/kg diet, but prepregnancy diet had no effect on fetal weight (table 2). There were no significant differences in litter size or incidence of resorptions among the groups, although resorptions tended to occur to a slightly greater extent in animals fed the 1.0 mg/kg prepregnancy diet than in those fed the 2.5 mg/kg diet, regardless of the diet fed during pregnancy. All animals carried some young to term, although a few mothers resorbed more than half of their litter.

Serum protein, hematocrit and hemoglobin. Total serum protein was similar for all groups (table 3) and the prepregnancy level of thiamine was without influence on the various serum protein components. The only serum protein component that was significantly influenced by the thiamine level during pregnancy was γ-globulin which was present in lower

TABLE 1

Influence of level of dietary thiamine prior to and during pregnancy on food intake and weight gain

Dietary thiamine		No. of animals	Average food intake			Average maternal wt gain				Final body wt
Pre-pregnancy	Pregnancy		Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Total	
mg/kg	*mg/kg*		*g*	*g*	*g*	*g*	*g*	*g*	*g*	*g*
2.5	5.0	8	107	118	130	29	30	74	132	362
1.0	5.0	7	104	107	132	33	28	68	129	329
2.5	0.5	9	108	88	39	26	15	− 5	37	257
1.0	0.5	7	63	41	17	0	−7	−15	−22	195

TABLE 2

Influence of level of dietary thiamine prior to and during pregnancy on reproductive performance

Dietary thiamine		No. of litters	Avg no. young	Avg fetal wt	Avg no. resorptions [1]
Pre-pregnancy	Pregnancy				
mg/kg	*mg/kg*			*g*	
2.5	5.0	8	10.1	4.9	0.9
1.0	5.0	7	9.0	4.9	1.4
2.5	0.5	9	8.8	3.7	1.0
1.0	0.5	7	9.0	3.9	1.4

[1] $\frac{\text{Total number of resorptions}}{\text{Number of animals in group}}$

TABLE 3

Serum proteins, hematocrit, and hemoglobin of pregnant rats fed at different levels of thiamine prior to and during pregnancy

Dietary thiamine		No. of sera analyzed	Serum proteins						Hematocrit	Hemoglobin
Pre-pregnancy	Pregnancy		Total	Albumin	Globulin					
					α_1-	α_2-	β-	γ-		
mg/kg	*mg/kg*		*g/100 ml*	% [1]	%	%	%	%	%	*g/100 ml*
2.5	5.0	8	5.4	43.5	26.6	11.3	14.0	4.7	35.8	12.6
1.0	5.0	6	5.3	40.6	28.0	10.0	16.5	4.8	33.4	11.6
2.5	0.5	7	5.8	41.2	26.8	10.8	15.3	6.0	42.0	15.0
1.0	0.5	7	5.2	40.9	25.8	10.6	14.9	7.8	41.5	14.5

[1] Per cent of total protein.

percentages in the sera of rats fed the 5.0 mg/kg diet than in those receiving the 0.5 mg/kg diet ($P = 0.01$).

Hematocrit and hemoglobin levels were not significantly influenced by the thiamine intake prior to pregnancy but were significantly elevated in animals fed the low thiamine intake during pregnancy ($P = 0.01$).

Thiamine content of maternal livers and fetuses. Average thiamine content of maternal livers and fetuses was not influenced by thiamine intake prior to pregnancy but was significantly higher in animals fed diets containing 5.0 mg thiamine /kg during pregnancy than in those receiving 0.5 mg/kg (table 4).

DISCUSSION

Thiamine status of animals fed 1.0 mg/kg prior to pregnancy was not sufficiently low at conception to reduce fertility, in contrast with results reported for rats fed diets devoid of thiamine (3) or containing a low level of 0.5 mg thiamine/kg at weaning with a gradual increase to 1.0 mg/kg (1). The effect of prepregnancy thiamine intake on food intake and weight gain during pregnancy was apparent only when the diet contained inadequate thiamine during pregnancy. The failure for prepregnancy level of thiamine to influence fetal weight provided further evidence of the importance of plane of nutrition during pregnancy. However, when the pregnancy level of thiamine was low (0.5 mg/kg), fetal tissue was maintained at considerable expense to the mother, and the adverse effect on the mother was greater when the prepregnancy level of thiamine also was low.

High percentages of serum γ-globulin have been observed in animals deprived of thiamine prior to mating but that were fed an adequate diet during pregnancy, presumably a delayed effect of the early diet (1). In the present study, increases in serum γ-globulin concentration occurred only in animals deprived of thiamine during gestation. Apparently the 1.0 mg/kg diet prior to pregnancy was adequate to prevent this presumably abnormal response. A decrease in γ-globulin concentration appears to be the normal response to pregnancy in the rat (4). The reason for the increase in γ-globulin concentration in thiamine-deprived rats in the present study is difficult to explain but does not appear to be due to the reduced food intake which occurred when thiamine level was low. The relatively constant concentration of α_1-globulin as well as the increased concentration of γ-globulin in the sera of these rats is in contrast with the observations of Weimer and Godfrey (5) that α_1-globulin is the fraction most susceptible to change when the dietary stress is starvation, semistarvation or protein depletion. Increased serum γ-globulin concentrations have been reported for thiamine-deficient pigeons (6). Similar results have been obtained in riboflavin-deficient rats in which an increased serum γ-globulin appeared to be a nonspecific effect of the deficiency and not significantly related to reduction in food intake (7).

The higher levels of hemoglobin and hematocrit in animals fed the low thiamine diet in the present study may be a result of hemoconcentration resulting from weight loss associated with the deficiency. Hemoglobin and hematocrit levels for ani-

TABLE 4

Thiamine content of maternal liver and litters

Dietary thiamine		Maternal livers			Fetuses		
Pre-pregnancy	Pregnancy	No. analyzed	Avg wt	Thiamine	No. of litters analyzed	Avg litter wt	Thiamine
mg/kg	*mg/kg*		*g*	*μg/g*		*g*	*μg/g*
2.5	5.0	7	12.6	8.42 ± 1.21 [1]	8	49.4	2.19 ± 0.13 [1]
1.0	5.0	6	11.8	8.17 ± 0.75	5	38.9	2.23 ± 0.09
2.5	0.5	9	7.9	0.98 ± 0.21	9	29.5	0.50 ± 0.07
1.0	0.5	7	6.2	0.62 ± 0.23	6	22.1	0.30 ± 0.05

[1] SE of mean.

mals fed 5.0 mg thiamine/kg during gestation are consistent with the hemodilution known to occur in pregnant animals of most species and which has been demonstrated for the rat (4). Assuming a blood volume of roughly 5 ml/100 g of body weight, total circulating hemoglobin may be estimated from average body weight and hemoglobin values. Thus, total hemoglobin in animals fed the 1.0 mg/kg diet prior to pregnancy and the 0.5 mg/kg diet during pregnancy was 1.4 g as compared with 1.9 for animals maintained with 2.5 mg/kg prior to pregnancy. Comparable levels for animals fed the pregnancy diet containing 5.0 mg/kg were 2.3 g for those fed 2.5 mg/kg prior to pregnancy and 1.8 for those fed 1.0 mg/kg. Consequently, there does not appear to be a profound effect on the hematopoietic system except in the animals with extremely low food intakes accompanied by severe weight loss.

If the assumption that hemoconcentration and a reduction in total blood volume may have occurred in animals fed the 0.5 mg/kg diet during pregnancy is considered further, speculations also may be made with respect to serum protein data. Absolute values for the total circulating γ-globulin, thus, would be expected to be equal or slightly less in animals fed the 0.5 mg/kg diet during pregnancy than in those fed 5.0 mg/kg; albumin and globulin fractions would be markedly less. It appears, then, that the apparently abnormal increase in γ-globulin concentration possibly may be more nearly normal than are the apparently normal concentrations of other serum protein fractions.

Thiamine content of maternal livers and fetuses appears to be related to thiamine intake during pregnancy and to be little influenced by level prior to pregnancy. The concentration in maternal livers is similar to a total thiamine concentration of 0.74 μg/g of tissue reported by Byerrum and Flokstra (8) for rats maintained with a diet devoid of thiamine during the early growth period. Pregnant animals in the present study received little thiamine during the last week of gestation, approximately 2.8 μg/day for animals fed 2.5 mg/kg prior to pregnancy and 1.2 μg/day for animals fed 1.0 mg/kg. For practical consideration these animals were starving.

LITERATURE CITED

1. Brown, M. L., and C. H. Snodgrass 1965 Effect of dietary level of thiamine on reproduction in the rat. J. Nutrition, *85:* 102.
2. Brown, R. A., and M. Sturtevant 1949 The vitamin requirements of the growing rat. Vitamins and Hormones, *7:* 171.
3. Nelson, M. M., and H. M. Evans 1955 Relation of thiamine to reproduction in the rat. J. Nutrition, *55:* 151.
4. Brown, M. L., and R. L. Pike 1960 Blood volume and serum protein in the deoxypyridoxine-fed rat during pregnancy. J. Nutrition, *71:* 191.
5. Weimer, H. E., and J. F. Godfrey 1964 Effects of inanition, semistarvation, and protein-free diets on serum proteins. Am. J. Physiol., *206:* 331.
6. LaCommare, A. F. 1960 Serum protein of the pigeon in experimental beriberi. Boll. Soc. Ital. Biol. Sper., *37:* 121.
7. Jacquot-Armand, Y., F. Gaudin-Harding, J. C. Blum and C. A. Boffa 1963 Effect of riboflavin deficiency on the serum proteins of the white rat. C. R. Acad. Sci., *236:* 2936.
8. Byerrum, R. U., and J. H. Flokstra 1951 Cocarboxylase and thiamine in tissues of rats receiving different concentrations of vitamin B_1 in the diet. J. Nutrition, *43:* 17.

Metabolism of Glucose and Acetate in Obese Rats [1,2]

MARJORIE LAVERS REYNOLDS [3] AND DOROTHY JUTTON PRINGLE
School of Home Economics, University of Wisconsin, Madison, Wisconsin

ABSTRACT To determine the mechanism leading to obesity when Osborne-Mendel rats are fed a high fat diet, the effect of dietary fat level on glucose and acetate metabolism was studied. Metabolism was followed at 3- or 6-week intervals by measurement of ^{14}C in expired carbon dioxide after intraperitoneal administration of 2 μc of glucose-1-^{14}C, glucose-6-^{14}C, or sodium acetate-1-^{14}C. Differences between the groups fed at the 2 levels of fat were observed, but showed no trends which might explain the obesity of rats fed the high fat diet, although at 15 and 21 weeks after weaning this group excreted less of the dose of acetate-1-^{14}C than did rats fed the 5% fat diet. The percentage of dose of glucose-1-^{14}C expired as CO_2 decreased sharply between 1 and 6 weeks in both diet groups. This decrease was also noted after administration of glucose-6-^{14}C to rats fed the 60% fat diet, and the decrease continued until 21 weeks. The percentage of dose expired after administration of sodium acetate-1-^{14}C did not change with increasing age in either diet group. However, the rats fed the 60% fat diet excreted carbon dioxide of lower specific activity than those fed the 5% fat diet at 9, 15, and 21 weeks after weaning.

Nutritional obesity has been produced in rats of the Osborne-Mendel strain (1) by means of a high fat diet. It has been suggested (2) that this tendency to obesity may be a genetic predisposition in this strain of rats. Genetic obesity has been studied extensively in mice, and differences in metabolism of carbohydrates and lipids have been observed. Guggenheim and Mayer (3) observed that obese-hyperglycemic mice incorporated less ^{14}C into carbon dioxide in 3 hours than did nonobese mice after injection of sodium acetate-1-^{14}C, and concluded that a large fraction of the acetate which the obese mice appear unable to oxidize is converted to fatty acids. Subrahmanyan (4) studied metabolism of labeled glucose and acetate in the New Zealand strain of obese mice. His observations suggested the possibility that increased lipogenesis from carbohydrate may contribute to the development of the obese condition in these animals. The present investigation was undertaken to study certain aspects of metabolism and growth of Osborne-Mendel rats fed diets differing greatly in fat content.

METHODS

One hundred and twenty male, weanling rats of the Osborne-Mendel strain, weighing between 40 and 50 g, were divided into 12 groups. Six groups were fed a 5% fat diet, and the remaining groups received a 60% fat diet ad libitum. The diets are described in table 1. The isotope experiments were carried out during the first, third, sixth, ninth, fifteenth, and twenty-first weeks that the rats were fed the diets.

TABLE 1
Composition of diets

	High CHO	High fat
Casein, %	25.00	25.00
Hydrogenated cottonseed oil [1]	4.50	60.00
Sucrose, %	64.35	8.15
Choline, %	0.15	0.15
Water-soluble vitamins, % [2]	0.50	0.85
Fat-soluble vitamins, % [3]	0.50	0.85
Salts, % [4]	5.00	5.00
Kilocalories/g	4.00	6.76
% calories from protein	25.10	14.80
% calories from CHO	64.70	5.20
% calories from fat	10.20	80.00
Casein, mg/kcal	63	37

[1] Crisco, Procter and Gamble Company, Cincinnati.
[2] Mixture contained in each gram: 2 mg thiamine, 2 mg riboflavin, 10 mg niacin, 8 mg Ca pantothenate, 1 mg pyridoxine·HCl, 80 μg folic acid, 200 μg menadione, 40 μg biotin, 8 μg vitamin B_{12}, 80 mg inositol, 20 mg ascorbic acid and 0.91 g sucrose.
[3] Mazola Oil, Corn Products Company, Argo, Illinois; contained in each gram: 800 IU vitamin A, 400 IU vitamin D and 20 mg α-tocopherol.
[4] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138*: 459; obtained from Nutritional Biochemicals Corporation, Cleveland.

Received for publication July 19, 1965.
[1] Supported in part by the Arctic Aeromedical Laboratory under Contract AF 41(657)-301.
[2] The experiments were conducted according to "Rules Regarding Animal Care" as established by the American Medical Association.
[3] General Foods Fellow. Present address: Department of Biochemistry, University of Tennessee, Knoxville, Tennessee.

TABLE 2

Recovery of ^{14}C in expired carbon dioxide 4.5 hours after injection of 2 μc of glucose-1-^{14}C intraperitoneally [1]

Weeks fed diet	Per cent of dose expired		P value	Specific activity [2]		P value
	5% fat diet	60% fat diet		5% fat diet	60% fat diet	
1	47.8 ± 1.7 [3a]	43.7 ± 5 [e]	ns [4]	87.7 ± 3.7 [j]	83.0 ± 1.5 [l]	ns
3	43.8 ± 1.8 [ab]	36.2 ± 1.9 [fg]	< 0.05	49.8 ± 4.3 [d]	39.6 ± 4.1 [m]	ns
6	31.1 ± 2.1 [d]	30.9 ± 0.7 [gh]	ns	25.7 ± 1.8 [k]	25.8 ± 2.1 [h]	ns
9	37.8 ± 1.5 [c]	30.1 ± 1.0 [h]	< 0.01	28.4 ± 1.7 [k]	22.9 ± 1.0 [n]	< 0.05
15	38.3 ± 2.2 [bc]	38.9 ± 2.6 [f]	ns	26.5 ± 2.3 [k]	22.8 ± 2.2 [n]	ns
21	34.4 ± 0.9 [cd]	33.4 ± 1.8 [gh]	ns	21.2 ± 1.4 [k]	17.5 ± 0.5 [n]	ns

[1] Within feeding regimens, means in the same column and having the same letter superscript do not differ significantly from each other ($P > 0.05$).

[2] $\frac{\text{dis/min} \times 10^{-3}}{\text{mmole } CO_2}$

[3] Mean ± SE of mean.

[4] Not significant.

Five rats from each group were fasted 24 hours before intraperitoneal injection of 2 μc of glucose-1-^{14}C in 1 ml solution. Each rat was then placed in a metabolism chamber, and air was drawn at the rate of 500 ml/minute through a trap containing either 100 ml of 1 N sodium hydroxide or 25 ml of 2.5 N sodium hydroxide. At the end of 0.5, 1.0, 1.5, 2.5, 3.5 and 4.5 hours the contents of the traps were changed. After 4.5 hours the animal was returned to its cage. Forty-eight to 72 hours later the procedure was repeated with the exception that glucose-6-^{14}C replaced glucose-1-^{14}C.

The sodium hydroxide solutions were analyzed for radioactivity and for the quantity of carbon dioxide dissolved. A solution of ethanolamine in ethylene glycol monomethyl ether (1:2 v/v) was used to trap carbon dioxide which was released from the alkaline solution by the addition of 4 N HCl in a closed system, as described by Towne et al. (5). The scintillation medium as suggested by Jeffay and Alvarez (6) was a toluene, ethylene glycol monomethyl ether solution (2:1 v/v) containing 5.5 g/liter of 2.5-dinitrophenyloxazole (DPO). The samples were counted in a Tri-carb Liquid Scintillation counter. A correction was made for background. Carbonate as millimoles of carbon dioxide was determined on aliquots of the alkali by titration with 0.1 N HCl using phenolphthalein and bromphenol blue as indicators. Specific activity was expressed as disintegrations per minute per millimole of carbon dioxide. The percentage of dose expired was also calculated.

The second 5 animals from each diet group received an intraperitoneal dose of 2 μc sodium acetate-1-^{14}C at the same time intervals except that the first-week test was omitted. The collection and counting of $^{14}CO_2$ was the same as for the ^{14}C glucose experiments.

Statistical comparison of the results between rats fed the 2 diets was made by the *t* test (7). To compare values for rats of different age groups the data were analyzed by analysis of variance and by Duncan's new multiple-range test (7).

RESULTS

The cumulative excretion of labeled carbon in $^{14}CO_2$ after injection of glucose-1-^{14}C is shown in table 2. The percentage of the dose incorporated into expired carbon dioxide differed significantly between rats receiving the 2 diets only during the third and ninth weeks of the study. Rats fed the 5% fat diet excreted a higher percentage of the dose in 4.5 hours than did rats fed the 60% fat diet at these ages. The total counts recovered in 4.5 hours decreased with age for all rats. Both diet groups excreted significantly greater amounts of label during the first week than they did during the following weeks.

Figure 1 and table 2 give the average specific activity of carbon dioxide expired after injection of glucose-1-^{14}C. There was a difference between the groups fed the 2 diets only at the ninth week when the control rats excreted carbon dioxide of greater specific activity. The average spe-

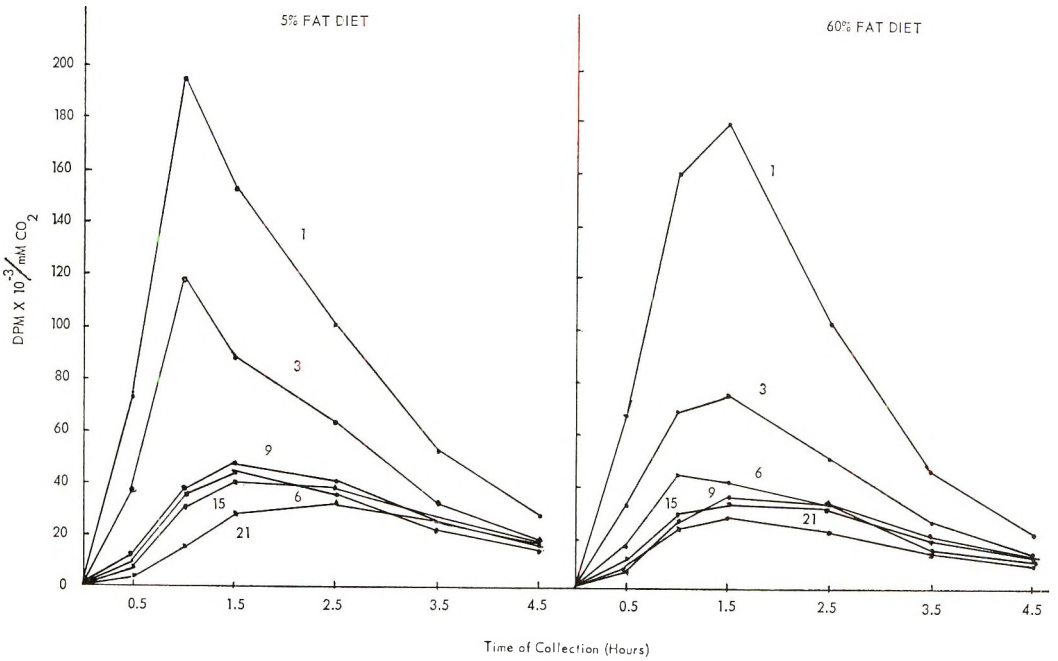

Fig. 1 Specific activity of $^{14}CO_2$ recovered after administration of an intraperitoneal dose of 2 μc glucose-1-^{14}C. Tests were made after the diet had been fed for 1, 3, 6, 9, 15 and 21 weeks.

TABLE 3

Recovery of ^{14}C in expired carbon dioxide 4.5 hours after injection of 2 μc of glucose-6-^{14}C intraperitoneally [1]

Weeks fed diet	Per cent of dose expired		P value	Specific activity [2]		P value
	5% fat diet	60% fat diet		5% fat diet	60% fat diet	
1	37.4 ± 2.8 [3a]	46.1 ± 0.4 [b]	< 0.05	68.1 ± 6.2 [g]	67.4 ± 2.1 [j]	ns
3	33.6 ± 2.9 [a]	40.5 ± 1.6 [c]	ns [4]	38.8 ± 5.2 [h]	48.3 ± 3.4 [k]	ns
6	31.3 ± 1.8 [a]	36.6 ± 0.9 [d]	< 0.05	29.1 ± 1.9 [hi]	27.1 ± 1.0 [l]	ns
9	34.8 ± 0.8 [a]	32.3 ± 2.0 [e]	ns	30.6 ± 0.8 [hi]	25.1 ± 1.4 [l]	< 0.01
15	31.5 ± 1.1 [a]	36.2 ± 0.2 [de]	< 0.01	23.2 ± 1.0 [i]	24.0 ± 1.3 [l]	ns
21	31.1 ± 1.8 [a]	28.3 ± 0.5 [f]	ns	20.4 ± 0.8 [e]	15.4 ± 0.5 [m]	< 0.001

[1] Within feeding regimens, means in the same column and having the same letter superscript do not differ significantly from each other ($P > 0.05$).

[2] $\frac{\text{dis/min} \times 10^{-3}}{\text{mmole } CO_2}$.

[3] Mean ± SE of mean.

[4] Not significant.

cific activity for 4.5 hours was highest at 1 and 3 weeks for rats fed both diets.

In table 3 is given the cumulative excretion of ^{14}C in expired carbon dioxide after injection of glucose-6-^{14}C. Rats fed the high fat diet incorporated significantly more label into expired carbon dioxide than did the control group at 1, 6 and 15 weeks. There was no significant difference between the percentage of dose expired at different ages by rats fed the 5% fat diet. Rats fed the high fat diet, however, excreted a higher percentage of the dose at 1 and 3 weeks than they did during later weeks.

In figure 2 and table 3 are shown the average specific activities of carbon dioxide recovered after administration of glucose-

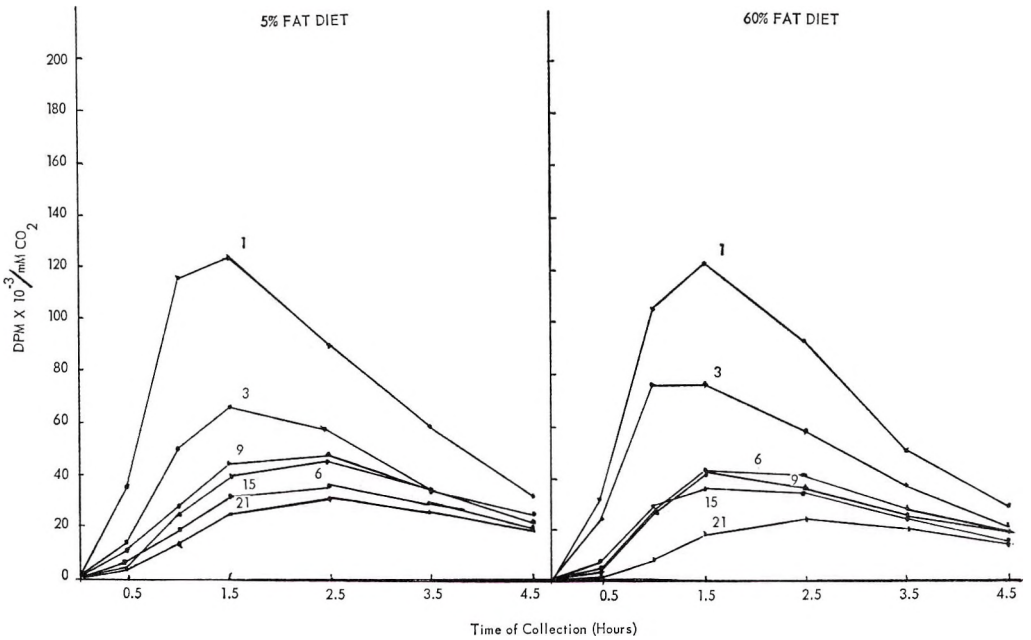

Fig. 2 Specific activity of $^{14}CO_2$ recovered after administration of an intraperitoneal dose of 2 μc glucose-6-^{14}C. Tests were made after the diet had been fed for 1, 3, 6, 9, 15 and 21 weeks.

6-^{14}C. Rats fed the low fat diet excreted carbon dioxide of higher specific activity during the ninth and twenty-first weeks than did those fed the 60% fat diet. The average specific activity was higher during the first week than during later weeks for all rats.

TABLE 4

Recovery of radioactivity in expired carbon dioxide after administration of glucose-1-^{14}C and glucose-6-^{14}C expressed as ratio of counts recovered in 4 to 5 hours.

Diet	Weeks fed diet	Weight	Total counts from glucose-1-^{14}C / Total counts from glucose-6-^{14}C
		g	
5% fat	1	66.6	1.28
60% fat	1	68.5	0.95
5% fat	3	153.8	1.30
60% fat	3	134.2	0.89
5% fat	6	271.4	0.99
60% fat	6	269.0	0.84
5% fat	9	361.4	1.09
60% fat	9	306.8	0.93
5% fat	15	472.2	1.22
60% fat	15	447.6	1.07
5% fat	21	512.0	1.11
60% fat	21	618.0	1.18

The ratio of $^{14}CO_2$ from glucose-1-^{14}C to $^{14}CO_2$ from glucose-6-^{14}C was calculated for each rat and the values for each group are shown in table 4. The ratio was greater than 1.0 for rats fed the low fat diet at all ages except 6 weeks when it was approximately unity. The ratio was lower than 1.0 for rats fed the high fat diet at all ages except the fifteenth and twenty-first weeks of the diet, when it was slightly greater than 1.0.

The percentage of the dose of sodium acetate-1-^{14}C incorporated into expired carbon dioxide is shown in table 5. Rats fed the 5% fat diet excreted a significantly higher percentage of the dose at 15 and 21 weeks than did rats fed the high fat diet. There were no differences due to age in either diet group.

The average specific activity of the carbon dioxide collected in 4.5 hours after injection of sodium acetate-1-^{14}C is shown in table 5. Rats fed the 5% fat diet showed higher specific activities at 9, 15, and 21 weeks than rats fed the 60% fat diet. The specific activity of carbon dioxide was

TABLE 5

Recovery of ^{14}C in expired carbon dioxide 4.5 hours after injection of 2 μc of acetate-1-^{14}C intraperitoneally [1]

Weeks fed diet	Per cent of dose expired		P value	Specific activity [2]		P value
	5% fat diet	60% fat diet		5% fat diet	60% fat diet	
3	75.1 ± 3.0 [3a]	76.1 ± 2.6 [b]	ns	81.2 ± 5.1 [c]	95.4 ± 6.0 [e]	ns
6	69.1 ± 3.2 [a]	69.3 ± 5.2 [b]	ns	61.6 ± 3.0 [d]	65.6 ± 3.9 [f]	ns
9	76.5 ± 1.1 [a]	65.1 ± 5.0 [b]	ns	61.5 ± 1.1 [d]	44.2 ± 3.2 [g]	< 0.001
15	76.0 ± 2.5 [a]	67.2 ± 1.9 [b]	< 0.05	54.2 ± 1.9 [d]	42.9 ± 2.7 [g]	< 0.02
21	76.6 ± 1.4 [a]	70.3 ± 1.9 [b]	< 0.05	52.7 ± 1.3 [d]	42.5 ± 2.5 [g]	< 0.01

[1] Within feeding regimens, means in the same column and having the same letter superscript do not differ significantly from each other ($P > 0.05$).

[2] $\frac{\text{dis/min} \times 10^{-3}}{\text{mmole } CO_2}$.

[3] Mean ± SE of mean.

[4] Not significant.

higher during the third week of the diet than during later weeks for rats receiving both diets.

DISCUSSION

Significant differences in some parameters measured between the groups of rats fed at the 2 levels of fat were observed, but no trends were noted which would give any indication that the rats fed the different diets were metabolizing the labeled compounds differently. Variations within some of the groups were quite large.

Rats fed the 5% fat diet incorporated more ^{14}C from glucose-1-^{14}C into $^{14}CO_2$ than rats fed the 60% fat diet at 3 and 9 weeks. However, the percentage of the dose expired by the latter group was greater at 1, 6 and 15 weeks after glucose-6-^{14}C was injected. When acetate was given, the percentage of dose expired by the control group was greater at 15 and 21 weeks than the percentage of dose expired by the group fed 60% fat diet.

When specific activities were considered, it was found that when a significant difference existed between groups fed the different diets, the group fed the low fat diet always excreted carbon dioxide of higher specific activity than rats fed the high fat diet. This was noted after injection of glucose-1-^{14}C at 9 weeks, after injection of glucose-6-^{14}C at 9 and 21 weeks, and after injection of acetate-1-^{14}C at 9, 15, and 21 weeks.

The differences in specific activity of expired carbon dioxide in rats of different ages may be related to their rate of growth. The sixth and ninth weeks appear to be turning points in glucose metabolism for the low and high fat diet groups, respectively. After the sixth week the rate of growth of the control group was no longer linear indicating a decrease in rate of gain. Rats fed the high fat diet continued to gain at the same rate until about the thirteenth week, and then showed a decline in the rate of gain. According to Brody (8) the resting metabolism of rats in terms of calories per unit area increases markedly from birth to 40 days, and decreases thereafter. It is possible that the decrease in specific activity took place after the tests were made during the third week and we did not observe the change until the next tests were made at the sixth week.

The relative contributions of carbons 1 and 6 of glucose to $^{14}CO_2$ formation have been widely interpreted as evidence for an extra-glycolytic pathway of glucose metabolism despite difficulties encountered in interpretation (9). The ratio (C_1/C_6) increased to greater than 1.0 when the weights of rats fed the high fat diet began to reach the weights of rats fed the low fat diet. At the same time rats fed the high fat diet were retaining a higher percentage of the dose of acetate-1-^{14}C, perhaps indicating greater fat depositions. Thus an increase in the production of NADP by the shunt mechanism coincided with increased deposition of fat in the animal.

Rats fed the high fat diet for 21 weeks attained an average weight of 586 g of which 37% was body fat. Rats fed the high carbohydrate diet, however, had a much lower percentage of their body

weight as fat. Their average weight was 501 g of which 20% was fat.[4]

Brice and Okey (10) studied the effect of varying the fat intake in relation to the excretion of $^{14}CO_2$ and synthesis of labeled liver lipids following a single intraperitoneal injection acetate-2-^{14}C. They observed that for about one hour after the injection, the specific activity of expired carbon dioxide was significantly lower in the group fed a diet containing 5% fat ad libitum than in the groups fed a 40% fat diet in amounts adjusted to match the calorie and protein intake of the first group or in the group fed the 40% fat diet ad libitum. We observed no difference in specific activity after administration of acetate to rats of the same age (3 weeks after weaning).

It had been suggested (2) that the tendency to obesity in Osborne-Mendel rats may be a genetic factor. In view of differences observed between non-obese and obese mice in metabolism of labeled compounds, our data were inspected to determine whether differences occurred between individual animals which could be related to weight. No correlation between weight of rat and percentage of dose expired or between weight of rat and specific activity of the carbon dioxide was noted within groups of rats of the same age.

LITERATURE CITED

1. Mickelsen, O., S. Takahashi and C. Craig 1955 Experimental obesity. I. Production of obesity in rats by feeding high-fat diets. J. Nutrition, *57:* 541.
2. Zucker, T. F., and L. M. Zucker 1963 Fat accretion and growth in the rat. J. Nutrition, *80:* 6.
3. Guggenheim, K., and J. Mayer 1952 Studies on pyruvate and acetate metabolism in the hereditary obesity-diabetes syndrome of mice. J. Biol. Chem., *198:* 259.
4. Subrahmanyam, K. 1960 Metabolism in the New Zealand strain of obese mice. Biochem. J., *76:* 548.
5. Towne, J. C., E. F. Krohn and M. Brandfonbrener 1964 A simplified diffusion apparatus for liquid scintillation counting. Clin. Chem. Acta, *9:* 566.
6. Jeffay, H., and J. Alvarez 1961 Liquid-scintillation counting of carbon-14. Anal. Chem., *33:* 612.
7. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
8. Brody, S. 1945 Bioenergetics and Growth. Reinhold Publishing Corporation, New York.
9. Katz, J., and H. G. Wood 1960 The use of glucose-C^{14} for the evaluation of the pathways of glucose metabolism. J. Biol. Chem., *235:* 2165.
10. Brice, E. G., and R. Okey 1956 The effect of fat intake on incorporation of acetate-2-C^{14} into liver lipide and expired carbon dioxide. J. Biol. Chem., *218:* 107.

[4] Unpublished data, M. L. Reynolds and D. J. Pringle.

ERRATUM

Grande, F., J. T. Anderson, D. Chlouverakis, M. Proja and A. Keys 1965 Effect of dietary cholesterol on man's serum lipids. J. Nutrition, *87:* 52. In table 6, the heading should have read: *Comparison of different methods of adding 1500 mg of cholesterol to a low cholesterol (50 mg/day) diet.*[1]

To correct the heading of table 6 in your copy of volume 87, number 1, please cut along lines of reprinted heading below and paste over the heading of table 6 on page 60.

TABLE 6

Comparison of different methods of adding 1500 mg of cholesterol to a low cholesterol (50 mg/day) diet [1]
