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A well-balanced moderate low-fat breakfast for teen-age girls, 16-19 years

When a moderate reduction of dietary fat is indicated for girls in their late teens, the basic cereal and milk breakfast merits consideration. *This breakfast is moderately low in fat because its fat content of 10.9 gm. provides 20 per cent of the total calories.* As shown in the table below, it contributes *well-balanced nourishment.* It provides "Girls, 16 to 19 Years" with approximately one-fourth of the recommended dietary allowances[1] of complete protein, important B vitamins, essential minerals; and provides quick and lasting energy.

recommended dietary allowances and the nutritional contribution of a moderate low-fat breakfast*

Menu: Orange Juice—4 oz.;
Cereal, dry weight—1 oz.;
Whole Milk—4 oz.; Sugar—1 teaspoon;
Toast (white, enriched)—2 slices;
Butter—5 gm. (about 1 teaspoon);
Nonfat Milk—8 oz.

Nutrients	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Niacin equiv.	Ascorbic Acid
Totals supplied by Basic Breakfast**	503	20.9 gm.	0.532 gm.	2.7 mg.	588 I.U.	0.46 mg.	0.80 mg.	7.36 mg.	65.5 mg.
Recommended Dietary[1] Allowances—Girls, 16 to 19 Years (54 kg.—120 lb.)	2400	75 gm.	1.3 gm.	15 mg.	5000 I.U.	1.2 mg.	1.9 mg.	16 mg.	80 mg.
Percentage Contributed by Basic Breakfast	20.9%	27.9%	40.9%	18.0%	11.8%	38.3%	42.1%	46.0%	81.9%

*Revised 1958. Food and Nutrition Board, National Research Council, Washington, D.C.

**Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1959.
Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

[1]*The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. Calorie allowances apply to individuals usually engaged in moderate physical activity. For office workers or others in sedentary occupations they are excessive. Adjustments must be made for variations in body size, age, physical activity, and environmental temperature.*

CEREAL INSTITUTE, INC.
135 South La Salle Street, Chicago 3

A research and educational endeavor devoted to the betterment of national nutrition

GUIDE FOR CONTRIBUTORS

TO

THE JOURNAL OF NUTRITION

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RICHARD H. BARNES, EDITOR
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The following information should be listed on page 1:

1. Complete title of paper.
2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
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(Place footnotes on a separate sheet, typed double-spaced.)

The text should begin on page 2. The manuscript should be submitted in complete and finished form exactly as the author wishes it to appear in type. Citations of the literature in the text should be made by author and year of publication, as (Jones, '58). The words et al. are not underscored, nor is ad libitum.

Never divide a word at the end of a line, for, it might be set incorrectly with a hyphen. The repeated use of a unit in a sentence may be avoided by stating the common unit used as, "the diet consisted of the following in milligrams: sucrose, 6; etc." Or, in other cases, as "8, 12 and 14%."

A common error occurs in describing the percentage of a compound. Use the form, "15% of calcium" when the percentage is not an adjective. Number all pages consecutively in the following order: title page, text, tables, figures and legends, literature cited and footnotes.

Start each of the following sections on a new page and typed double-spaced:

1. Tables, with appropriate heading (a separate sheet for each table)
2. Explanation of figures, numbered and listed consecutively
3. Literature cited, arranged alphabetically by authors—see current issue for exact form, and
4. Footnotes.

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on page 1. In a table, footnotes should be made part of the table, typed directly beneath it, and numbered 1, 2, 3, etc. These are independent of other footnote numbers used in the manscript.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will follow the summary.

Summary. This should represent a brief recapitulation of the problem and results.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports, personal communications or unedited reports should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evalua-

tion of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited.

Numbers. If possible, avoid using a number as the first word in a sentence. Numbers so used should always be spelled out, but no abbreviations should follow such numbers. Numbers indicating time, weight and measurements are to be expressed in Arabic figures when followed by an abbreviation (e.g., 2 mm; 1 sec.; 3 gm; 10 mm^3). Except as indicated, the numbers *one*, *two* and *three* will be spelled out in the text. All higher numbers will be given in Arabic figures. Use Arabic numerals throughout, including tables.

Abbreviations. The word *figure,* written in the text, is not abbreviated except when appearing in parentheses, as (fig. 2) (figs. 4–6).

The words *pounds*, *weeks* and *days* are not abbreviated.

The metric system should be used in preference to others for measurements, weights, etc. Temperatures should always be expressed in Centigrade scale. Metric abbreviations listed below are shown in the approved lower-case style, *without periods:*

Weight		*Length*	
kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
μg	microgram	mm	millimeter
mμg	millimicrogram	μ	micron
μμg	micromicrogram	mμ	millimicron
m^3	cubic meter	μμ	micromicron

Volume		*Area*	
cm^3	cubic centimeter		
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

Symbols. When preceded by a figure, the following symbols are used:

A	angstrom units	°	degree
%	per cent	ppm	parts per million

Tables and figures. Follow form in current issues for the use of upper and lower case letters and italics. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or when necessary, two column widths (5½ inches). A charge will be made for that space used for tables and figures which exceeds one-half of the space used for the manuscript exclusive of tables and figures. A table or figure should be constructed so as to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied.

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Modifications of Diets Responsible for Induction of Coronary Thromboses and Myocardial Infarcts in Rats[1,2]

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The production of occlusive thromboses of major branches of coronary arteries with associated myocardial infarction in intact rats by dietary means alone was reported from this laboratory in 1957 (Hartroft and Thomas, '57; Thomas and Hartroft, '59). Renal arterial thrombi with infarcts of the kidneys in some animals were also described. Most of the diets[3] employed contained large amounts of saturated fats (40% butter or lard), and three hypercholesteremic agents (0.3% propylthiouracil, 2% sodium cholate and 5% cholesterol). Preliminary reports of the effects of a few modifications of the fat content, amount of choline supplement and level of protein in this diet were also given (Thomas and Hartroft, '59). A later series of experiments, involving 775 rats fed 38 modifications and 11 repetitions of this dietary regimen, will be reported herein.

Although it has been possible for many years to produce certain types of atherosclerosis in animals by a variety of methods, our report of the deliberate induction of occlusive arterial thromboses and infarction by diet alone was without much precedent at that time. The value of this experimental model in studying related conditions in man needs little elaboration. It seemed mandatory that the initial studies be extended in several directions: (1) to repeat the original experiments, producing the lesions in larger number of animals than employed in our initial studies, (2) to modify the diet with the object of increasing the incidence of infarcts from that originally reported (10 to 60%) to approach 100% and (3) to modify the diet with the object of testing the ability of substances to prevent development of infarcts. Our efforts have been directed primarily to the first two objectives in the series of experiments herein reported. We have successfully repeated induction of infarcts by this dietary method and have established their presence by both gross and microscopic examinations in a total of 73 rats. But we have not approximated an incidence of 100% infarction in any single group. The percentage of animals developing infarcts is variable, despite many modifications of the diets, and averages approximately 25% of rats surviving on the "basal" diet for two months or longer. We have not completed our attempts to achieve the third objective (mod-

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[1] These investigations were supported by U.S. Public Health Service Grant no. H-1820 from the National Heart Institute, Institutes of Health, Bethesda, Maryland and by a grant from the Nutrition Foundation.

[2] Presented in part before the 56th Annual Meeting of the American Association of Pathologists and Bacteriologists, Boston, Massachusetts, April 23, 1959.

[3] The diet we now regard as our standard basal thrombogenic mixture is as follows in per cent by weight: casein, 20; sucrose, 20.5; salt mixture, 4; Alphacel, 6; vitamin mixture, 2; propylthiouracil, 0.3; choline, 0.2; sodium cholate, 2; cholesterol, 5; butter, 40. The salt mix is Wesson's modification of Osborne and Mendel's salt mixture. The vitamin mixture is Nutritional Biochemical's Diet Fortification Mixture without choline chloride, each kilogram of which contains the following, in grams, triturated in dextrose: vitamin A concentrate (200,000 units/gm), 4.5; vitamin D concentrate (400,000 units/gm), 0.25; α-tocopherol, 5; ascorbic acid, 45; inositol, 5; menadione, 2.25; *p*-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; calcium pantothenate, 3; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.00135.

ifications of the diet that will give complete protection to the entire group) and only a few of those results will be included. Perhaps most remarkable in this connection is that up to the present, no *single* ingredient of our basal diet studied to date has proven essential to the production of at least a small percentage of infarcts.

MATERIALS AND METHODS

Six separate experiments, involving 775 rats divided into 49 groups, were carried out between March 1957 and February 1959. Starting weights are recorded in table 1. All were males except group 15. They were housed in individual wire-bottom cages and offered water ad libitum. All were weighed weekly. Fresh food was provided each day in amounts greater than consumed on previous days. During most of the period the food not consumed was weighed daily, thus providing a daily record of intake. Because of the time required to provide a record of the daily intake, we have recently limited the determination to alternate days. Animals that died during the course of the experiments were placed in a refrigerator until autopsied (usually in less than 10 hours). A few from many groups were killed in the experimental period to provide fresh material for histologic and electron microscopic study. All experiments were terminated at 18 to 26 weeks (usually nearer 18) and survivors killed and autopsied.

Diets. Diets were prepared as indicated in the text footnote[3,4] and in table 1. Dry ingredients were thoroughly blended in a Hobart food mixer. The various supplements were mixed in a mortar with small portions of dry ingredients and blended with the remainder in the mixer. Fats were added last and if solid they were warmed until they became liquid. Diets were kept in closed plastic containers and refrigerated at 4°C for no longer than two weeks; under these conditions the food did not become obviously rancid.

Microscopic studies. Sections of major viscera were fixed in cobalt-formalin and selected pieces, prepared in carbowax, were sectioned at 4 μ thickness. Adjacent sections were stained with hematoxylin and eosin, Oil Red O for fats and aldehyde fuchsin—van Gieson for elastic and connective tissues.

Criteria for diagnosis of infarcts in rats. Since our original report in 1957 others have described the production of lesions variously referred to as "infarctoid," "infarct-like," etc. (Selye and Renaud, '57, '58; Selye et al., '58). The criteria for recording a lesion as an infarct should therefore be clarified so that results from various centers will be comparable. In this laboratory we have adopted the following criteria: (1) an area of demonstrable necrosis visible grossly and confirmed microscopically must occupy a sharply demarcated area in the organ (heart or kidney in our material) corresponding to that supplied by a nutrient artery; (2) the remainder of the myocardium must be free of "spotty" or diffuse distributed areas of necrosis in order eliminate the possibility that the apparently infarcted area really represents coalescence of "metabolic" foci of necrosis that are not directly the result of arterial occlusion and ischemia; and (3) ideally, the site of thrombotic occlusion in the nutrient artery should be found.

In rats the demonstration of sites of occlusion is difficult, since the thrombi are too small to be seen grossly. By utilizing serial sections in many of the animals we have improved our ability to demonstrate the obstructing thrombi. Since the infarcts were similar pathologically whether or not the associated thrombus was demonstrated, we see no reason to exclude those in which the thrombus was not found. The rats' coronary arteries are small and an occluding thrombus, while extending proportionately for a distance that corresponds to that encountered in man, may only be 100 μ or less in length. This fact, as well as the possibility that a thrombus may be lost in sectioning or lysed after the development of the infarct, probably accounts for our failure to demonstrate thrombi in coronary arteries of all infarcted hearts. In our experiments the first two criteria were fulfilled, and in 10 instances all three, before an infarct was recorded.

[3] See footnote 3, page 325.

[4] Lederle Laboratories kindly supplied the propylthiouracil used in the diets.

Criteria for diagnosis of an occlusive thrombosis need statement also. Coagulation by fixative of the highly lipemic plasma of this type rat, mixed with erythrocytes, may superficially give the appearance of recently formed ante-mortem thrombi in sections, particularly the frozen sections stained for fat. Clear demonstration of at least fibrin strands and nuclear disintegration were required in our series and in practically every instance we also saw early invasion of the thrombi by young fibroblasts, constituting clear evidence of the ante-mortem nature of the thrombi.

Calculation of percentage infarcts in the groups. In only 6 rats have we observed development of thromboses and infarcts before the animals have been maintained at least two months on the experimental diets. From two months until the termination of an experiment fatalities and the demonstration of infarcts appear to occur at a steady rate. In most experiments a number of the animals have died without demonstrable infarcts during the first several weeks of the regimen. Weight loss, decreased food intake and absence of infection at autopsy are compatible with the likelihood that most deaths at this time resulted from thiouracil intoxication before the animals had developed sufficient tolerance to the drug. For this reason the calculations of percentages of infarcts, presented in table 1, are based on the number of animals in each group surviving two months or longer. When this calculation is used for repeated runs (groups 1, 10, 17, 29, 36 and 42) of positive control groups of similar starting weights fed the standard basal diet the percentage-infarction is reasonably consistent (21 to 35%). This degree of consistency was not apparent when the calculations were based on the number of rats used at the start of the dietary regimen. For this reason and because some of the modifications of the diet increased the number of deaths during this preliminary period of adjustment to the food mixtures, the first type of calculation, using the numbers surviving two months or longer as denominators, appears more useful for evaluating effects of these diets than does the latter method. The method used eliminates at least one variable throughout the series: early toxic effects of the several regimens studied.

Cholesterol determinations. Blood was obtained by clipping the tail and the total plasma cholesterol content determined by the method of Pearson, Stern and McGavack ('53). In the recent experiments three or 4 samples were taken from each group, usually twice weekly. Rats to be sampled were alternated as long as the number remaining in the group allowed it.

RESULTS

Results are presented in detail in table 1 and are illustrated in the photomicrographs (figs. 1-8). As in the original experiments, most diets resulted in immediate cessation of growth and body weights remained close to the starting levels. Fur of the rats rapidly lost its shine and lustre and became coarse, dull and tended to stand out from the body. A few lost hair over their flanks, but this was not common.

The anatomical features did not differ from those described in detail previously (Thomas and Hartroft, '59; O'Neal, Thomas and Hartroft, '59). Infections in the form of pneumonia, empyema or widespread abscesses were encountered in approximately 10%. These were scattered through nearly all groups and had no apparent relation to the incidence of infarcts. Marked accumulations of fat were found in the liver, spleen, lymph nodes, lungs and kidneys of rats in all groups.

Eighty-one infarcts were found in 73 rats. Of these infarcts, 47 were cardiac and 34 were renal. These were indistinguishable from those seen in man. Thrombi were demonstrated in the supplying arteries in many. The thrombi observed were in arteries showing lipid deposits but in general lipidosis was not prominent. None contained conspicuous plaques, although beginning plaques were demonstrated in occasional aortas and in some other vessels but none of these had overlying thrombi. Mural thrombi were found in hearts over many of the myocardial infarcts. All thrombi contained stainable lipid and in some of the mural thrombi, ceroid was demonstrated.

TABLE 1

Incidence of myocardial and renal infarcts and other data[1]

Group	No. rats	Weights		Av. food intake[2]	Survivors 2 mos.	Av. serum cholesterol 2–5 mos.[3]	Infarcts		Principal dietary and other variations[4]
		Start	2 mos.				Cardiac and renal	In 2 mos. survivors	
		gm	*gm*	*gm*		*mg%*		*%*	
						Experiment 1			
1	25	118	109	6.4	22	—	6	27	Basal (see text footnote 3)
2	25	121	122	6.1	25	—	0	0	20% Crisco; 20% butter
3	25	118	137	6.5	23	—	1	4	20% Crisco; 20% corn oil
4	25	122	132	6.1	24	—	3	13	20% butter; 20% Crisco
5	10	117	103	8.4	7	—	0	0	Fat-free
6	10	115	164	7.0	10	—	1	10	No cholesterol or sodium cholate
7	10	119	385	12.6	10	—	0	0	No thiouracil, cholesterol or sodium cholate
8	10	115	122	5.7	10	—	0	0	20% Crisco; 20% cod liver oil
9	10	115	127	5.7	10	—	0	0	10% corn oil; 30% butter
						Experiment 2			
10	25	107	107	6.8	17	3064 (3)	6	35	Basal—starting wt. 107 gm
11	24	68	88	6.3	11	1953 (1)	2	18	Basal—starting wt. 68 gm
12	10	58	73	7.5	3	—	1	33	Basal—starting wt. 58 gm
13	15	223	165	9.4	11	2313 (3)	1	9	Basal—starting wt. 223 gm
14	14	429	301	12.6	14	1895 (14)	5	36	Basal—starting wt. 429 gm
15	25	97	94	6.5	9	2709 (2)	3	22	Basal—female
16	24	108	99	8.1	19	2590	5	26	Bile salts 2%; no sodium cholate
						Experiment 3			
17	18	129	112	6.6	11	1641 (12)	3	27	Basal
18	12	129	99	—	5	—	0	0	Sodium cholate raised to 4% after 45 days
19	10	129	129	5.4	9	1130 (25)	1	11	Butter oil, 40%
20	10	128	118	8.0	7	1330 (24)	1	14	Butter residue, after removal of oil, 40%
21	10	132	120	7.2	7	1620 (24)	0	0	Crisco 40%; no calcium and vitamin D
22	10	128	110	5.9	6	3315 (6)	4	50	Salt mix, 12%
23	10	125	—	6.3	0	—	0	0	Crisco, 40%; Vit. D concentrate, 0.7%; Ca_3PO_4, 6%; $CaCO_3$, 8%
24	10	134	—	6.5	0	—	0	0	Vit. D concentrate, 0.7%; Ca_3PO_4, 6%; $CaCO_3$, 8%
25	10	125	—	4.5	0	—	0	0	Vit. D concentrate, 0.7%; Ca lactate, 14%
26	10	130	—	3.1	0	—	0	0	Vit. D concentrate, 0.7%; Ca citrate, 14%
27	20	131	100	6.5	5	1415 (1)	2	20	Fat-free; choline, 1%
28	10	131	115	6.7	2	1190 (7)	0	0	Non-fat milk, 38%; corn oil, 2%

						Experiment 4			
29	30	80	85	5.3	9	2570 (11)	2	22	Basal
30	24	80	101	4.6	9	2246 (20)	3	33	Corn oil, 10%; choline, 1%; no butter
31	25	80	93	4.1	15	1529 (17)	4	20	Crisco, 40%
32	24	80	75	6.6	18	717 (20)	0	0	Corn oil, 40%
33	23	80	89	5.4	11	—	1	9	No cholesterol
34	10	80	—	2.9	0	—	0	0	Cod liver oil, 40%
35	10	80	—	4.1	0	—	0	0	Ca citrate, 14%; Vit. D conc. 0.7%; salt mix without $MgSO_4$
						Experiment 5			
36	18	129	120	8.3	8	1396 (21)	2	25	Basal
37	10	112	122	6.9	6	1396 (12)	2	17	Lard, 40%
38	10	123	122	5.5	8	1327 (16)	2	25	Butter oil, 40%
39	10	118	—	10.3	0	—	0	0	Non-fat fraction of butter, 40%
40	10	120	136	9.1	6	724 (17)	1	17	Non-fat milk, 20%; corn oil, 2%; no butter
41	19	112	111	7.9	1	—	0	0	Sodium cholate, 10%; no thiouracil
						Experiment 6			
42	29	98	103	6.8	29	2140 (34)	6	21	Basal
43	29	100	171	7.8	20	1742 (15)	1	5	No thiouracil; sodium cholate increased slowly to 12%
44	29	93	107	7.0	10	2003 (22)	3	30	Salt mix, 12%
45	10	93	134	9.2	6	1165 (27)	1	0	Tween 80 substituted for sodium cholate
46	6	93	133	9.2	2	—	0	0	Lipase substituted for sodium cholate
47	6	95	—	7.6	0	—	0	0	Calcium lactate, 1%
48	10	99	—	8.5	0	—	0	0	Sodium chloride, 1%
49	7	95	—	12.7	0	—	0	0	Sodium cholate, 5%

[1] More complete tables will be included with reprints if requested.
[2] Dietary intake given is the average daily consumption during the fifth week of the experiment.
[3] The numbers within parentheses refer to the number of determinations from which the average was derived.
[4] Unless noted otherwise, the percentage of sucrose in the diet was adjusted to compensate for addition or removal of any other component of the basal diet. Fats were never given at a level of greater than 40%, and were substituted for the butter in the basal diet.

DISCUSSION

Causes of deaths. In the early weeks of the experiments, we have attributed deaths to thiouracil intoxication or to other severe metabolic stress and collapse, because infections (bronchopneumonia, etc.) cannot account for more than 10% of fatalities at this stage. After two months a few more animals on the dietary regimen died in which infarcts were not demonstrable and in serial sections of these hearts even very recently formed thrombi have not been found. We know of no way to exclude the possibility that suddenly developing thromboses, infarction and rapid (within a few hours) death may be the explanation for these unexplained fatalities. In previous studies we have found thrombi difficult to distinguish from post-mortem clots (Thomas, O'Neal and Lee, '56) and even by electron microscopy infarcts of less than 12 hours duration cannot be recognized (Bryant, Thomas and O'Neal, '58).

In the animals in which thromboses and infarcts have been found, it is impossible in any individual animal to determine the part played by the infarct in causation of death. Some infarcts have been sufficiently large to suggest that they actually were responsible for death.

Importance of various dietary constituents in the thrombogenic diet. The important ingredients appear to be fat, cholesterol, choline chloride, propylthiouracil and bile salts, although no single one of these supplements, even when fed in large quantities, has proved sufficient alone to produce infarcts. On the other hand, no single ingredient has proved to be essential and infarcts have been produced with a variety of combinations.

Rats whose diets included large amounts of corn oil had fewer infarcts, lived longer and had much lower cholesterol levels in the blood than rats whose diets included butter, hydrogenated vegetable oil or lard (compare groups 2, 29, 31, 32, and 37 in table 1). Infarcts were not observed in rats fed 40% corn oil plus thiouracil, bile salts, and a moderate amount of choline (group 32). However, infarcts were produced in one rat fed lesser amounts of corn oil in combination with saturated fats (group 3) and were produced in many rats, in this (group 30) and previous experiments, fed corn oil at the 10% level with an excess of choline plus thiouracil, bile salts, etc.

The presence of fatty acids was not absolutely necessary for the production of infarcts since one appeared in a rat on a fat-free diet (group 27). Corn oil in the diet seemed to decrease mortality throughout the experimental period, particularly when given at the 10% level (groups 9 and 30). On a "well-balanced" synthetic diet 2% corn oil is usually satisfactory for the maintenance of good health in rats, but in the presence of various deleterious ingredients more seemed to be required (groups 28 and 40 compared to 2 and 9).

The role of lipotropic agents in these experiments needs further clarification. As already mentioned, it appears that an excess of choline chloride can take the place of saturated fats in the infarct-producing diet (group 30). This perhaps provides a clue as to the mode of action of the diet, but we have not as yet been able to interpret it.

Pathogenesis of dietary-induced infarcts. In general, cholesterol levels in the plasma were only slightly higher in rats fed infarct-producing diets than in animals fed diets used by others that have usually produced only atherosclerosis (Fillios et al., '56). Likewise the average serum cholesterol level in rats with infarcts (1,994 mg %) was not significantly higher than in rats of the same groups that did not have infarcts (1,824 mg %). It is possible that hypercholesterolemia is an essential feature but further evidence is needed, and at present the role of cholesterol in thrombosis is unknown. Certainly the hypercholesterolemia did not produce an effect by resulting in bigger and better local lesions, since thrombosis actually occurred before plaque formation. The rising cholesterol level in the plasma may simply parallel, *pari passu*, a rise in some substance we have not measured. Alterations in this latter, unknown substance may be the essential feature leading to thrombosis with the rise in cholesterol being only an indicator.

Cardiac necroses (as distinguished from infarcts) have been produced in rats in

many ways. For example, areas of myocardial necrosis can be produced in a few days with a single massive dose of vitamin D (Ham, '32). In our animals the necrosis is clearly that of infarction following occlusion of the nutrient arteries by thrombi. For this reason we have called these lesions *infarcts* and not infarctoid lesions or infarct-like changes as have others in somewhat different models (Selye and Renaud, '57, '58; Selye et al., '58). In our animals it is logical to concentrate on factors that are important for the development of thrombi.

In our opinion coronary thrombosis in man results from the interplay of two factors: (1) a local factor, usually atherosclerosis, and (2) a hematologic factor, either procoagulative or antifibrinolytic or both. For many years we have had an experimental model with which to study atherosclerosis, but thrombosis almost never occurred. The experimental model reported herein is the first to reliably and repeatedly offer the means to study, in animals, the multiple factors involved in coronary thrombosis.

SUMMARY

The dietary production of experimental arterial thrombosis, with resultant myocardial and renal infarction, has been accomplished in 6 separate experiments. In each experiment approximately one-fifth of the animals on the "basal thrombogenic diet" surviving two months developed infarcts. Although 38 modifications of the basal thrombogenic diet have been fed to separate groups of rats, none has resulted in a clearly higher percentage of infarcts than that obtained with the basal diet. In individual dietary groups no appreciable differences in longevity, within the experimental period, or serum cholesterol levels have been noted between the rats with and without infarcts.

Of the many dietary constituents omitted, none was found to be absolutely essential to the development of infarcts, but omission of any of the important ingredients (propylthiouracil, sodium cholate, cholesterol and fat) lowered the incidence of infarcts.

The areas of infarction in these rats are grossly visible, well circumscribed, almost always single, and often associated with thrombi in the supplying artery. These infarcts are to be distinguished from "metabolic" areas of necrosis or "infarctoid" lesions obtained in other experimental models of myocardial disease.

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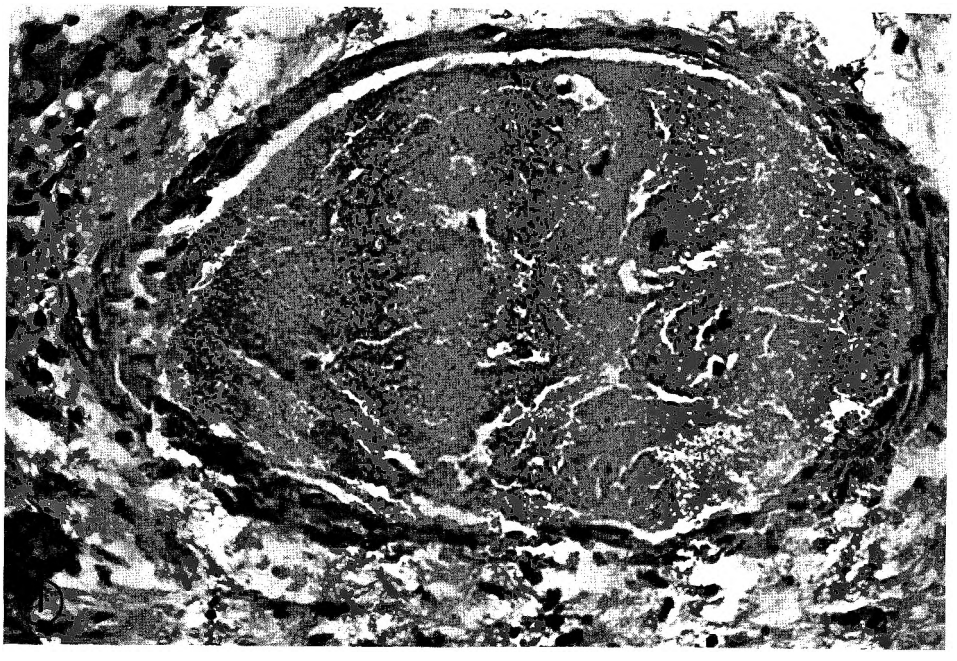
PLATE 1

EXPLANATION OF FIGURES

1 A recent, occluding thrombus in a coronary artery of a rat. A few young fibroblasts lie in the spaces between the heavy strands of fibrin that fill the arterial lumen. No intimal lesion is seen. Hematoxylin and eosin. × 620.

2 Photomicrograph of the entire thickness of the left ventricular wall in one of the rats with a large infarct. The subendocardial myocardium has been almost completely replaced by fibrous tissue. The loss of muscle has produced marked thinning of the ventricular wall. Oil red O-hematoxylin. × 200.



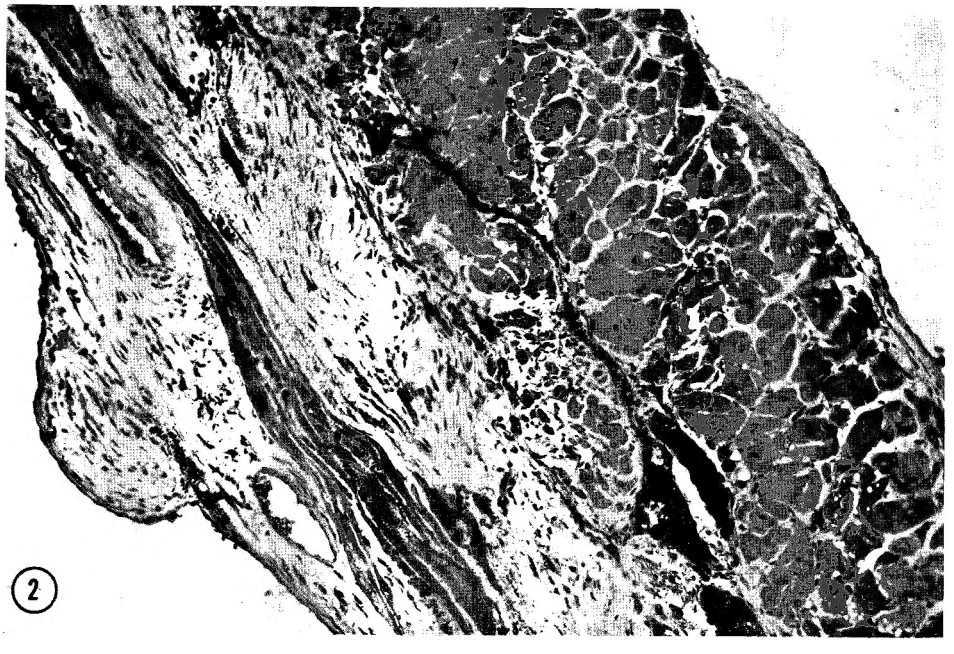
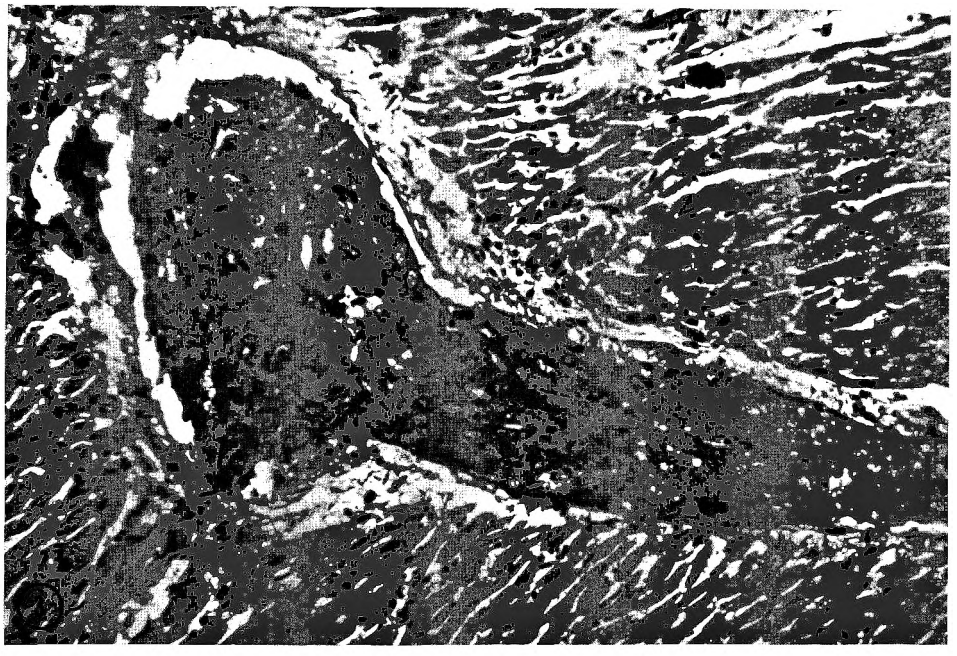


PLATE 2

EXPLANATION OF FIGURES

3 An occlusive thrombus of a coronary artery extending into a smaller branch. Many of the clear, slit-like spaces within the thrombus contain fibroblasts, indicating early organization. Oil red O-hematoxylin. × 425.

4 The artery partially seen at the right is occluded by a recent thrombus which extends along the endothelial surface of a branch running to lower left. This thrombus contained a large amount of lipid, seen as very fine black particles. Fat is also present in the arterial media. The myocardium in the lower center is necrotic and muscle cells are no longer seen. Oil red O-hematoxylin. × 750.



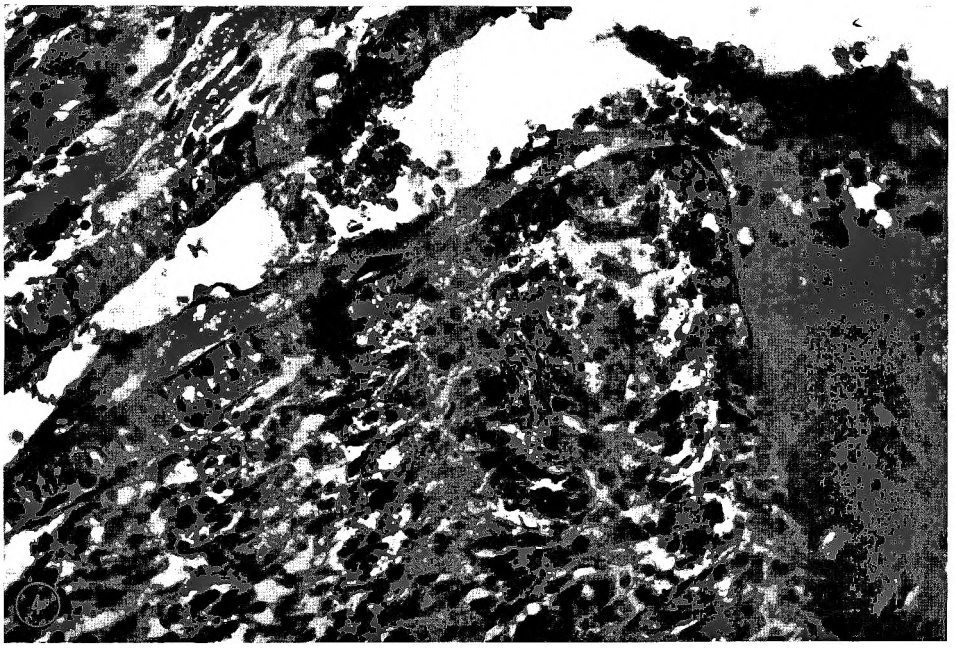


PLATE 3

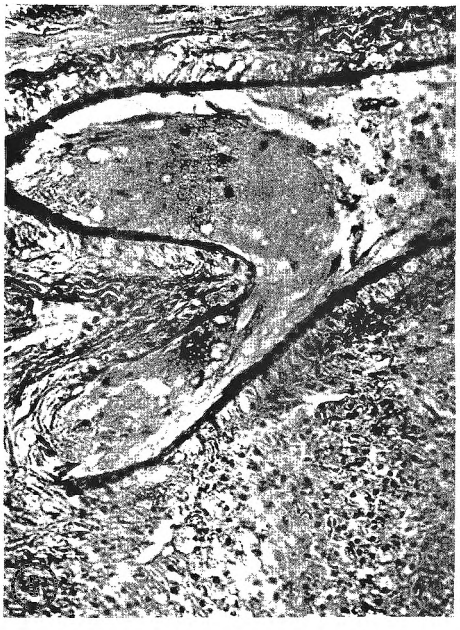
EXPLANATION OF FIGURES

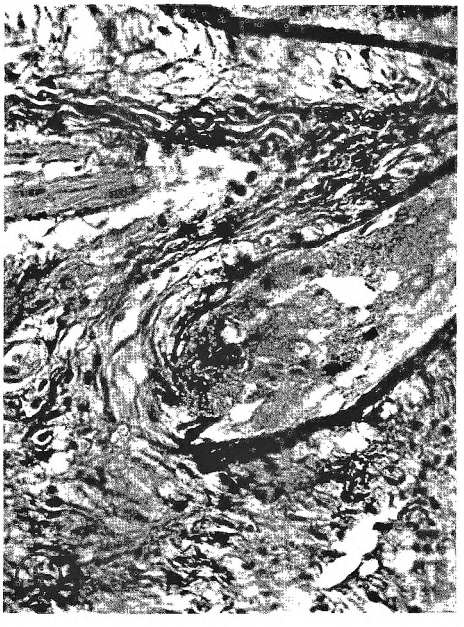
5 A thrombus "straddling" the point of branching of a coronary artery. The portion of the thrombus lying in the larger branch appears to be retracted from the arterial wall on one side, but this could be an artefact resulting from tissue preparation, as is also often seen in human material. Aldehyde fuchsin-van Gieson-hematoxylin. × 250.

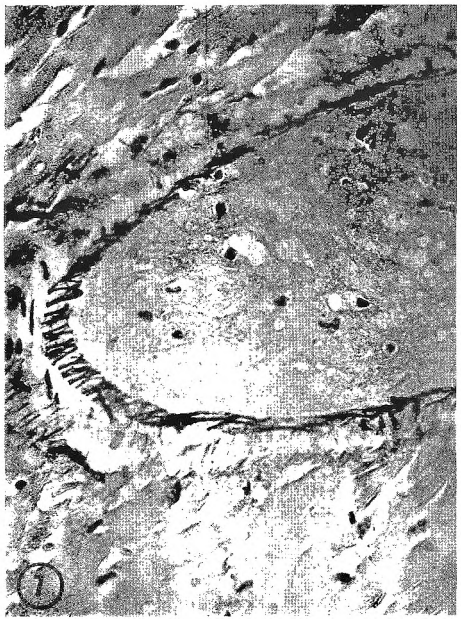
6 A higher magnification of the smaller branch shown in figure 5, demonstrating the presence of fibroblasts and the granular nature of the thrombotic material. Aldehyde fuchsin-van Gieson-hematoxylin. × 420.

7 Another recent thrombus in a coronary artery. The myocardium was not necrotic in this immediate area, the infarct lying distal to the occlusion as was usual in the rats. Again note the absence of intimal lesion. The thrombus appears adjacent to the internal elastica which shows as a black band. Aldehyde fuchsin-van Gieson-hematoxylin. × 420.

8 Another portion of the same thrombus seen in figure 7. Serial sections of hearts often allowed the demonstration of extension of thrombi at several levels, but the extension was never great. Aldehyde fuchsin-van Gieson-hematoxylin. × 420.



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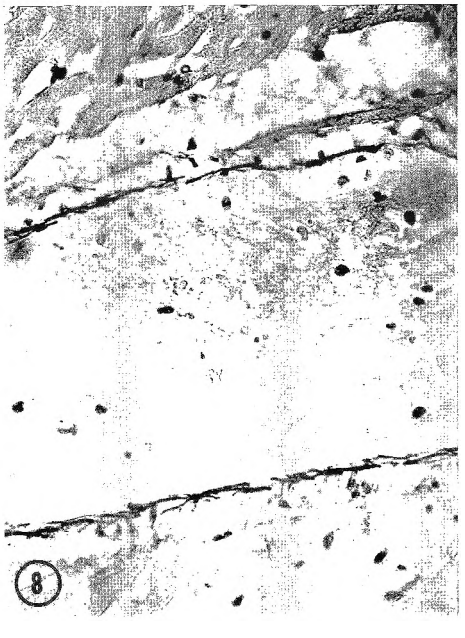
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The Absorbability of Stearic Acid when Fed as a Simple or Mixed Triglyceride

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From the extensive studies carried out in the Department of Agriculture a number of years ago, Langworthy ('23) concluded that the absorbability of dietary fat is determined by its melting point. This concept received further support from the work of Deuel (Crockett and Deuel, '47; Cheng et al., '49). On the other hand, Hoagland and Snider ('43a) proposed that the absorbability of a fat is determined by its content of saturated fatty acids having 18 or more carbon atoms. Work in this area was subsequently reviewed by Mattil ('46). His conclusions were in general agreement with the theory proposed by Hoagland and Snider. However, in all of the studies to date it has been impossible to distinguish between the effect of melting point and the saturated fatty acid content of a fat, since the melting point increases with the content of saturated long chain fatty acids. Yet, the melting point of a fat is not proportional to its content of saturated long chain acids, since these acids when present as trisaturated glycerides will give the fat a higher melting point than will the same amount of saturated acids distributed randomly among the glycerides.

Actually, melting point is not a good criterion for describing a fat, since fats usually contain a variety of triglycerides and hence do not have a sharp melting point but melt over a temperature range. Thus, a typical natural fat may contain triglycerides having melting points from minus 50°C or less up to 70°C or more. At any intermediate temperature there will be both solid and liquid fat present, the amount of each depending upon the glyceride composition of the fat. The hypothesis of Langworthy and Deuel can be re-examined with respect to the amount of high melting triglycerides that was present in the fats they used Thus, rather than melting point, the amount of unmelted fat at body temperature (or perhaps 50°C as suggested by Deuel) may serve as a better criterion.

Studies by Calloway et al. ('56) and Mattil et al. ('45) using a limited number of fats led them to the conclusion that the absorption of stearic acid depended in part at least on the nature of the fatty acids making up the rest of the triglyceride molecule. In general, stearic acid was better assimilated when it was fed as a mixed glyceride than when fed as tristearin.

The experiment that is reported here was designed so that it would be possible to distinguish between the effect of saturated fatty acid content and saturated triglyceride content on the coefficient of absorbability of a fat. For this purpose pairs of fats containing the same amount of stearic acid, but differing in the amount present as tristearin, were prepared and the coefficients of absorbability of these pairs determined. From the results obtained it is apparent that the coefficient of absorbability of a fat is inversely proportional to its content of simple triglycerides made up of saturated fatty acids having a chain length of 18 carbon atoms or greater, and is influenced by the amount of such saturated fatty acids only insofar as they are present as saturated triglycerides.

METHODS

The test fats used in this study were prepared from safflower seed oil and hydrogenated linseed oil. The safflower seed

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oil which by analysis was found to contain 13% of saturated fatty acids, 78% of linoleic acid, and 8% of oleic acid was rearranged randomly. At this level of saturated fatty acid content this processing step assured that an insignificant amount of saturated triglycerides would be present in this fat. The hydrogenated linseed oil had an iodine value of 3. The fatty acid composition of this fat was 97% of saturated fatty acids and 3% of oleic acid. Since linseed oil consists almost entirely of fatty acids containing 18 carbon atoms, the saturated fatty acids originally present and those formed as a result of hydrogenation will consist almost entirely of stearic acid. This is a convenient method for preparing essentially pure tristearin.

These two fats were mixed together so that a series containing 5, 10, 20, 40, and 70% of the hydrogenated linseed oil in the randomly rearranged safflower seed oil was obtained. Each of these mixtures was divided into two parts. One part was rearranged randomly while the other was used as the simple mixture. The fatty acid composition of the final mixtures was determined by analysis. The values obtained were in agreement with those calculated from the proportions of the two fats used in the preparation of these mixtures.

The tristearin content of each fat was calculated as follows: the original safflower seed oil, since it has been rearranged randomly, could contain only a fraction of a per cent of saturated triglycerides. The 3% of oleic acid in the hydrogenated linseed oil was assumed to be distributed maximally. Thus, this fat was considered to consist of 91% of tristearin. For the randomly rearranged mixtures, the saturated triglyceride values were obtained by a calculation according to random distribution. The values for the simple mixes are based on the amount of hydrogenated linseed oil they contained and the tristearin content of this fat.

Calculation of the disaturated-monounsaturated triglyceride contents was as follows: as in the case of the calculation of the tristearin content, the saturated acids in the randomly rearranged safflower seed oil were considered to be randomly distributed, while the unsaturated acids in the hydrogenated linseed were assumed to be distributed maximally. The content of disaturated-monounsaturated triglycerides in the simple mixtures of fat was calculated from these bases. The disaturated-monounsaturated triglyceride content of the randomly rearranged mixes was obtained by calculation based on random distribution of the fatty acids.

These fats were incorporated into a semi-purified diet, the composition of which was casein, 27%; sucrose, 50%; salt mix U.S.P. XIV, 5%; Cellu Flour, 3%; and fat, 15%. The diet also contained all of the necessary water soluble vitamins. The animals were supplied orally once each week with the necessary fat-soluble vitamins.

Seventy-two young adult male rats were distributed according to body weight into 12 groups of 6 animals each. The animals were housed in individual cages with raised screen bottoms and fed their respective diets ad libitum. One week was allowed for orientation to the diets. During the succeeding 10 days, the food consumption of each animal was recorded and the feces of each animal were collected separately. The feces were dried, ground in a Wiley mill so as to pass a 60-mesh screen, saponified with alcoholic KOH, acidified, and the weight of the fatty acids extracted with petroleum ether was determined.

RESULTS AND DISCUSSION

The experimental values obtained and the coefficient of absorbability of each of the dietary fats are given in table 1. In figure 1 the relationship between the stearic acid content of the fats and their coefficients of absorbability is given. In figure 2 the relationship between the tristearin content of the fats and their coefficients of absorbability is given.

From figure 1 it can be seen that the coefficients of absorbability of the randomly rearranged fats were essentially the same until the stearic acid content reached 35% and only then did they decrease. Since these were randomly rearranged fats, this is about the lowest level of saturated fatty acids at which saturated triglycerides would be present in significant

TABLE 1

Proportion of dietary fatty acids absorbed by rats fed diets containing fats which varied in their content of stearic acid and simple and mixed triglycerides into which the stearic acid was incorporated

	Characteristics of diet fat											
Hydrogenated linseed oil, %	0	5	5	10	10	20	20	40	40	70	70	100
Safflower seed oil, %	100	95	95	90	90	80	80	60	60	30	30	0
Preparations of fat[1]	RR	Mix	RR	Mix	RR	Mix	RR	Mix	RR	Mix	RR	—
Stearic acid, %	13	16	16	20	22	30	28	45	46	72	71	97
Tristearin, %	0	4	0	9	1	18	2	36	9	63	36	91
Distearin monounsaturated glycerides, %	4	9	6	8	11	7	17	5	34	3	44	9
	Animal data[2]											
Food eaten, *gm*	12.4	13.3	14.3	13.5	12.3	12.9	13.4	15.0	12.1	16.9	12.8	17.0
Total fatty acids eaten, *gm*	1.79	1.92	2.07	1.95	1.78	1.87	1.94	2.17	1.75	2.44	1.85	2.46
Feces egested, *gm*	0.618	0.767	0.719	0.891	0.698	1.05	0.799	1.51	0.789	2.26	1.53	3.25
Total fatty acids in feces, %	10.2	16.0	12.1	22.1	12.9	32.1	16.5	38.4	28.9	53.9	44.1	63.8
Total fatty acids egested, *gm*	0.063	0.123	0.087	0.197	0.090	0.337	0.132	0.580	0.228	1.22	0.68	2.08
Diet total fatty acids absorbed, *gm*	1.73	1.80	1.98	1.75	1.69	1.53	1.81	1.59	1.52	1.22	1.17	0.38
Coefficient of absorbability, %	96.7	93.8	95.7	89.8	95.0	81.8	93.3	73.2	86.8	50.0	63.8	15.4
Standard error of the mean, ±	0.5	0.3	0.3	0.3	0.3	0.9	0.3	0.8	1.0	1.3	2.2	4.0

[1] RR = fat mixture randomly rearranged; Mix = simple mixture of fats.

[2] Average values per rat per day.

amounts. The further decrease in assimilation in the randomly rearranged mixes containing larger amounts of stearic acid can be attributed to the tristearin that was present.

Figure 1 also shows that in the case of the simple mixes the coefficients of absorbability decreased linearly with increasing amounts of stearic acid. Since this increase in saturated fatty acids was obtained by adding tristearin, this decrease in assimilation again was probably due to the tristearin rather than the stearic acid content.

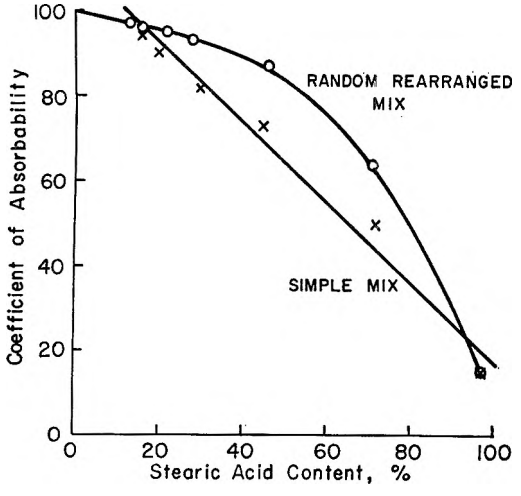

Fig. 1 The relationship between the stearic acid content of a fat and its coefficient of absorbability.

Figure 2 shows that there was essentially an inverse linear relationship between the assimilation of a fat and the amount of tristearin that was present. All of these observations then lead to the conclusion that the coefficient of absorbability of these fats was determined by their tristearin content. The level of stearic acid influenced absorption only insofar as it contributed to the content of tristearin.

These conclusions are probably equally applicable to saturated triglycerides made up of fatty acids containing more than 18 carbon atoms, since there is no reason to presuppose that such fats would be better absorbed. However, the conclusions are not necessarily applicable when the chain length of the fatty acid is less than 18 carbon atoms. Tripalmitin, which is absorbed to a considerably greater extent than tristearin (Hoagland and Snider, '43b), may follow this same pattern. However, trimyristin and trilaurin are almost completely absorbed (Cheng et al., '49) and therefore may not follow this pattern.

Examination of the data in table 1 shows that little or no correlation can be seen between the distearin-monounsaturated triglyceride content of a fat and its coefficient of absorbability. Since any decrease in utilization of a fat can be accounted for almost entirely by a corresponding increase in tristearin content, it appears that the stearic acid of the distearin-monounsaturated glycerides is essentially completely absorbed. This is particularly striking in the case of the randomly rearranged 40/60 mixture of hydrogenated linseed oil and safflower seed oil. Forty-six per cent of the fatty acids of this fat was stearic acid. Yet, only 13% of this fat was not absorbed. Nine per cent of that which was not absorbed can be attributed to the tristearin. Thus, if one does not consider the tristearin which was present, 96% of this fat was absorbed—essentially the same figure that was obtained when safflower seed oil alone was fed. This high absorbability was obtained on a fat which contained 34% of distearin-monounsaturated glycerides. Thus

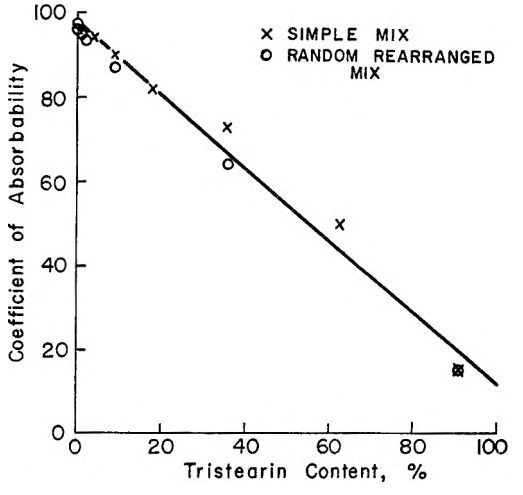

Fig. 2 The relationship between the tristearin content of a fat and its coefficient of absorbability.

stearic acid, when present as a disaturated-monounsaturated glyceride, is essentially completely absorbed. Such glycerides of stearic acid have melting points of 38° and 43°C which are above body temperature but below 50°C which Deuel (Cheng et al., '49) considered to be the critical temperature in determining whether a fat was well utilized.

On the same basis it is apparent that the stearic acid of a monostearin-diunsaturated glyceride is well absorbed also.

SUMMARY

Rats were fed a series of fats in which the level of stearic acid and the distribution of the stearic acid among simple and mixed triglycerides were varied. The coefficients of absorbability of these fats were determined. From the results obtained it is shown that the stearic acid of tristearin is not absorbed. On the other hand, the stearic acid of distearin-monounsaturated or monostearin-diunsaturated triglycerides is almost completely absorbed.

These results show that the theory which holds that the coefficient of absorbability of a fat is a function of its content of saturated fatty acids containing 18 or more carbon atoms is not tenable.

Since natural fats do not have a sharp melting point but melt over a temperature range, it is not reasonable to continue to use melting point as a parameter in fat utilization studies.

It is concluded that the coefficient of absorbability of a fat is inversely proportional to its content of simple triglycerides made up of saturated fatty acids having a chain length of 18 carbon atoms or greater and is influenced by the level of such saturated fatty acids only insofar as they are present as saturated triglycerides.

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All-Vegetable Protein Mixtures for Human Feeding

I. USE OF RATS AND BABY CHICKS FOR EVALUATING CORN-BASED VEGETABLE MIXTURES[1]

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Underdeveloped areas of the world where malnutrition is prevalent may themselves contain potential resources for the prevention of human nutritional disease. It is generally accepted, however, that diets compounded from indigenous sources, which are little known or completely unknown, must be thoroughly tested in animals before human feeding is attempted. The results of animal studies are used to assess possible toxicity as well as the nutritional quality of the food or diet as measured by growth, nutrient utilization and pathology of tissues.

Scrimshaw et al. ('57) have presented data on a corn-based, simplified all-vegetable protein mixture developed for the prevention of protein malnutrition in Central American children. The present paper describes, in part, the biological testing in rats and baby chicks of various food ingredients and combinations which preceded the trials with children.

MATERIALS AND METHODS

Diets and ingredients. The selection of the ingredients and the basic proportions used in these studies were based on observations over a 7-year period during which all of the ingredients had been fed in various combinations in the laboratories of the Instituto Agropecuario Nacional (IAN), Guatemala, to rats, chicks, laying hens and swine. No evidence of toxicity was observed from growth, reproduction or autopsy records (Squibb et al., '50, '51, '52, '53, '58).

Chemical data and detailed specifications for the sesame flour, the cottonseed flour and the other ingredients of the basic formula have been given by Scrimshaw et al. ('57). The crude sesame oil meal and the cottonseed oil meal were protein concentrates produced locally by the screw-press process. Because of its high protein and excellent carotene content (Squibb et al., '53), kikuyu leaf meal was used as a source of vitamin A activity in all of the biological trials. The diets were formulated to contain approximately 25% of crude protein, since this was the protein level desired in a product for infant and child feeding. The essential amino acid content of the final formula was determined microbiologically using *Streptococcus faecalis* for threonine and *Leuconostoc mesenteroides* for arginine and histidine with media of Steele and coworkers ('49); *Leuconostoc mesenteroides* and Difco[4] media were used for the remaining amino acid assays.

Experimental animals. Four independent trials were carried out with weanling white rats of the IAN Wistar strain colony, which were housed at 74°F in individual all-wire cages with raised screen bottoms. Three independent trials were carried out with three-day-old, straight-run baby New Hampshire chicks. Further details on test treatment, distribution of experimental animals and duration are presented with the tabulation of the results.

Water and the experimental diets for both the rat and baby chick were offered ad libitum and all animals were weighed individually each week. Feed consumption records were kept individually for the rats

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and as a group for all the chick experiments.

RESULTS

Rat feeding experiments

Trial 1. Comparison of crude sesame oil meal and refined sesame flour. Since sesame was to contribute the largest percentage of protein in all diets to be tested, this first trial compared a local 16.5% fat sesame oil meal with a refined sesame flour suitable for human feeding which contained 33% of fat and was used in the subsequent trials. The comparison was made for a 9-week period with and without the addition of skim milk. Each group of 10 male rats was replicated three times to give 30 rats per treatment. The calculated protein content of the diets tested, final weights of the rats and the efficiencies of feed conversion expressed as grams of feed per gram of gain in weight are presented in table 1. Analysis of the data showed that there was no significant difference in the weights of the rats fed the two sesame products, with and without the addition of skim milk powder. The refined sesame product without skim milk, however, resulted in efficiency of feed utilization superior to that with the crude sesame oil meal, a difference which disappeared when skim milk was added to both types of sesame.

Trial 2. Supplementation of corn and sesame flour with cottonseed flour and two levels of dried skim milk. Cottonseed flour with a free gossypol content of 0.045% and two levels of dried skim milk were tested as supplements to the sesame flour to determine whether this would improve the nutritive value of the mixture.

Forty-eight weanling rats of the same age were distributed by sex and weight among 4 experimental groups. Group 1 received the sesame flour ration; group 2, sesame flour plus 9% of cottonseed flour; group 3, sesame flour plus 9% of skim milk; and group 4, sesame flour plus 14.3% of skim milk. The protein supplements tested were substituted for a part of the protein of the sesame flour. The rations fed, final weight and efficiencies of feed utilization of the rats are presented in table 1. Although the rats receiving 14.3% of a supplement of skim milk gained slightly more and showed a better feed efficiency, the differences in growth were not significant in this trial.

Trial 3. Supplementation of corn and sesame flour with cottonseed flour, dried skim milk and L-*lysine.* In this experiment a further attempt was made to improve the nutritional value of an all-vegetable mixture containing sesame flour as the principal source of protein by the addition

TABLE 1

Growth and feed efficiency of rats fed simplified all-vegetable protein mixtures supplemented with cottonseed flour or skim milk

Ingredient	Trial 1—9 weeks[1] Group				Trial 2—8 weeks[1] Group			
	1	2	3	4	1	2	3	4
Sesame oil meal	—	—	49.0	38.0	—	—	—	—
Sesame meal	44.0	35.0	—	—	44.0	35.0	35.0	30.8
Cottonseed flour	—	—	—	—	—	9.0	—	—
Skim milk	—	9.0	—	9.0	—	—	9.0	14.3
Lime-treated corn	46.98	46.98	41.98	43.98	46.98	46.98	46.98	45.88
Kikuyu leaf meal	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Torula yeast	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Minerals[2]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin D[3]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Crude protein, %	25	25	25	25	25	25	25	25
Number of rats	30	30	30	30	12	12	12	12
Initial weight, gm	57	57	57	57	54	54	54	54
Final weight, gm	274	272	238	274	250	238	237	264
Feed efficiency[4]	3.59	3.94	4.92	4.10	2.58	2.55	2.55	2.50

[1] F test = no significance.
[2] Squibb and Wyld ('52).
[3] Delsterol.
[4] Grams of feed per gram of gain.

of cottonseed flour and dried skim milk, with and without L-lysine.

Seventy-two weanling rats of the same age were allotted on the basis of weight and sex to 6 experimental groups. Groups 1 and 2, 3 and 4, and 5 and 6 were designed to compare the effect of a lysine supplement without cottonseed flour, with a cottonseed flour supplement, and with the addition of skim milk respectively. The rations used, growth of the rats and the efficiencies of feed utilization are presented in table 2. The data show that there were no significant differences in the growth of the rats in the 6 experimental groups. While cottonseed flour, skim milk and L-lysine supplementation did not improve the growth rate of the rats, in each case the addition of L-lysine improved the efficiency of feed utilization. The lack of difference in growth upon lysine addition was due to the high protein percentage of the diet and the fact that the experiment was continued beyond the usual 4-week period of rapid growth response.

Trial 4. Effect of L-*lysine and* DL-*valine in an all-vegetable protein mixture containing sesame and cottonseed flour.* From the results and the apparent deficiency of lysine and valine in the amino acid pattern of the vegetable mixture of the previous experiment compared with that of milk, the effect of lysine and valine addition was tested at a 15% level of protein in the diet.

Thirty weanling rats of the same age and sex were distributed by weight among three experimental groups. The rations fed, gains in weight of the rats and their efficiencies of feed utilization are presented in table 2. Although DL-valine appeared to depress the rate of growth of the rats, the effect was not significant at the 5% level. At this 15% level of protein in the diet, the addition of lysine significantly increased the growth rate of the rats and improved the efficiency of feed utilization.

Chick feeding trials

Trial 1. Effect of the lime treatment of corn on chick growth. In this experiment a lime-treated corn, similar to that consumed by humans, was compared to raw corn, with and without the addition of B complex vitamins. Forty-eight chicks were distributed by weight among 4 ex-

TABLE 2

Growth and feed efficiency of rats fed simplified all-vegetable protein mixtures supplemented with L-*lysine and* DL-*valine*

Ingredients	Trial 3—8 weeks[1] Group						Trial 4—5 weeks[2] Group		
	1	2	3	4	5	6	1	2	3
Sesame meal	44.0	44.0	35.0	35.0	35.0	35.0	21.0	21.0	21.0
Cottonseed flour	—	—	9.0	9.0	—	—	5.4	5.4	5.4
Skim milk	—	—	—	—	9.0	9.0	—	—	—
Lime-treated corn	46.98	46.53	46.98	46.53	46.98	46.53	30.18	30.18	30.18
Dextrin	—	—	—	—	—	—	36.8	36.0	36.4
Kikuyu leaf meal	3.0	3.0	3.0	3.0	3.0	3.0	1.8	1.8	1.8
Torula yeast	3.0	3.0	3.0	3.0	3.0	3.0	1.8	1.8	1.8
L-lysine	—	0.45	—	0.45	—	0.45	—	—	0.4
DL-valine	—	—	—	—	—	—	—	0.8	—
Minerals[3]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin D[4]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Crude protein, %	25	25	25	25	25	25	15	15	15
Number of rats	12	12	12	12	12	12	10	10	10
Initial weight, *gm*	44	44	44	44	44	44	49	49	49
Final weight, *gm*	233	232	223	230	236	235	179	169	215
Feed efficiency[5]	2.08	1.90	2.71	2.14	2.29	1.98	3.57	3.68	2.77

[1] F test = no significance.
[2] Lysine effect, P = < 0.01.
[3] Squibb and Wyld ('52).
[4] Delsterol.
[5] Grams of feed per gram of gain.

TABLE 3

Growth and feed efficiency of New Hampshire chicks fed simplified all-vegetable protein mixtures supplemented with gelatin, arginine and L-lysine

Ingredient	Trial 1—6 weeks[1] Group				Trial 2—6 weeks[2] Group						
	1	2	3	4	1	2	3	4	5	6	7
Sesame oil meal	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Cottonseed flour	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Kikuyu leaf meal	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Torula yeast	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Lime-treated corn	46.7	—	46.7	—	46.7	—	45.7	45.7	46.25	44.25	—
Ground raw corn	—	46.7	—	46.7	—	46.7	—	—	—	—	44.25
Corn cob meal	—	—	—	—	—	—	—	—	—	—	—
Minerals[4]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Cod liver oil[5]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
B-complex vitamins[4]	—	—	+	+	+	+	+	+	+	+	+
Gelatin	—	—	—	—	—	—	1.0	—	—	1.0	1.0
Arginine	—	—	—	—	—	—	—	1.0	—	1.0	1.0
L-lysine	—	—	—	—	—	—	—	—	0.45	0.45	0.45
Protein, %	25	25	25	25	25	25	25	25	25	25	25
Number of chicks	12	12	12	12	12	12	12	12	12	12	12
Initial weight, *gm*	39	39	39	39	39	39	39	39	39	39	39
Final weight, *gm*	181	255	202	282	221	272	200	292	368	348	352
Feed efficiency[6]	3.16	2.70	3.10	2.88	2.95	2.75	3.74	2.50	2.49	2.59	2.80

TABLE 3 (*continued*)

Ingredient	Trial 3--5 weeks[3] Group							
	1	2	3	4	5	6	7	8
Sesame oil meal	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Cottonseed flour	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Kikuyu leaf meal	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Torula yeast	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Lime-treated corn	46.7	—	44.2	41.7	39.2	44.5	46.3	46.1
Ground raw corn	—	46.7	—	—	—	—	—	—
Corn cob meal	—	—	2.5	5.0	7.5	—	—	—
Minerals[4]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Cod liver oil[5]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
B-complex vitamins[4]	+	+	+	+	+	+	+	+
Gelatin	—	—	—	—	—	—	—	—
Arginine	—	—	—	—	—	—	—	—
L-lysine	—	—	—	—	—	0.2	0.4	0.6
Protein, %	25	25	25	25	25	25	25	25
Number of chicks	12	12	12	12	12	12	12	12
Initial weight, *gm*	43	43	43	43	43	43	43	43
Final weight, *gm*	204	232	228	236	233	306	380	374
Feed efficiency[6]	2.76	2.68	2.54	2.85	2.74	2.24	2.18	2.15

[1] F = 6.37, least significant difference 1% = 43, 5% = 28.
[2] F = 7.38, LSD 1% = 98, 5% = 74.
[3] F = 2.90, LSD 1% = 46, 5% = 35.
[4] Squibb and Wyld ('52).
[5] Contains 1800 I.U. vitamin A per gram.
[6] Grams of feed per gram of gain.

perimental groups. The rations fed, growth and efficiency of feed utilization of the chicks are presented in table 3. As may be seen from the table, raw corn resulted in significantly greater growth and efficiency of feed utilization than lime-treated corn, whether B vitamins were added or not. The greater growth which resulted from the addition of B complex vitamins was not statistically significant.

Trial 2. Effect of gelatin, arginine and lysine in the all-vegetable protein mixture. In this experiment the value of adding gelatin, arginine and lysine to a sesame flour-cottonseed flour-lime treated corn or raw ground corn combination for chicks was determined. Gelatin was tested because it is a good source of arginine for the chick and it was thought that the lower growth obtained with the lime-treated corn might be due to the loss of 18% of arginine taking place as a result of the lime treatment (Bressani and Scrimshaw, '58). Eighty-four chicks were distributed among 7 experimental groups. In group 2, raw corn replaced the lime-treated corn of the control group 1; in groups 3, 4 and 5 gelatin, arginine and lysine, respectively, were added singly to the ration, while in groups 6 and 7 these three supplements were added in combination with either raw or lime-treated corn.

The rations fed and the growth and feed efficiency of the chicks are presented in table 3. The chicks fed the lime-treated corn again failed to grow as well as those receiving the raw corn. While the addition of gelatin tended to depress the growth rate, arginine improved growth. None of these differences, however, reached the 5% level of significance, although the increase in growth resulting from the addition of L-lysine was highly significant. The chicks fed a combination of the two amino acids and gelatin with lime-treated or raw corn also grew at the same rate as the group fed the lysine supplement alone and significantly faster than the other groups.

Better efficiencies of feed utilization were consistently observed with the diets containing raw corn than those with lime-treated corn. The supplementation of the lime-treated corn diets with either L-lysine or arginine improved the efficiencies of feed utilization. The simultaneous addition of gelatin, L-lysine and arginine to either lime-treated corn or raw corn diets resulted in a better efficiency of feed utilization only for the lime-treated corn ration.

Trial 3. Supplementation of lime-treated corn diets with corn cob meal and L-lysine. The results of chick trials 1 and 2 indicated that the sesame flour-cottonseed flour-lime treated corn combination was lysine-deficient for chicks and that the use of lime-treated corn resulted in lower growth rates. In an attempt to improve digestion and absorption through a purely mechanical effect, corn cob meal was given at three levels to three of 8 groups whose diets contained lime-treated corn. In three groups the supplemental value of L-lysine was tested at 0.2, 0.3 and 0.4% of the diet.

The composition of the diets, number of chicks per group, the weights of the chicks and efficiencies of feed utilization are presented in table 3. The lime-treated corn again produced lower growth of the chicks although the differences were not statistically significant. The addition of the ground corn cob to the lime-treated corn diets apparently improved growth of the chicks to values similar to those obtained with raw ground corn. At all levels the addition of L-lysine produced significantly greater growth and efficiency of feed utilization, but was most efficient at the 0.4% level. This suggested that, rather than any direct nutritional effect, the physical characteristics of the diets containing raw corn were responsible for their superiority for chicks over diets containing lime-treated corn.

Amino acid composition of final formula. On the basis of these experiments and previous laboratory data, a final formula designated INCAP Vegetable Mixture 8 was adopted for human trials which consisted in per cent of dry corn masa, 50; sesame flour, 35; cottonseed flour, 9; kikuyu leaf meal, 3; and torula yeast, 3. The essential amino acid composition of this formula is shown in table 4, together with the amino acid patterns of milk (Orr and Watt, '57) and the FAO Reference Protein (FAO, '55). Comparison of the patterns suggests that the

TABLE 4

Amino acid content of INCAP Vegetable Mixture 8, milk and the FAO reference protein

Amino acid	Vegetable mixture 8		Milk	FAO reference protein
	gm%	*mg/amino acid/ gm nitrogen*	*mg/amino acid/ gm nitrogen*	*mg/gm nitrogen*
Arginine	2.15	537	233	—
Histidine	0.91	227	168	—
Isoleucine	1.26	315	407	270
Leucine	2.24	560	626	306
Lysine	1.12	280	496	270
Methionine[1]	0.69	173	213	270
Phenylalanine	1.50	375	309	180
Threonine	0.80	200	294	180
Tryptophan	0.21	53	90	90
Valine	1.18	295	438	270
Nitrogen	4.00	—	—	—

[1] Methionine plus cystine.

INCAP Vegetable Mixture 8 is limiting in lysine and that tryptophan is the second most limiting amino acid. Nevertheless, the protein score of the mixture is around 67%. The vitamin and mineral content of the formula has already been published (Scrimshaw et al., '57).

DISCUSSION

These biological studies of corn-based all-vegetable protein mixtures are of special interest since they resulted in the designation of a formula, INCAP Vegetable Mixture 8, which was subsequently employed with success in feeding trials with normal children and in the experimental treatment of young children with severe protein malnutrition (kwashiorkor) (Scrimshaw et al., '57; Béhar et al., '58).

Evaluation of the different feed combinations indicated that cottonseed meal definitely improved the biological value of the corn-sesame mixtures, presumably by increasing the proportion of lysine per gram of protein. The addition of free lysine to the corn-sesame-cottonseed mixture increased further the efficiency of feed utilization by rats and chicks. Lysine did not, however, improve the growth of rats fed the different rations until the total crude protein content was reduced to 15%. The response at 15% of protein in the diet confirms the analytical evidence that when compared with either milk or the FAO Reference Protein, the most limiting amino acid in the mixture is lysine. Furthermore, the growth of the baby chicks was improved with the addition of free lysine at the 25% level of protein intake. This difference was probably due to a higher absolute lysine requirement in the chicks fed the diets tested.

When lime-treated corn was substituted for raw corn, the growth rates of baby chicks were poorer. This was the reverse of the increased growth of rats fed lime-treated instead of raw corn as part of low tryptophan niacin-deficient diets (Laguna and Carpenter, '51; Cravioto et al., '52; Squibb et al., '59). As the trials in which the addition of crude fiber in the form of corn cob meal suggest, this may be due to poorer physical characteristics for poultry feeding of mixtures containing lime-treated corn.

The results of the biological trials in rats fed the INCAP Vegetable Mixture 8 formula at the 25% level intended for human consumption were excellent and were not significantly improved by the substitution of dried skim milk for the cottonseed, or even by the addition of lysine. Similarly, the results in the chick were very satisfactory, although the addition of lysine brought about some further improvement; the computation of protein score, on the basis of analytical data, indicated that the mixture is within the lower range of protein of animal origin.

Since this mixture was based on materials easily produced in Central America and potentially low in cost, its testing in the feeding of young children appeared

indicated. Analysis showed that the formula selected contained less than 0.0017 gm % of free gossypol,[5] a negligible level (Altschul, '58). While the biological data cited may not be entirely comparable to future human performance, it was reasonable to assume that if the nutritional requirements of fast-growing animal species were met by this formula, those of the slower-growing child would also be likely to be satisfied. Furthermore, the use of both the rat and the chick helped to reduce the possibility of important species differences giving misleading results.

Knowledge that none of the experiments reported in this paper or run previously in this laboratory had given any indication of toxicity of any of the ingredients of this mixture, either singly or in combination, was a necessary preliminary to clinical trials. As will be reported in a subsequent paper in this series, the thorough biochemical and biological testing to which the mixture was subjected resulted in a product of highly satisfactory nutritional characteristics for the feeding of infants and young children.

SUMMARY

Corn-based, simplified all-vegetable protein mixtures containing corn, sesame, cottonseed, torula yeast and green leaf meal were evaluated by amino acid analysis and by growth and feed efficiency studies using both rats and baby chicks. The data indicated that a combination designated as INCAP Vegetable Mixture 8, composed in per cent of lime-treated corn, 50; sesame flour, 35; cottonseed flour, 9; kikuyu leaf meal, 3; and torula yeast, 3, was palatable and gave good growth and efficiency of feed utilization in rat trials. Neither the addition of 0.45% of free lysine nor the substitution of skim milk for part of the corn improved the growth or feed efficiency of rats fed the mixture, although addition of lysine did improve growth and feed utilization of the mixture by chicks. When the mixture was diluted with cornstarch to feed rats at a 15% protein level, added lysine improved growth and feed efficiency. On the basis of the studies, INCAP Vegetable Mixture 8 was recommended for clinical feeding trials in children.

[5] Result of analysis obtained through the courtesy of Dr. A. Altschul, Southern Regional Research Laboratories, New Orleans, Louisiana.

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All-Vegetable Protein Mixtures for Human Feeding

II. THE NUTRITIVE VALUE OF CORN, SORGHUM, RICE AND BUCKWHEAT SUBSTITUTED FOR LIME-TREATED CORN IN INCAP VEGETABLE MIXTURE EIGHT[1]

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The first paper in this series described biological trials in chicks and rats leading to the development of an all-vegetable protein mixture for the supplementary and mixed feeding of young children (Squibb et al., '59b). The formula recommended for clinical trials was designated INCAP Vegetable Mixture 8 and contained corn masa, sesame flour, cottonseed flour, torula yeast and kikuyu leaf meal. Simultaneously with studies in children, additional experiments using this mixture were carried out with chicks, in which the lime-treated corn in the original formula was replaced by raw corn, sorghum, rice or buckwheat.

Although corn is the most important staple food consumed by the rural population in Central America, its production is far below the level needed for human and animal consumption. For this reason other cereal grains are becoming important as substitutes for corn. Grain sorghum is of particular interest since it produces well under environmental conditions which are too dry for high yields of corn (Hillier et al., '54; Pond et al., '58).

Even though rice is not an agricultural product found in surplus quantities in Central America, it is very important in Panama (Sogandares and de Barrios, '55; Sogandares et al., '55) where it replaces corn in most human diets. In this study, rice and grain sorghum are compared with corn in feeding experiments with chicks, as components in all-vegetable protein diets.

Wyld et al. ('58) have recently reported on the nutritive value of buckwheat as a possible component of vegetable protein mixtures. They found that buckwheat was a useful component of the ration used in their chick experiments because it contributed significant amounts of lysine, the amino acid most limiting for chick growth in diets based on sesame meal and cottonseed oil meal. In the present experiments buckwheat was studied to obtain further information on its possible value in areas where protein is in short supply.

MATERIALS AND METHODS

Four-day-old New Hampshire chicks of both sexes were distributed by weight and confined in battery brooders. Temperature was thermostatically controlled as required by the age of the birds. Feed and water were provided ad libitum. The chicks were weighed individually every week for a total of 35 days and weekly records were kept of group diet consumption.

Although the raw materials for the rations used are, with the exception of torula yeast, currently produced in Central America, the sesame and cottonseed flours were imported because these are not as yet locally processed in a form suitable for human consumption. The basic chemical composition including the lysine content of the major constituents of the diets is shown in table 1. The percentage composition of the basal diet, INCAP Vegetable Mixture 8 (Scrimshaw et al., '57; Béhar et al., '58; Squibb et al., '59b), was as follows: sesame flour, 35; cottonseed flour, 9; torula yeast, 3; kikuyu leaf meal, 3; corn masa flour, 50. Each cereal grain tested was substituted completely for 50% of the corn masa flour of the basal diet.

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TABLE 1

Proximate composition of major diet constituents

Ingredient	Moisture	Protein	Ether extract	Crude fiber	Nitrogen-free extract[1]	Ash	Lysine
	%	%	%	%	%	%	%
Sesame meal[2]	7.2	46.3	19.6	7.9	13.4	5.6	0.43
Cottonseed flour[3]	7.4	53.2	5.5	3.0	24.5	6.4	2.10
Torula yeast[4]	6.7	48.3	2.5	2.4	29.9	7.8	3.80
Dehydrated kikuyu leaf meal	6.1	20.7	3.8	24.3	34.2	10.9	0.58
Masa flour	7.5	10.6	3.7	1.6	73.6	3.0	0.26
Yellow corn	13.9	9.2	3.5	2.6	69.6	1.2	0.26
Sorghum	14.2	10.2	2.7	4.0	67.9	1.0	0.29
Rice	17.0	8.0	0.2	0.7	73.8	0.3	0.30
Buckwheat	14.0	9.2	2.4	13.4	53.0	8.4	0.54

[1] Calculated by difference.
[2] American Sesame Products, Inc. Paris, Texas.
[3] Traders Oil Mill Co., Fort Worth, Texas.
[4] Lake States Yeast Corp., Rhinelander, Wisconsin.

TABLE 2

Growth response of chicks to various cereals in all-vegetable protein mixtures

Cereal	Crude protein in diet	Number of chicks initial/ final	Initial weight	Final weight[1]	Feed conversion[2]	Protein efficiency[3]
	%		*gm*	*gm*		
			Experiment 1			
Basal diet (INCAP Vegetable Mixture 8)	21.3	12/12	49	223 ± 31.8	2.68	1.75
Yellow corn	20.8	12/10	49	264 ± 57.5	2.60	1.85
Sorghum	21.1	12/10	49	196 ± 36.1	2.89	1.64
Rice	20.3	12/12	49	237 ± 35.5	2.55	1.93
Buckwheat	20.8	12/12	49	332 ± 34.8[4]	2.40	2.00
			Experiment 2			
Basal diet (INCAP Vegetable Mixture 8)	21.3	12/12	54	240 ± 38.3	2.50	1.88
Yellow corn	20.8	12/12	54	262 ± 57.2	2.74	1.76
Sorghum	21.1	12/12	54	247 ± 47.5	2.92	1.62
Rice	20.3	12/11	54	260 ± 30.7	2.75	1.79
Buckwheat	20.8	12/12	54	341 ± 33.1[4]	2.60	1.85

[1] Final weight ± standard deviation.
[2] Grams of feed per gram of weight gained.
[3] Grams of weight gained per gram of protein consumed.
[4] Highly significant; $P = < 0.01$.

The term "masa" is given to the product obtained by cooking corn in a lime solution and subsequently drying and grinding it (Bressani et al., '58; Bressani and Scrimshaw, '58).

In the first and second feeding trials, the basal diet and the experimental diets containing whole ground yellow corn, whole ground sorghum, ground polished rice, or whole ground buckwheat were fed to groups of 12 birds. The complete diets were diluted with cornstarch to 75% of their original value to give protein concentrations of approximately 21%. In the third trial, 24 chicks per ration were used and the complete diets were diluted with cornstarch to 80% to give a protein concentration of about 22%. The experimental rations used in trial 3 were supplemented with 0.4% L-lysine hydrochloride. One group received a complete chick stock ration[2] containing 22.1% protein.

[2] "Ace-Hi," manufactured by Compañía Riverside, Guatemala.

All of the rations tested contained 3% of a mineral supplement,[3] 0.3% of cod liver oil,[4] and 1 ml of a vitamin solution to provide the following in milligrams per 100 gm of ration: thiamine hydrochloride, 2; riboflavin, 2; niacin, 10; inositol, 10; choline chloride, 160; vitamin K, 5; *p*-aminobenzoic acid, 10; pyridoxine hydrochloride, 2; calcium pantothenate, 6; biotin, 0.04, and vitamin B_{12}, 0.003.

RESULTS

Table 2 summarizes the first and second trials by listing the protein content of the rations, the initial and final weights of the chicks, and the feed conversion and protein efficiency values. From these results, it is evident that buckwheat resulted in better growth, feed conversion and protein efficiency than any of the other cereal grains substituted for part of the corn. Yellow corn and rice were slightly more effective than sorghum and the basal diet of INCAP Vegetable Mixture 8. Feed conversion values for the basal diet were, however, somewhat better than those obtained when yellow corn and rice were utilized. The feed conversion and protein efficiency values of the rations containing sorghum were the lowest. The protein efficiency values of the basal diet, yellow corn and rice were essentially equal.

The results of the third trial are shown in table 3. From these it is evident that when all of the cereal combinations were supplemented with lysine, rice substitution produced the best growth response, feed conversion and protein efficiency. The differences observed were not, however, statistically significant. The growth, feed conversion and protein efficiency values of all the experimental rations were improved by supplementation with 0.4% of lysine when compared with the results of experiments 1 and 2. They also gave better results than the stock ration which contained the recommended amount of animal protein for growing chicks.

DISCUSSION

In the rations used in this study, as in those of Wyld et al. ('58), Squibb and Braham ('55) and Squibb et al. ('59b), lysine was the most limiting amino acid. When the vegetable protein mixtures were supplemented with this amino acid alone, marked gains were observed and the apparent differences among the cereal grains tested disappeared. This is evidence that the formulas tested do not contain sufficient lysine for good growth in chicks although they may still prove useful for the feeding of children. In fact, a preliminary report indicating good results from the feeding of the basal ration, INCAP Vegetable Mixture 8, to young children has already appeared (Scrimshaw et al., '57), and the next paper in this series will de-

[3] Mineral supplement of 33% bone meal, 33% calcium carbonate, 33% iodized salt, and 1% minor elements.

[4] Obtained through the courtesy of Mead Johnson & Co., Evansville, Indiana.

TABLE 3

Growth response of chicks to various cereals in all-vegetable protein mixtures[1]

Experiment 3

Cereals	Crude protein in diet	Number of chicks initial/final	Initial weight	Final weight	Feed conversion[2]	Protein efficiency[3]
	%		*gm*	*gm*		
Basal diet (INCAP Vegetable Mixture 8)	23.1	24/24	52	503 ± 46.4	1.97	2.20
Yellow corn	22.5	24/23	52	537 ± 65.0	1.90	2.33
Sorghum	22.9	24/24	52	524 ± 70.1	1.92	2.27
Rice	22.1	24/24	52	546 ± 56.7	1.82	2.48
Control diet[4]	22.1	24/24	52	520 ± 51.6	2.10	2.16

[1] Supplemented with 0.4% lysine.

[2] Grams of feed per gram of weight gained.

[3] Grams of weight gained per gram of protein consumed.

[4] Ace-Hi. Compañía Riverside, Guatemala.

scribe in detail the supporting clinical data.

It has been shown by several workers (Laguna and Carpenter, '51; Cravioto et al., '52; Squibb et al., '59a) that rats fed raw corn do not gain as much weight as rats fed lime-treated corn. Nevertheless, in chicks the basal diet which contained lime-treated corn produced consistently less growth than when either raw corn or rice was substituted. This has been attributed to differences in the physical suitability of the diets for chick feeding (Squibb et al., '59b). When the cereal grains were not supplemented with lysine, however, slightly better feed conversion values were obtained with the diet containing masa flour than with those containing ground yellow corn, ground sorghum or rice. Furthermore, protein efficiencies of the diets containing masa flour and raw corn were similar. An alternate explanation may lie in the altered dietary amino acid proportions since during the preparation of masa from corn, 18.7% of the arginine, 11.7% of the histidine, 21% of the leucine and 12.5% of the cystine are lost, as well as lower percentages of other amino acids (Bressani and Scrimshaw, '58).

It is significant that grain sorghum is not as effective as corn in promoting growth of chicks, although both their chemical composition and amino acid content are very similar. It would be of practical interest to investigate whether this is the result of poorer amino acid availability in sorghum, a less favorable amino acid pattern, or lower digestibility.

The results obtained in this series of experiments indicate that any of the 4 cereals tested, ground yellow corn, buckwheat, sorghum or rice, could be substituted for all or part of the masa flour (from lime-treated corn) in INCAP Vegetable Mixture 8 if economic and agricultural factors make this desirable. They also provide further evidence that the basic formula contains sufficiently good protein to produce excellent growth in rats and satisfactory growth in chicks, although the latter is improved when lysine is added.

SUMMARY

Baby New Hampshire chicks were used to measure the nutritive value of ground yellow corn, grain sorghum, rice, and whole buckwheat substituted for masa flour (from lime-treated corn) in INCAP Vegetable Mixture 8, a formula designed for the supplementary and mixed feeding of infants and young children and containing corn masa flour, sesame flour, cottonseed flour, torula yeast and kikuyu leaf meal. In two experiments, buckwheat produced significantly better growth and feed conversion than any of the cereal grains. Yellow corn gave the next best growth response, followed by rice and sorghum. Substitution of each of the cereal grains resulted in better growth than with masa flour, though the masa flour produced better feed conversions in most cases. In a third experiment in which all rations were supplemented with 0.4% lysine, equally excellent growth and feed conversion as well as protein efficiency values were obtained with all of the diets tested. These were equal or superior to those obtained with a complete stock ration for growing chicks which contained animal protein. The results obtained in this series of experiments indicate that any of the 4 cereals tested, ground yellow corn, buckwheat, sorghum or rice, could be substituted for all or part of the masa flour (from lime-treated corn) in INCAP Vegetable Mixture 8 if factors of economy and agriculture make this desirable.

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Relation of Dietary Fat and Supplementary Riboflavin to Tissue Levels of Cholesterol, Riboflavin and Total Lipids in the Rat[1,2]

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Several investigators (Gofman et al., '50; Keys et al., '50; Hildreth et al., '51a, b; Keys, '52) have reported a correlation between total fat intake and level of cholesterol in serum. Since the requirement for riboflavin in the diet of experimental animals is influenced by the amount of fat in the diet (Mannering et al., '41, '44; Shaw and Phillips, '41; Czaczkes and Guggenheim, '46; Reiser and Pearson, '49; Kaunitz et al., '54), a relation between riboflavin intake and cholesterol metabolism seemed possible. The following experiment was designed to investigate the effect of varying levels of dietary fat and riboflavin on the concentration of cholesterol, riboflavin-containing coenzymes and total lipids in tissues of the rat.

EXPERIMENTAL

Two riboflavin-deficient rations, A and B, differing only in the relative proportions of fat and carbohydrate, were used. The percentage composition of ration A was: sucrose, 69; vitamin-free casein,[4] 18; salt mixture (U. S. Pharmacopoeia XIV, '50), 4; cellulose,[5] 4; and cottonseed oil,[6] 5. Ration B, with 20% fat, was prepared by adding cottonseed oil to ration A at the expense of the carbohydrate. The following weights in milligrams of water-soluble vitamins were added per kilogram of ration: thiamine·HCl, 5.0; pyridoxine·HCl, 2.5; Ca pantothenate, 20.0; niacin, 10.0; biotin, 0.1; folic acid, 2.0; vitamin B_{12}, 0.02; inositol, 100.0; *p*-aminobenzoic acid, 100.0; and choline chloride, 1300.0. Three drops of a cottonseed oil fat-soluble vitamin mixture were fed each rat once a week to provide per week: vitamin A palmitate, 1000 I.U.; viosterol, 100 I.U.; α-tocopherol, 0.8 mg; and menadione, 0.04 mg. Six days per week, 10, 30 or 100 μg of riboflavin in aqueous solution were given by mouth. The rations and vitamin supplements were stored in the refrigerator.

Fifty- to seventy-gram rats[7] were allotted according to litter and body weight to 7 groups of 8 males and 8 females per group. One group was killed immediately for analyses of cholesterol and riboflavin-containing coenzymes in serum and liver and total lipids in liver. The remaining 6 groups were assigned to the experimental diets so that one group on each level of fat received one of the three levels of supplementary riboflavin.

The animals were housed individually in cages with raised-wire bottoms. Food and distilled water were provided ad libitum during the 12-week experimental period. Prior to decapitation, the animals were fasted for 4 hours and the riboflavin supplement was withheld for 48 hours. The liver was removed quickly, blotted, frozen with solid carbon dioxide and stored at

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[2] Portions of the data were taken from a thesis submitted by Anne M. Kylen to the Graduate School of Colorado State University in partial fulfillment of the requirements for the degree of Master of Science.

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[7] Sprague-Dawley.

– 20°C until analyzed. The blood was collected in centrifuge tubes, centrifuged at 4°C, and the serum was stored at – 20°C.

Serum and liver were analyzed for cholesterol by the revised method of Sperry and Webb ('50). The fluorometric method of Burch et al. ('48) was used to determine the concentrations of flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) plus free riboflavin in the serum. Except for minor modifications in the preparation of the sample, the riboflavin-containing coenzymes in liver were determined by the method of Bessey et al. ('49). The concentrations of riboflavin-containing coenzymes in liver or serum were calculated as riboflavin. Total lipids of liver were measured by the method of Bloor ('28) as modified by Okey and Lyman ('54).

RESULTS

Serum cholesterol. Table 1 shows the average cholesterol in serum of weanling rats and rats maintained on rations A or B. In general, the mean total serum cholesterol increased with increasing ingestion of riboflavin. The mean total serum cholesterol of rats fed the highest level (100 μg) of riboflavin was significantly higher than that of corresponding animals fed the lowest level (10 μg) in both males and females fed ration B and in males fed ration A ($P < 0.01$). An increase in the riboflavin intake from 10 to 30 μg produced a significant increase in the mean total serum cholesterol only in males fed ration A ($P < 0.05$).

At a given level of riboflavin intake, the females fed ration A had slightly higher mean total serum cholesterol than females fed ration B, but the mean difference observed at the intermediate level (30 μg) of riboflavin intake was the only one of significance ($P < 0.01$). The fat level of the ration had no significant effect on the average total serum cholesterol of males.

Although the mean total serum cholesterol values observed in weanling rats were about the same in both sexes, the mean serum cholesterol observed in all female rats was significantly higher than that for all male rats at the end of the experimental period. Free cholesterol showed the same trend as total cholesterol. The percentage of free cholesterol in relation to total serum cholesterol for any level of riboflavin or fat decreased for males and increased for females during the experimental period. The percentages within a sex for different levels of riboflavin or fat differed very little at the end of the experiment.

Liver cholesterol. There were no significant differences among the values for the mean cholesterol per gram of fresh tissue in livers (table 1) of rats fed ration A. At the higher level of fat intake, the mean liver cholesterol of males or females fed 100 μg of riboflavin was significantly lower than that of similar animals fed 10 μg of riboflavin ($P < 0.01$). The ingestion of 30 μg of riboflavin produced a significant decrease in mean liver cholesterol of males fed ration B ($P < 0.01$).

The mean cholesterol per gram of fresh liver tissue of rats fed ration B was significantly greater than that of corresponding animals fed ration A supplemented with 10 or 30 μg of riboflavin ($P < 0.01$). The augmenting effect of the higher level of fat intake on liver cholesterol was less pronounced at the highest level of riboflavin intake ($P < 0.05$). The mean cholesterol in liver of weanling rats was slightly higher than that of the experimental animals. At the higher level of fat intake, the average liver cholesterol in all male rats was significantly higher ($P < 0.01$) than that in all female rats.

Serum riboflavin. Table 2 shows the mean free riboflavin + FMN, FAD and total riboflavin of the serum of weanling and experimental rats. The total riboflavin in serum was increased significantly ($P < 0.01$) in males or females fed either fat level by increasing the dietary riboflavin from 10 to 30 μg or 30 to 100 μg, in all cases except in males fed 20% of fat supplemented with the lower levels of riboflavin.

Increasing the riboflavin supplement from 10 to 30 μg resulted in a significantly higher mean free riboflavin + FMN in the serum of females fed 5 or 20% of fat ($P < 0.01$) or males fed 20% of fat ($P < 0.05$). The mean increases in FAD were not statistically significant. The mean concentration of both fractions in

TABLE 1

The average cholesterol levels in serum and liver tissue of weanling rats and rats fed for 12 weeks, rations containing 5 or 20% of cottonseed oil supplemented with graded levels of riboflavin

		Weanling rats	Rats fed 5% cottonseed oil			Rats fed 20% cottonseed oil		
			Supplementary riboflavin			Supplementary riboflavin		
			10 μg	30 μg	100 μg	10 μg	30 μg	100 μg
	Sex							
Serum cholesterol, *mg %*[1]								
Total	F	85.2	97.2 ± 6.5	108.1 ± 6.3	115.3 ± 6.1	89.0 ± 3.8	82.2 ± 2.0	107.2 ± 3.2
	M	89.6	70.6 ± 4.1	89.7 ± 4.8	99.0 ± 4.9	79.7 ± 4.6	86.8 ± 6.4	93.1 ± 6.1
Free	F	17.2	27.4 ± 2.5	29.0 ± 2.0	30.7 ± 1.8	23.4 ± 1.0	20.3 ± 1.3	26.7 ± 1.2
	M	23.0	15.4 ± 1.1	17.3 ± 1.4	19.5 ± 1.5	16.0 ± 1.1	16.9 ± 1.7	18.4 ± 1.4
% of total	F	20.1	28.0	27.0	26.6	26.5	24.6	25.1
	M	25.7	22.0	19.3	19.6	20.3	19.2	19.7
Liver cholesterol								
Total								
mg/gm fresh liver tissue[1]	F	2.61 ± 0.05	2.26 ± 0.07	2.16 ± 0.07	2.13 ± 0.04	2.64 ± 0.09	2.47 ± 0.04	2.34 ± 0.06
	M	2.41 ± 0.04	2.25 ± 0.11	2.27 ± 0.04	2.19 ± 0.12	3.19 ± 0.10	2.64 ± 0.08	2.55 ± 0.07
mg/liver[1]	F	13.06 ± 0.71	12.48 ± 0.32	15.18 ± 0.49	14.81 ± 0.36	15.74 ± 0.83	15.26 ± 0.58	16.44 ± 0.44
	M	13.46 ± 0.49	15.16 ± 0.96	22.81 ± 0.81	25.45 ± 1.57	20.30 ± 0.90	23.65 ± 1.31	26.25 ± 0.97
Liver								
% body weight	F	5.5	3.1	3.1	3.0	3.5	2.9	3.0
	M	4.8	3.2	3.2	3.3	3.4	3.1	2.9

[1] Standard error of mean included.

TABLE 2

The riboflavin-containing coenzymes and total riboflavin in serum of weanling rats and rats fed for 12 weeks, rations containing 5 or 20% of cottonseed oil supplemented with graded levels of riboflavin

		Weanling rats	Rats fed 5% cottonseed oil			Rats fed 20% cottonseed oil		
			Supplementary riboflavin			Supplementary riboflavin		
			10 μg	30 μg	100 μg	10 μg	30 μg	100 μg
	Sex							
Riboflavin, μg %[1]								
Free + FMN	F	1.54	0.81 ± 0.07	1.32 ± 0.11	2.31 ± 0.33	0.56 ± 0.08	1.38 ± 0.13	2.12 ± 0.14
	M	1.32	0.90 ± 0.04	1.14 ± 0.13	1.66 ± 0.02	0.71 ± 0.05	0.98 ± 0.11	1.66 ± 0.09
FAD	F	2.34	1.69 ± 0.11	1.98 ± 0.10	2.21 ± 0.06	1.59 ± 0.07	1.74 ± 0.11	1.96 ± 0.13
	M	2.58	1.33 ± 0.05	1.48 ± 0.09	2.08 ± 0.10	1.42 ± 0.11	1.44 ± 0.08	1.86 ± 0.05
Total riboflavin, μg %[1]	F	3.89	2.50 ± 0.15	3.30 ± 0.12	4.52 ± 0.34	2.15 ± 0.12	3.12 ± 0.13	4.08 ± 0.20
	M	3.90	2.23 ± 0.06	2.62 ± 0.08	3.74 ± 0.09	2.13 ± 0.12	2.42 ± 0.08	3.52 ± 0.06

[1] Standard error of mean included.

TABLE 3

The riboflavin-containing coenzymes, total riboflavin and total lipids in liver of weanling rats and rats fed for 12 weeks, rations containing 5 or 20% of cottonseed oil supplemented with graded levels of riboflavin

		Weanling rats	Rats fed 5% cottonseed oil			Rats fed 20% cottonseed oil		
			Supplementary riboflavin			Supplementary riboflavin		
			10 μg	30 μg	100 μg	10 μg	30 μg	100 μg
	Sex							
Riboflavin[1]								
μg/gm fresh tissue								
Free + FMN	F	3.68	3.92 ± 0.40	5.51 ± 0.55	7.08 ± 0.63	2.62 ± 0.38	5.47 ± 0.59	7.21 ± 0.57
	M	3.72	3.87 ± 0.92	6.79 ± 0.40	7.61 ± 0.70	3.21 ± 0.62	4.81 ± 0.59	9.07 ± 0.63
FAD	F	19.70	12.29 ± 0.42	18.42 ± 0.86	21.45 ± 0.91	11.08 ± 0.70	17.66 ± 1.13	22.01 ± 1.74
	M	19.16	14.07 ± 0.82	19.46 ± 0.42	22.97 ± 1.33	13.72 ± 0.73	20.28 ± 0.69	23.69 ± 0.71
Total	F	23.37	16.21 ± 0.62	23.93 ± 0.61	28.53 ± 1.01	13.71 ± 0.70	23.13 ± 1.47	29.22 ± 2.02
	M	22.88	17.93 ± 0.68	26.25 ± 0.52	30.58 ± 1.20	16.93 ± 1.12	25.09 ± 1.01	32.76 ± 1.04
FAD, % of total	F	84%	76%	77%	75%	80%	77%	75%
	M	84%	79%	73%	75%	81%	81%	72%
Total lipids[1]								
mg/gm fresh tissue	F	32.6	41.4 ± 2.7	40.1 ± 1.1	37.1 ± 1.8	53.8 ± 3.4	48.6 ± 1.5	46.1 ± 1.6
	M	30.6	35.4 ± 2.7	37.8 ± 0.8	35.3 ± 1.8	46.5 ± 3.3	45.9 ± 1.0	41.2 ± 3.0

[1] Standard error of mean included.

males ($P < 0.01$) and free riboflavin + FMN in females ($P < 0.01$ for ration B) fed 100 μg of riboflavin was significantly higher than that of corresponding animals fed 30 μg of the vitamin.

The higher fat intake significantly decreased ($P < 0.05$) the mean free riboflavin + FMN in serum of males or females fed 10 μg of riboflavin and did not significantly alter the mean concentration of FAD in any instance. The mean total serum riboflavin was significantly higher in females fed 30 ($P < 0.01$) or 100 μg ($P < 0.05$) of riboflavin than that of corresponding males. The mean FAD in serum of weanling rats was higher than that for the animals fed the experimental diets.

Liver riboflavin. A summary of the mean free riboflavin + FMN, FAD and total riboflavin in the liver tissue is shown in table 3. Increasing the level of supplementary riboflavin from 10 to 30 μg produced significantly higher mean free riboflavin + FMN in liver in females fed 5 ($P < 0.05$) or 20% ($P < 0.01$) of fat and in males fed 5% of fat ($P < 0.01$). The mean increase in FAD was significant ($P < 0.01$). Feeding riboflavin at the 100 μg level resulted in a further significant increase in the mean free riboflavin + FMN only in males fed 20% of fat ($P < 0.01$). The mean FAD increased significantly in males or females fed 5% of fat ($P < 0.05$) and in males fed 20% of fat ($P < 0.01$).

Increasing the intake of dietary fat from 5 to 20% significantly decreased the mean free riboflavin + FMN in liver of males fed 30 μg and of females fed 10 μg of riboflavin ($P < 0.05$). The level of dietary fat had no significant effect on the mean FAD in liver. Although male livers were consistently higher in riboflavin than were female livers, the differences were significant only in animals fed ration A with 30 μg or ration B with 10 μg of riboflavin ($P < 0.05$). The mean FAD or total riboflavin in liver of weanling rats was similar to that of the experimental animals fed 30 μg of riboflavin, but the mean level of free riboflavin + FMN was somewhat less than that of the animals receiving 30 μg of the vitamin. The FAD expressed as a percentage of the total was slightly lower in experimental than weanling rats. The decrease in percentage for males fed the high-fat, high-riboflavin diet was greatest.

Total lipids in liver. Table 3 shows the mean total lipids in liver of weanling and experimental rats. Although the mean total lipids in liver tended to decrease with increased riboflavin intake, differences were not significant. Significant increases in the mean total lipds due to the higher intake of dietary fat were observed in liver of males or females fed 10 ($P < 0.05$) or 30 μg ($P < 0.01$) of riboflavin and in females fed 100 μg ($P < 0.01$) of the vitamin. The mean concentration of total liver lipids of females was slightly higher than that of corresponding males, but the differences were not significant.

Growth. A summary of the growth data is presented in table 4. The supplementation of rations A or B with 30 μg of riboflavin significantly increased the mean weight gain of males or females above that

TABLE 4

The effect of graded levels of supplemental riboflavin on growth of rats fed rations containing 5 or 20% of cottonseed oil for 12 weeks

Supplementary riboflavin		5% cottonseed oil		20% cottonseed oil	
		Mean final body weight	Mean total weight gain	Mean final body weight	Mean total weight gain
μg	*Sex*	*gm*	*gm*	*gm*	*gm*
10	F	183	127	174	118
	M	209	144	188	123
30	F	227	171	216	159
	M	311	246	287	222
100	F	232	176	236	180
	M	355	290	354	288

attained by corresponding animals fed 10 μg of the vitamin. One hundred micrograms of riboflavin resulted in a further significant increase in the mean weight gain of males fed either ration and females fed 20% of fat. The rats receiving 10 or 30 μg of riboflavin gained more in weight on the 5% fat ration than did the corresponding groups fed ration B but the differences were not significant. The mean weight gains of either sex maintained on 100 μg of riboflavin were essentially the same at both levels of fat intake. Those animals maintained on ration B supplemented with 10 μg of riboflavin developed symptoms characteristic of riboflavin deficiency. Five males and 4 females fed 30 μg of riboflavin at the 20% fat level developed less pronounced deficiency symptoms.

DISCUSSION

Investigators have postulated that the increased need for dietary riboflavin in experimental animals fed high-fat diets may be due to a "metabolic effect" or decreased synthesis of riboflavin by intestinal flora (Mannering et al., '41; Shaw and Phillips, '41; Czaczkes and Guggenheim, '46; Reiser and Pearson, '49; Kaunitz et al., '54). Since the elevating effect on serum cholesterol of increased dietary riboflavin was observed at both levels of fat intake it would seem that the results observed were not due to an inhibitory effect of the higher fat level on intestinal flora.

Essential fatty acids may function in normal distribution and metabolism of cholesterol as proposed by Alfin-Slater et al. ('54). In this study, using rations low in riboflavin and high in essential fatty acid content, the association of essential fatty acids and serum cholesterol may depend on riboflavin-containing coenzymes. It appears that in riboflavin deficiency, there is an accumulation of cholesterol in liver and the effect is more marked on the higher fat level. Alfin-Slater et al. ('54) found that cholesterol accumulated in liver of rats fed purified rations which contained no added fat but were not limiting in riboflavin.

Mahler ('54) has shown that riboflavin functions as a part of butyryl coenzyme A dehydrogenase which is essential to fatty acid metabolism. If the metabolism of fatty acids takes priority over cholesterol transport, riboflavin may become the limiting factor for cholesterol metabolism when diets high in fat and low in riboflavin are fed. This may account for the accumulation of cholesterol in liver of the rats fed 20% of cottonseed oil. Riboflavin may function in a specific enzyme needed for the catabolism of cholesterol, since it is known that the cholesterol molecule may be metabolized to fatty acids (Kritchevsky et al., '52).

The increased level of fat in ration B replaced an equal weight of sucrose and reduced the caloric contribution from protein by 2.9%. The average food intake of the rats on ration B was less than that of similar animals fed ration A. Since most of the plasma cholesterol is transported as lipoproteins (Byers et al., '52), the results reported here may give rise to the questions of the effect of protein intake and protein-riboflavin relationships to cholesterol concentration in tissues. It is possible that the results apparently due to increased fat intake were influenced partially by decreased protein intake. Kokatnur et al. ('58) reported that increased protein levels were more effective than decreased fat levels in reducing serum cholesterol concentration in chicks when added cholesterol was withdrawn. Kaunitz et al. ('54) reported that "high dietary protein levels cannot be utilized if riboflavin is rigidly restricted." It is planned to investigate further the possible effect of riboflavin and protein on cholesterol concentration in tissue of the rat.

Burch et al. ('48) fed graded levels of dietary riboflavin and concluded that (1) changes in the concentration of FAD in serum were small relative to those of corresponding changes in dietary riboflavin, (2) FMN in serum was small and fairly constant and (3) free riboflavin in serum paralleled increased levels of dietary riboflavin. In the present study the free riboflavin + FMN fraction accounted for most of the increase in serum riboflavin. The riboflavin supplement was withheld 48 hours prior to killing. This may explain the smaller increase in free riboflavin in serum compared to the results reported by Burch et al. ('48).

Decker and Byerrum ('54) reported that 30 μg of riboflavin per day incorporated in-

to the ration produced a maximum concentration of FAD or FMN in tissues of rats. Bessey et al. ('58) reported that approximately 40 μg of riboflavin per day were required for maximum concentration of riboflavin in tissues. These authors measured total riboflavin in the liver and carcass and found that at a given level of riboflavin intake, riboflavin incorporated into purified rations was more effective than supplementary riboflavin. These results may explain partially the findings of the present study which indicate that 30 μg of supplementary riboflavin fed 6 days per week did not produce maximum concentration of FAD in the liver. Decker and Byerrum ('54) measured riboflavin-containing coenzymes, xanthine oxidase activity and D-amino acid oxidase activity in tissues of rats fed rations containing graded levels of riboflavin. These authors concluded that "whereas the total coenzyme concentration does not increase with increasing ingestion of riboflavin, the activity of some specific riboflavin-requiring enzymes may increase, as was exemplified by liver D-amino acid activity." Since the findings of the present study indicate that there is no clear-cut relationship between the level of dietary fat and the concentration of riboflavin-containing coenzymes in serum or liver, it is possible that diets high in fat may intensify riboflavin deficiency in several ways. The increased intake of fat may increase the need for a given enzyme or enzymes, the concentration of which does not correspond to riboflavin intake. Therefore, increased fat intake may decrease a given enzyme without affecting the total coenzyme concentration. Burch et al. ('56) reported that flavin enzymes decreased at various rates during riboflavin deficiency.

In this study the mean concentrations of total liver lipids in females were slightly higher than those in males as reported by Deuel ('55) whereas Okey and Lyman ('54) found that male rats accumulated more fat in the liver than do females.

SUMMARY

Rats fed a ration containing 20% of fat and varying levels of riboflavin had a higher concentration of cholesterol in liver than did corresponding animals fed a ration containing 5% of fat. An increase of supplementary riboflavin from 10 to 30 μg or 30 to 100 μg per day decreased the deposition of cholesterol in liver for rats on 20% of fat. The level of dietary fat had no effect on serum cholesterol which increased with increased riboflavin intake at both fat levels.

Increased levels of supplementary riboflavin significantly increased the mean total serum riboflavin. The free riboflavin + FMN fraction accounted for most of the increase. The mean level of FAD remained fairly constant. Mean FAD in liver increased significantly with increased ingestion of riboflavin but increased concentrations of free riboflavin + FMN were not consistently significant. Increased fat intake affected riboflavin-containing coenzymes in serum and liver only in isolated cases.

Total lipids in liver decreased with increased riboflavin intake but the differences were not significant. The higher fat level significantly increased the total lipids in liver.

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The Use of Casual Urine Specimens in the Evaluation of the Excretion Rates of Thiamine, Riboflavin and N^1-Methylnicotinamide

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For biochemical evaluation of an individual's nutritional status one must measure the rate of excretion of certain vitamins in the urine. Ideally, a 24-hour urine specimen is used, but satisfactory data can be obtained from timed collections of 4 or 6 hours. However, in certain large field surveys it has proved difficult or impossible to obtain timed collections (Adamson et al., '45; Aykroyd et al., '49; Interdepartmental Committee on Nutrition for National Defense (ICNND), '57b, '58). Casual urine specimens were therefore collected and the vitamin excretion was expressed per gram of creatinine contained in the sample. Although this procedure is believed to be valid (Lowry, '52), the degree of error thus introduced has not been established. The present study was designed to estimate the amount of this error.

METHODS

The subjects were 10 healthy young men, 18 to 20 years of age, who were, on the average, 172 cm tall (range 165 to 178 cm), and weighed 66.4 kg (range 60.9 to 72.4 kg). They were housed in a metabolic ward but were permitted to go off the ward for supervised activity such as sports. They received a constant diet for a 4-day period during which all urine was collected every 6 hours.

The subjects completely consumed a constant weighed diet as the only source of food. Meals were eaten at 8 A.M., 12 noon and 5 P.M. The cooked diet contained, by analysis: protein, 137 gm; fat, 121 gm; carbohydrate, 363 gm; thiamine, 1.55 mg (Mickelsen et al., '45); and riboflavin 1.84 mg (Conner and Straub, '41). The diet was calculated to contain 20 mg of niacin. The diet included 305 gm of meat (205 gm of ground beef and 100 gm of tuna fish) which was presumably the source of the 0.80 gm of creatine and 0.37 gm of creatinine found by analysis (Hawk et al., '47) in the cooked diet. The breakfast contained no meat.

The urine was analyzed for creatinine (Hawk et al., '47), thiamine (Mickelsen et al., '45), riboflavin (Conner and Straub, '41) and N^1-methylnicotinamide (Huff et al., '45).

RESULTS

The rates of creatinine excretion during the 4 daily periods are recorded in table 1. As has been reported many times the lowest excretion rate occurred during the early morning while the highest was in the afternoon.

TABLE 1

Diurnal variation in creatinine excretion (10 men for 4 days)

Period	Mean	Standard deviation	Coefficient of variation
	gm	*gm*	*%*
1 A.M.–7 A.M.	0.446	0.047	11
7 A.M.–1 P.M.	0.489	0.083	17
1 P.M.–7 P.M.	0.587	0.080	14
7 P.M.–1 A.M.	0.569	0.071	12
7 A.M.–7 A.M.	2.094	0.170	8

The individual excretion rates of thiamine, riboflavin and N^1-methylnicotinamide expressed as micrograms per 6-hour period and as micrograms per gram of creatinine are compared graphically in figures 1, 2 and 3. These data were subjected to re-

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TABLE 2

Regression equations for the prediction of vitamin excretion per 6 hours from vitamin excretion per gram of creatinine

Vitamin	Period	Constants of regression			
		Linear equations		Log-log equations	
		Intercept	Slope	Intercept	Slope
Thiamine, μg	All	5	0.52[1]	− 0.27	0.99[2]
	1 A.M.–7 A.M.	23	0.39	− 0.22	0.95
	7 A.M.–1 P.M.	41	0.42	− 0.00	0.89
	1 P.M.–7 P.M.	95	0.49	0.06	0.90
	7 P.M.–1 A.M.	17	0.54	− 0.20	0.98
Riboflavin, μg	All	46	0.45	− 0.05	0.92
	1 A.M.–7 A.M.	32	0.40	− 0.16	0.93
	7 A.M.–1 P.M.	12	0.47	− 0.27	0.99
	1 P.M.–7 P.M.	107	0.42	− 0.02	0.92
	7 P.M.–1 A.M.	14	0.53	− 0.12	0.95
N^1-methyl-nicotinamide, mg	All	0.30	0.46	0.85	0.79[3]
	1 A.M.–7 A.M.	− 0.13	0.46	0.74	0.83
	7 A.M.–1 P.M.	− 0.09	0.51	0.66	1.03
	1 P.M.–7 P.M.	0.18	0.54	0.92	0.73
	7 P.M.–1 A.M.	0.45	0.49	1.04	0.64

[1] The linear equations have the following form:
thiamine, μg/6 hours = 5 + 0.52 (thiamine, μg/gm creatinine)

[2] The log-log equations have the following form:
log (thiamine, μg/6 hours) = −0.27 + 0.99 log (thiamine, μg/gm creatinine)

[3] In calculating the log-log equations for N^1-methylnicotinamide only, the observed values of N^1-methylnicotinamide per 6 hours were multiplied by 10 for ease in handling. These equations therefore have the following form:
log (10 × N^1MN, mg/6 hours) = 0.85 + 0.79 log (N^1MN, mg/gm creatinine)

gression analysis (table 2). Since the purpose of the study was to estimate the predictability of the 6-hour vitamin excretion from the excretion per gram of creatinine, the equations were calculated with the excretion per gram of creatinine as the independent variable.

As a further measure of predictability the 5% confidence limits were calculated from the data. Since the analysis compared one highly variable quantity (vitamin excretion per 6 hours) with that same quantity divided by a relatively constant quantity (creatinine excretion per 6 hours) the graphic comparisons were expected to show much closer grouping near the origin than at a distance therefrom; such is indeed the case. Therefore confidence limits were calculated from the logarithms of the data. The constants for linear regression equations relating the logarithms of the data are also given in table 2. Regression lines and 5% confidence limits, converted to the linear scale, are included in figures 1, 2 and 3. In the cases of thiamine and riboflavin the confidence limits fit the data very well. The wider spread for the N^1-methylnicotinamide data is due to a few aberrant values so far removed from the main body of data that they were omitted from the graph.

DISCUSSION

The only source of variation in the data as presented here is the creatinine excretion. One source of such variation is body size, although no obvious relation of this type was observed among the 10 fairly uniform subjects of this study. Variation in creatinine excretion may actually be a point in favor of expressing vitamin excretion per gram of creatinine. A man's vitamin requirement probably depends on his body size as does his creatinine excretion, so that comparisons of vitamin excretion per gram of creatinine should tend to correct for variations in vitamin requirement from man to man.

The mean creatinine excretion in this study, 2.09 gm per 24 hours, is rather high. The diet contained 0.37 gm of preformed creatinine which certainly contributed to

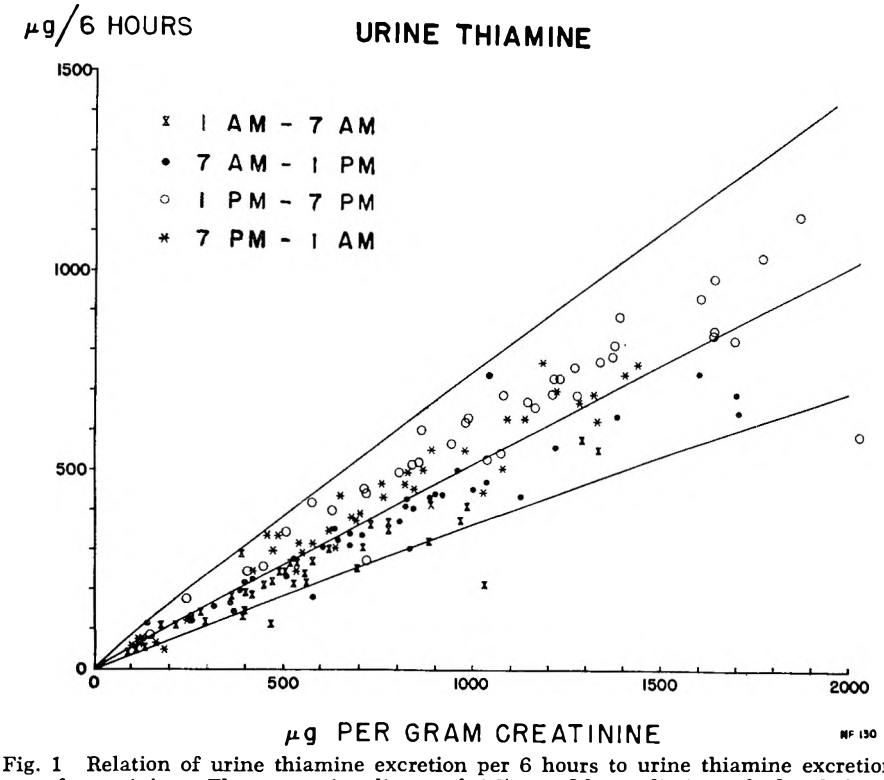

Fig. 1 Relation of urine thiamine excretion per 6 hours to urine thiamine excretion per gram of creatinine. The regression line and 5% confidence limits calculated from the logarithms of the data are included.

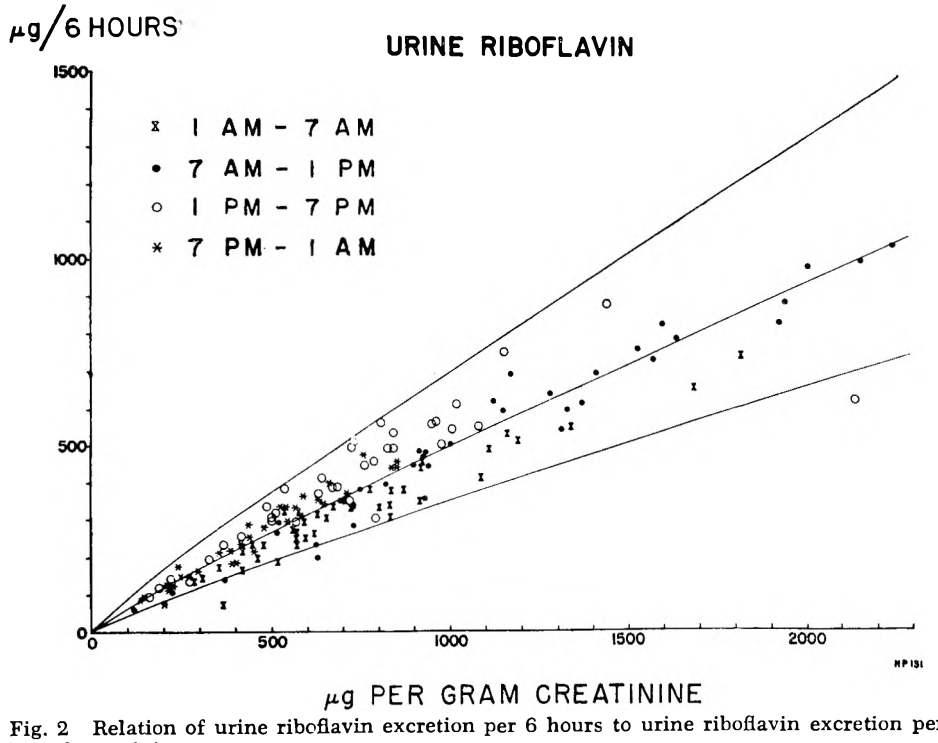

Fig. 2 Relation of urine riboflavin excretion per 6 hours to urine riboflavin excretion per gram of creatinine.

this high value. The difference between these figures, 1.72 gm, approaches the expected daily excretion rate for men of this body size, 1.5 gm, used by the ICNND ('57a). Obviously the consumption of sizeable amounts of preformed creatinine can cause considerable error in the conversion of vitamin excretion per gram of creatinine to vitamin excretion per unit time. This source of error may be more apparent than real for the major source of dietary creatinine is meat, and men consuming much meat are unlikely to be deficient in the vitamins measured in the urine.

Preformed dietary creatinine is probably the main cause of the diurnal variation in creatinine output, for the consumption of a meat-free diet reduces the diurnal variation (Best, '53), although this does not abolish it. In the present study the sum of the increases in excretion over the fasting rate (1 A.M. to 7 A.M.) was approximately the amount provided by the diet. It is, therefore, advisable to use fasting urine specimens if the vitamin excretion is to be measured per gram of creatinine. Actually the increase in creatinine output in the morning following a meat-free breakfast was small enough for such specimens to be satisfactory also. If the 6-hour vitamin excretion is calculated from the excreation per gram of creatinine, use should properly be made of the conversion equation appropriate to the time interval. However, little error would be introduced by the conversion factor of the ICNND ('57a), which assumes an intercept of zero and a slope of 0.375 (compare table 2).

The confidence limits calculated from the present data are rather wide. Over the range of the data, these limits, expressed as a percentage of the predicted vitamin excretion per 6 hours, are, for thiamine, 69 to 145%; for riboflavin, 71 to 141%; and for N^1-methylnicotinamide, 47 to 212%. These represent the limits of predictability, 19 times out of 20, of the 6-hour vitamin excretion from excretion per

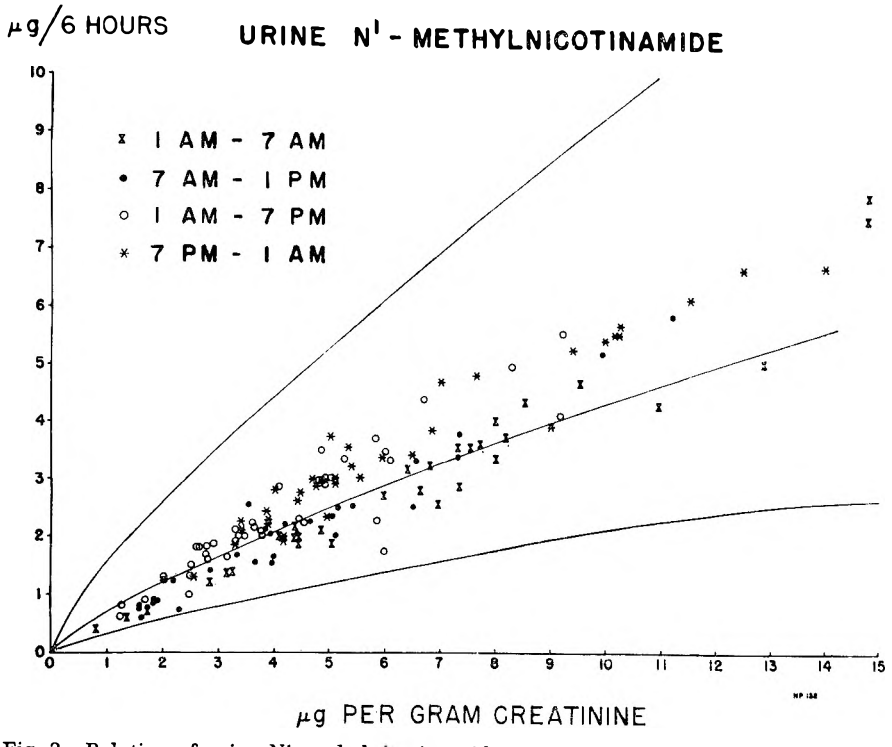

Fig. 3 Relation of urine N^1-methylnicotinamide excretion per 6 hours to urine N^1-methylnicotinamide excretion per gram of creatinine.

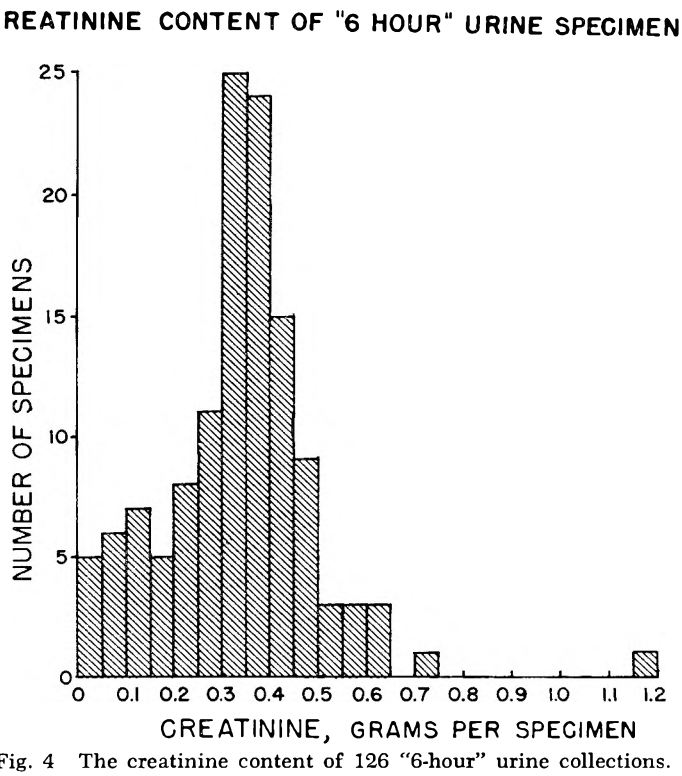

Fig. 4 The creatinine content of 126 "6-hour" urine collections.

gram of creatinine, from a single urine collection. Because of these wide limits, Hegsted et al. ('56) did not recommend the use of casual urine specimens in the evaluation of nutritional status. With more than one individual with the same vitamin excretion per gram of creatinine the confidence limits will be reduced by a factor equal to the reciprocal of the square root of the number of individuals. For 10 similar values of thiamine excretion per gram of creatinine, the 6-hour excretion would be within approximately 90 and 115% of predicted value, 19 times out of 20. This range of accuracy is entirely satisfactory for large surveys of populations.

The alternative to the measurement of vitamin excretion per unit of creatinine is the collection of timed urine specimens, which requires careful supervision of every individual. As witness to this problem, figure 4 presents the creatinine content of "6-hour" urine specimens collected from 126 men who had been carefully instructed, but were not completely supervised.[1] The urine collection times apparently varied from one to more than 12 hours. The interpretation of vitamin excretion rates from these urine specimens on the assumption that all were 6-hour collections could lead to considerable error.

SUMMARY AND CONCLUSIONS

A man's urinary excretion of thiamine or riboflavin per 6 hours can be predicted from the excretion per gram of creatinine within limits of plus or minus 30 to 40%. The limits are larger for N^1-methylnicotinamide. The predictability is affected by variations in body size, by diurnal variations in creatinine excretion, and by dietary intake of creatinine. The predictability is more accurate with fasting urine specimens. It is concluded, however, that for surveys of large groups of individuals the measurement of vitamin excretion rate per gram of creatinine in casual urine specimens is a satisfactory procedure in the biochemical evaluation of nutritional status.

[1] Authors' unpublished data.

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Effect of Dietary Fats and Carbohydrates on Digestibility of Nitrogen and Energy Supply, and on Growth, Body Composition and Serum Cholesterol of Rats

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Differences in the growth of rats fed various kinds and levels of fats and oils have been reported by many investigators (Hoagland and Snider, '40, '41; Deuel and co-workers, '47; Forbes et al., '46; Cayama, '55; Thomasson, '55; and Barki et al., '50). The obesity-producing effects in the rat of high levels of various fats and oils have also been investigated (Barboriak et al., '58). Less attention has been paid to the effect on body composition of feeding moderate levels of fats and oils. In one study, Scheer et al. ('47) determined body composition of male and female rats that had received purified diets containing several levels of cottonseed oil with sucrose as the carbohydrate or a stock diet containing 14% cottonseed oil for 18 weeks after weaning. The percentage of body fat varied from a low of 9.7 to a high of 25.5 for the various groups of females and from 9.9 to 24.7 for the various groups of male rats, but, in general, was not related to the fat content of the diet.

In the present study, an animal fat (lard), a vegetable oil[1] or a hydrogenated vegetable oil[2] was incorporated in diets at moderate levels and fed to rats from weaning or shortly thereafter until they were either 200 or 400 days old. Other groups were fed corresponding diets low in fat or containing a different carbohydrate. Data are reported on digestibility of nitrogen and energy supply and on gains in weight, carcass composition and serum cholesterol levels.

EXPERIMENTAL

A stock diet[3] and a purified diet were fed. The stock diet which contained 4% fat was ground in a mill and fed either as the ground powder, or with 11.5% of fat added (total fat 15%). The purified diet (P) contained three or 15% fat; lactalbumin;[4] salt mixture (Jones and Foster, '42) 4%; vitamin A and D concentrate,[5] 0.05%; inositol, 0.1%; choline chloride, 0.2%; and carbohydrate (either sucrose (S) or cornstarch (C)) to 100%. The following vitamins were added per kilogram of ration: thiamine·HCl, 5 mg; pyridoxine·HCl, 5 mg; niacin, 5 mg; riboflavin, 10 mg; Ca pantothenate (*d*), 25 mg; *p*-aminobenzoic acid, 300 mg; α-tocopherol acetate, 25 mg; 2-methyl-1, 4-naphthoquinone, 2 mg; folic acid, 2 mg; biotin, 100 μg; and vitamin B_{12}, 30 μg. The three fats used were lard (L), corn oil (CO), and hydrogenated vegetable oil (HVO).

Three hundred and twenty-five male and 52 female rats from the laboratory stock colony (Womack, Harlin and Lin, '53) were housed individually and fed one of the various diets ad libitum from weaning (or shortly thereafter) until 200 or 400 days old.[6] The animals were weighed weekly and records of weight changes and food intakes were kept for the entire ex-

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[1] Mazola.

[2] Crisco.

[3] Animal Foundation Laboratory Diet, Standard Brands, Inc., New York, N. Y.

[4] Lactalbumin was incorporated at a level of 26% in the diets containing 3% fat, and at a level of 30% in the diets containing 15% fat.

[5] Squibb's Navitol containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gram.

[6] Thirty-seven animals died from various causes, mostly respiratory diseases.

perimental period. All scattered food was collected and food intakes corrected accordingly. When some of the rats were nearing 200 or 400 days of age urine and feces were collected as previously described (Womack et al., '53) except that the feces were frozen until the end of the period, dried under infra-red lamps and ground in a Wiley mill. Nitrogen was determined in each sample of dried feces and in the food and urine by the Kjeldahl-Wilfarth-Gunning method (Association of Official Agricultural Chemists, '50) using mercury as a catalyst and distilling into boric acid. The caloric value of samples of the food and of pooled fecal samples from each group was determined in the Parr Bomb Adiabatic Calorimeter.

Half of the male animals fed the stock diets or the purified diets with sucrose as the carbohydrate were killed when 200 days old and the remainder when 400 days old. All of the female rats and the male rats fed the diets containing cornstarch were killed when 400 days old. The animals were not fasted. They were anesthetized with sodium amytal solution, the body cavity opened and blood removed by heart puncture for determination of cholesterol. The livers and kidneys were removed and weighed, the gastrointestinal contents were removed, the carcasses autoclaved at 15 pounds pressure for 15 minutes, ground three times in a meat grinder, and a weighed sample dried under infrared lamps. The livers were also dried and ground in a Wiley mill. Small samples of the livers, kidneys, adrenals, lungs and pancreas of the 400-day-old animals were preserved for histological examination. Nitrogen was determined in the dried carcasses and livers. The fat content of the carcasses was calculated from moisture and protein content, assuming 3% ash and 2% residual moisture. In selected samples of the carcasses and pooled samples of the livers the ether-extractable material was determined using the Bailey-Walker extraction apparatus. Cholesterol determinations were made on blood serum by the method of Zlatkis, Zak and Boyle ('53).

RESULTS AND DISCUSSION

In table 1 are the data showing the effect of the addition of moderate amounts of fats on the digestibility and retention of nitrogen and digestibility of the energy supply of the different diets by rats 200 and 400 days old. The food intakes during the periods after the animals were transferred to the metabolism cages were sometimes not in line with previous intakes; in order that the figures for nitrogen retention might be representative, data were deleted for any animal which ate more than 115% or less than 80% of its average food intake for 4 weeks prior to the collection. Due to the small number remaining, two groups were omitted completely from the 400-day-old animals.

Calculation of per cent apparent digestible energy (ADE)

$$\frac{(\text{gross calorie intake}-\text{fecal calories})}{\text{gross calorie intake}} \times 100$$

showed that the stock diet, without or with fat added, was less digestible than the purified diets.[7] The addition of fat to the stock diet was associated with a significant increase in digestibility of the energy supply. Increasing the level of fat from three to 15% in the purified diets had no apparent effect on the digestibility of the calories. The increase in the amount of fat in the diets had no consistent effect on the digestibility of the nitrogenous matter. Nitrogen retention *per se* or as per cent of the nitrogen intake did not appear to be related to kind or amount of fat in the diet.

As would be expected from the work of Schneider ('34), a linear relationship was observed when individual values for grams of food eaten during the two collection periods were plotted against grams of feces or milligrams of fecal nitrogen. However, for both fecal nitrogen and grams of feces, the slope of the line was greater for rats receiving the stock diet than for those receiving the purified diets.

[7] The values used for converting grams of food to gross calorie intake (average of 131 determinations on various batches of food) were 3.95 and 4.59 for the stock and stock-plus-fat diets; 4.35 and 5.07 for the low-fat and 15% fat diets containing sucrose; and 4.21 and 4.98 for the low-fat and 15% fat diets containing cornstarch. Theoretically the diets containing cornstarch should have had a higher calorie value than those containing sucrose, but the cornstarch had about 10% moisture, which accounted for the differences.

TABLE 1

The effect of fats on digestibility of nitrogen and energy supply

Diet[1]	No. rats	Intake		Fecal calories	ADE[2]	Nitrogen			Nitrogen retained		ADN[3]
		Food	Gross calories			Intake	In feces	In urine			
		gm/day	*Cal./day*	*Cal./day*	%	*mg/day*	*mg/day*	*mg/day*	*mg/day*	%	%
200 days											
Stock (ground)	22	16.5	65.2	11.2	82.8	767	185	550	32	4.2	76.2
Stock + corn oil	14	14.3	65.6	9.2	86.0	576	135	414	28	4.9	76.4
Stock + HVO	15	14.5	66.6	10.3	84.5	573	141	400	32	5.6	75.7
Stock + lard	14	16.3	74.8	12.3	83.6	656	156	453	47	7.2	77.7
PS 3% corn oil	13	15.0	65.2	2.7	95.9	490	46	412	33	6.7	90.3
PS 15% corn oil	9	13.7	69.5	3.0	95.7	513	46	438	29	5.7	90.9
PS 15% HVO	11	13.4	67.9	4.0	94.1	502	42	418	42	8.4	91.6
PS 15% lard	12	13.6	69.0	3.8	94.5	510	43	440	27	5.3	91.3
PC 3% corn oil	10	14.5	61.0	2.5	95.9	480	46	394	40	8.3	90.4
PC 15% corn oil	10	12.8	63.7	2.6	95.9	485	42	414	28	5.8	91.3
PC 15% HVO	8	12.1	60.3	2.8	95.4	457	38	382	37	8.1	91.6
PC 15% lard	8	12.4	61.8	2.9	95.3	468	41	385	42	9.0	91.3
400 days											
Stock (ground)	14	20.5	81.0	14.2	82.5	919	227	644	48	5.2	75.3
Stock + corn oil	9	16.4	75.3	11.2	85.1	642	162	462	18	2.8	74.7
Stock + HVO	9	17.5	80.3	12.9	83.9	688	177	474	37	5.4	74.4
Stock + lard	10	17.8	81.7	13.0	84.1	711	172	506	32	4.5	75.9
PS 3% corn oil	5	14.8	64.4	2.6	96.0	497	49	452	− 5	− 1.0	90.2
PS 15% HVO	6	13.2	66.9	3.2	95.2	496	41	442	13	2.6	91.5
PS 15% lard	4	15.9	80.6	5.2	93.5	596	51	526	19	3.2	91.4
PC 15% corn oil	6	14.4	71.7	3.2	95.5	541	51	485	4	0.7	90.7
PC 15% HVO	7	14.6	72.7	3.6	95.0	555	51	477	27	4.9	90.9
PC 15% lard	7	13.8	68.7	3.2	95.3	505	49	438	17	3.4	90.2

[1] HVO, hydrogenated vegetable oil; PS, purified, sucrose; PC, purified, cornstarch.
[2] ADE, apparent digestible energy.
[3] ADN, apparent digestible nitrogen.

TABLE 2

Average daily gross calorie intakes and gains per 100 calories of male rats fed various fat-containing or low-fat diets from weaning until 400 days of age

Diet[1]	Gross calorie intake per day per rat						Gain in weight per 100 gross calories (gm)					
	Weeks 1–4	Weeks 5–10	Weeks 11–20	Weeks 21–30	Weeks 31–40	Weeks 41–50	Weeks 1–4	Weeks 5–10	Weeks 11–20	Weeks 21–30	Weeks 31–40	Weeks 41–50
Stock (ground)	60.3	74.0	76.0	77.1	82.7	83.9	7.9	4.2	1.3	0.8	0.6	0.3
Stock + corn oil	60.1	71.9	75.5	77.2	79.0	82.8	8.9	3.8	1.8	0.5	0.8	0.6
Stock + HVO	61.5	70.8	71.5	78.1	84.5	87.1	8.2	3.9	1.6	1.1	0.5	0.6
Stock + lard	58.9	71.5	73.7	77.3	83.1	86.2	8.4	4.2	1.6	0.6	0.8	0.3
PS 3% corn oil	57.2	68.5	70.2	72.8	73.8	75.0	9.2	4.8	1.9	1.2	0.7	0.8
PS 15% corn oil	59.5	72.4	72.0	78.5	81.1	84.1	10.0	4.9	2.0	1.3	0.9	0.8
PS 15% HVO	58.1	70.0	68.4	69.1	71.0	73.1	9.1	4.6	1.9	0.6	0.9	0.7
PS 15% lard	59.3	72.6	72.4	73.8	77.6	78.2	9.5	4.6	2.0	0.9	0.9	0.3
PC 3% corn oil	46.1	58.4	61.1	64.8	68.0	70.5	10.1	5.0	2.0	1.1	0.6	0.2
PC 15% corn oil	56.1	66.6	67.4	70.5	71.8	74.1	9.5	5.1	1.4	1.1	0.9	0.5
PC 15% HVO	57.9	64.6	66.3	67.1	69.8	71.5	8.9	4.5	1.8	1.0	0.9	0.3
PC 15% lard	58.3	69.1	66.3	66.7	68.9	71.2	9.0	4.5	1.7	0.7	0.8	0.3

[1] See footnote 1, table 1.

TABLE 3

Body composition and serum cholesterol values of male and female rats fed various fat-containing or low-fat diets

Diet[1]	200-day-old rats				400-day-old rats			
	Weight	Body protein	Body fat (estimated)	Cholesterol[2]	Weight	Body protein	Body fat (estimated)	Cholesterol[2]
	gm	*%*	*%*	*mg%*	*gm*	*%*	*%*	*mg%*
				Male				
Stock (pelleted)	412	20.5	15.9	105 ± 5(12)	555[3]	19.4	23.2	210 ± 28(21)
Stock (ground)	398	21.2	14.9	112 ± 7(13)	486	19.1	22.3	175 ± 9(12)
Stock + corn oil	444	19.2	20.3	135 ± 5(13)	522	18.4	25.0	237 ± 31(14)
Stock + HVO	411	19.8	18.9	109 ± 5(14)	529	18.4	23.1	219 ± 28(14)
Stock + lard	432	19.8	18.7	147 ± 4(13)	510	18.5	24.2	192 ± 16(15)
PS 3% corn oil	456	19.2	20.9	155 ± 10(14)	579	17.0	30.5	241 ± 19(11)
PS 15% corn oil	500	17.9	25.4	155 ± 15(10)	663	16.2	33.2	356 ± 41(13)
PS 15% HVO	488	18.0	24.8	122 ± 8(14)	545	17.0	29.8	190 ± 27(14)
PS 15% lard	473	18.5	23.1	152 ± 8(14)	571	17.1	30.2	221 ± 20(11)
PC 3% corn oil					478	18.8	24.4	171 ± 17(10)
PC 15% corn oil					552	18.2	25.3	172 ± 10(12)
PC 15% HVO					528	17.7	26.3	146 ± 9(12)
PC 15% lard					513	17.6	26.3	170 ± 12(11)
				Female				
Stock (ground)					324	19.0	21.5	146(7)
Stock + corn oil					366	16.5	31.4	—
Stock + HVO					327	16.6	27.9	145(5)
Stock + lard					345	16.6	30.7	152(6)
PS 3% corn oil					428[4]	14.4	38.6	303(5)
PS 15% corn oil					446	15.1	38.1	—
PS 15% HVO					418	14.2	41.4	160(5)
PS 15% lard					429	15.1	37.5	199(5)

[1] See footnote 1, table 1.

[2] Includes standard error and number of rats.

[3] Not litter mates of any of the other animals, but included here to show that heavier rats fed the low-fat stock diet had only small changes in body composition.

[4] Does not include one rat which weighed 662 gm.

The growth curves of the groups of male animals during the first 50 weeks of feeding are shown in figure 1. The caloric intakes and gains in weight per 100 Cal. for the male rats were calculated for various periods and are shown in table 2. Calorie intake continued to increase even after the growth rate had slowed down and after the gain in weight per 100 Cal. had reached a very small figure.

Final weights (table 3) of the groups of male animals fed the stock diet with 15% fat were 529 (HVO), 510 (L), and 522 (CO) gm; of those fed the purified diet with 15% fat and cornstarch as the carbohydrate, 528 (HVO), 513 (L), and 552 (CO) gm; and of those fed the purified diet with 15% fat and sucrose as the carbohydrate, 545 (HVO), 571 (L), and 663 (CO) gm. Statistical analyses of the data by the method of Duncan and Bonner ('54) showed that the test difference for significance at the 5% level between any two means was 61 gm. Using this test difference it can be seen that the group which received the purified diet containing 15% corn oil and sucrose was significantly heavier than all other groups.

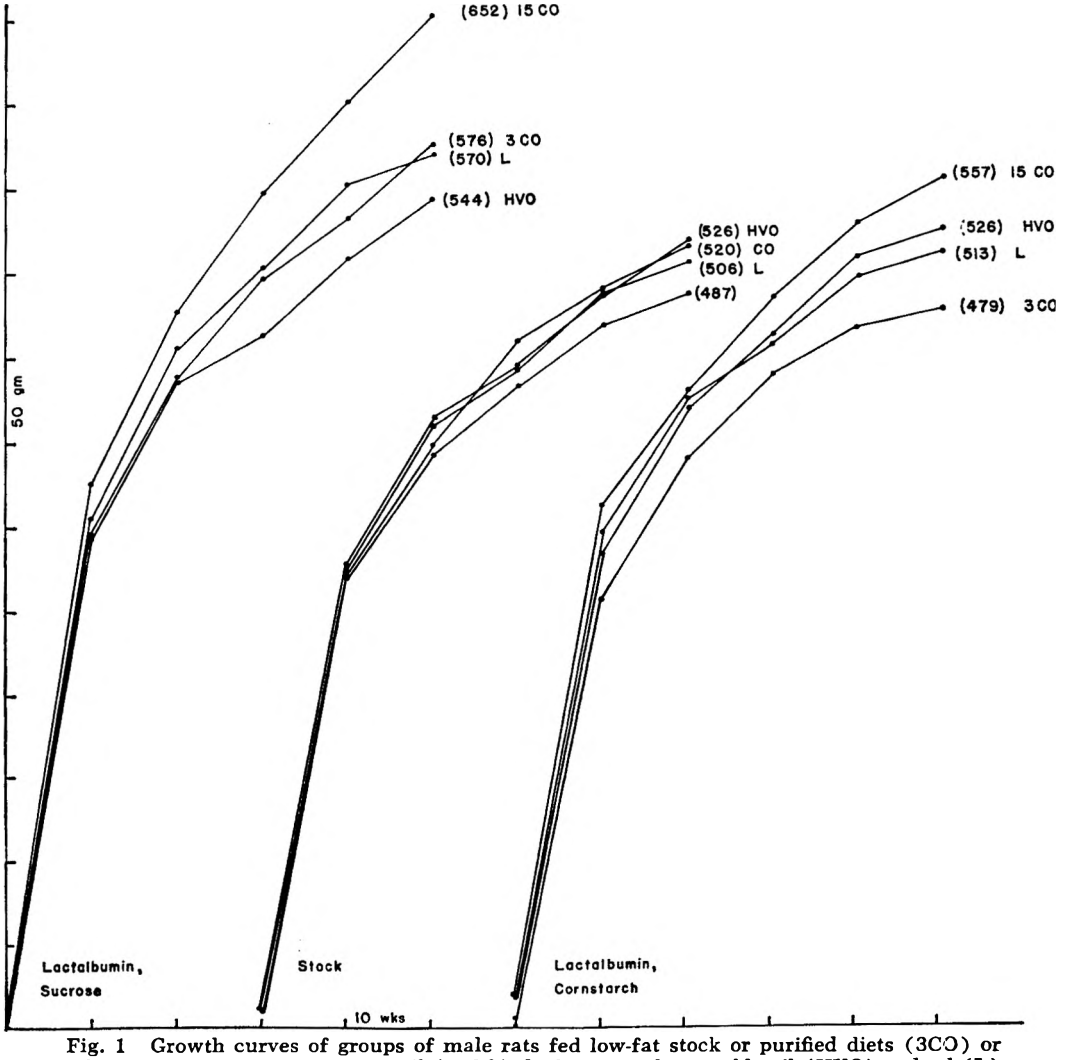

Fig. 1 Growth curves of groups of male rats fed low-fat stock or purified diets (3CO) or similar diets containing 15% corn oil (15CO), hydrogenated vegetable oil (HVO) or lard (L) for 50 weeks.

The test difference for significance at the 5% level for per cent body protein[8] (table 3) is 1.2%. It can be seen that there was a significant decrease in per cent body protein from 200 to 400 days in all groups but one (stock plus corn oil) as the per cent body fat increased. By 400 days the male animals receiving the purified diets containing sucrose had stored significantly more body fat than those receiving the stock diets (test difference, 4.6%), and there was a significant difference between the per cent fat of the animals receiving the purified diets containing sucrose or cornstarch with 3% corn oil, and also between the groups receiving sucrose or cornstarch with 15% corn oil.[9]

The ratios of grams of carcass fat to grams of carcass protein are shown in figure 2. The 200-day-old male rats receiving the stock diet plus corn oil had a significantly higher fat to protein ratio than those receiving the stock diet alone; those receiving the purified diet with 15% corn oil had a significantly higher fat to protein ratio than those receiving the purified diet low in fat (test difference, 0.35). There were no significant differences within the groups fed the stock diets, the purified diets containing sucrose, or the purified diets containing cornstarch for

[8] Figures for per cent body protein and fat do not include the liver.

[9] Calculated body fat was 33.2 and 25.3% for the groups of male rats receiving the purified diets containing 15% corn oil and sucrose or cornstarch, respectively. Analyzed values for the same groups were 33.6 and 24.5%.

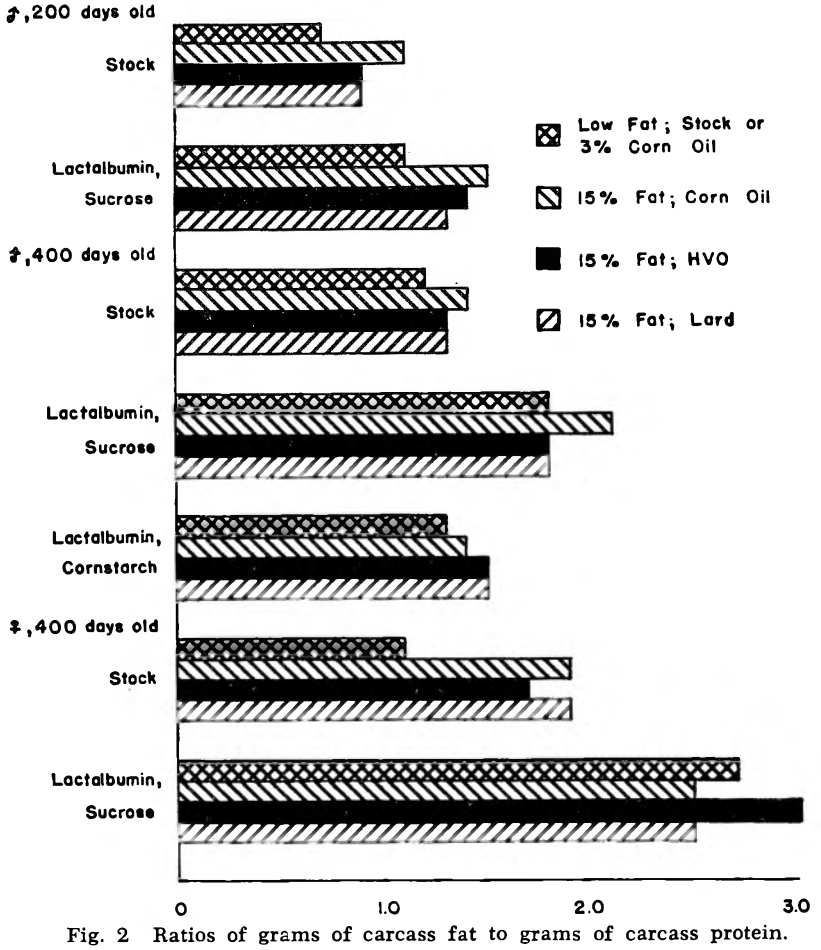

Fig. 2 Ratios of grams of carcass fat to grams of carcass protein.

400 days, but all of the groups of animals receiving the purified diets with sucrose as the carbohydrate had a higher fat to protein ratio than those fed the stock diet. In addition there was a significant difference between the fat to protein ratios of the animals receiving the purified diets containing sucrose or cornstarch with 3% corn oil, and also between those receiving sucrose or cornstarch with 15% corn oil. The group receiving the diet with sucrose and 15% corn oil had 2.1 gm of body fat for each gram of body protein. The female rats fed the purified diets had the highest fat to protein ratios.

The amounts of the experimental diets eaten by the 155 male and 44 female animals carried through the entire study and killed when 400 days old are given in table 4. The gross energy supply of the stock diet was calculated to be 82.7% digestible, of the stock diet plus the fats 84.5% digestible and of the purified diets 95.3% digestible (table 1, average for 200 and 400 days) and hence no comparison of the utilization of calories for body gain can be made between the stock diets and the others on the basis of gross calorie intake. Therefore, the digestible calorie intakes were calculated. There may be some question as to the validity of applying to the entire experimental period an average figure for digestibility based on determinations at two periods. However, the differences were small, and since no other data were available, the average figure was

TABLE 4

Total digestible calorie intake, fat gained, and per cent calorie intake stored as fat by male and female rats fed various fat-containing or low-fat diets from weaning until 400 days old

Diet[1]	No. rats	Food intake	DCI[2]	Gain	Body fat gained[3]	Calories stored as fat[4]	DCI stored as fat
		gm	*(1000)*	*gm*	*gm*		*%*
			Male				
Stock (ground)	13	7491	24.5	422	98	959	3.9
Stock + corn oil	14	6284	24.4	469	121	1153	4.7
Stock + HVO	15	6468	25.1	473	114	1081	4.3
Stock + lard	15	6348	24.6	446	113	1074	4.3
PS 3% corn oil	12	6151	25.5	523	168	1596	6.2
PS 15% corn oil	14	5847	28.2	605	209	1983	7.0
PS 15% HVO	14	5108	24.7	486	153	1457	5.9
PS 15% lard	13	5542	26.8	511	161	1534	5.8
PC 3% corn oil	10	5687	22.8	424	108	1027	4.5
PC 15% corn oil	12	5206	24.7	482	132	1255	5.0
PC 15% HVO	12	5079	24.1	460	131	1249	5.1
PC 15% lard	11	5023	23.8	442	127	1210	5.1
			Female				
Stock (ground)	7	6228	20.3	271	61	579	2.8
Stock + corn oil	4	5335	20.7	308	102	969	4.7
Stock + HVO	5	4743	18.4	274	82	779	4.2
Stock + lard	7	5005	19.4	294	96	912	4.7
PS 3% corn oil	6[5]	5350	22.2	375	152	1444	6.5
PS 15% corn oil	4	4546	22.0	389	156	1482	6.7
PS 15% HVO	5	4171	20.2	363	162	1539	7.6
PS 15% lard	6	4516	21.8	379	150	1425	6.5

[1] See footnote 1, table 1.

[2] Digestible calorie intake.

[3] Carcasses of weanling rats, litter mates of animals used in this study and saved for carcass analysis, were lost due to a defective freezer. The percentage of fat in these animals at the start of the study was therefore assumed to be 5% (Conrad and Miller, '56).

[4] Grams fat × 9.5.

[5] Does not include one animal which ate 6710 gm and reached a weight of 662 gm, a gain of 613 gm in weight and of 287 gm body fat.

used. As would be expected, growth rate was proportional to digestible calorie intake ($r = 0.91$). Except for the group receiving hydrogenated vegetable oil, digestible calorie intakes were higher for the groups receiving sucrose than for those receiving corresponding diets containing cornstarch or the stock or stock plus fat diets. The male animals which received the diet with 15% corn oil and sucrose as the carbohydrate had the highest digestible calorie intake.

The test difference for the per cent of the total digestible calorie intake stored as fat (table 4) for the 12 groups of male animals is 1.3. All of the groups which received sucrose had a higher per cent of their calorie intake stored as fat when compared with the animals receiving the stock diets. Moreover, the groups which received sucrose and three or 15% corn oil had a higher per cent of their calorie intake stored as fat than did the animals receiving comparable diets containing cornstarch. It would be expected that the per cent of the total digestible calorie intake stored as fat would be highest for the group with the highest digestible calorie intake and this was actually the case. However, there seemed to be a difference in the conversion to body fat of calories from the different diets even when the caloric intakes were comparable. For example, the digestible calorie intakes of the male animals receiving the stock diet plus lard and the purified diet containing sucrose and 15% hydrogenated vegetable oil were 24,600 and 24,700; one would expect that about the same per cent of the calories would be stored as fat. However, the corresponding values for per cent digestible calories stored as fat were 4.3 and 5.9. Even larger differences were found for the female rats, where the intakes of the groups were 20,300 and 20,200 digestible calories, respectively on the stock diet and the purified diet containing 15% hydrogenated vegetable oil with sucrose, whereas the per cent stored as fat was 2.8 and 7.6. In a final comparison, the data for all male animals receiving diets containing sucrose, regardless of level or kind of fat, were combined and compared with a similar combination of the data for the animals which received the diets containing cornstarch. To rule out as far as possible the effect of variations in caloric intake, only those animals from all groups which had digestible calorie intakes between 23,000 and 28,000 were compared. The differences were highly significant between the per cent of the digestible calorie intake stored as fat by the rats receiving cornstarch (5.0 ± 0.31, 24 animals, DCI 25,200) and those receiving sucrose (6.1 ± 0.27, 30 animals, DCI 25,600). These findings are in agreement with the work of Feyder ('35) who reported that groups of rats pair-fed low-fat diets gained more weight on diets containing sucrose than on those containing dextrose and that the difference in the weight gains was due principally to fat.

The liver weights (table 5) of the 200-day-old animals were roughly proportional to body weights, varying from 3.0 to 3.2% of body weight. For the 400-day-old rats, liver weight varied from 2.8 to 3.7% of body weight. Fat in liver (wet basis) varied from 1.9 to 5.8% for 400-day-old male rats and from 2.5 to 7.0% for the female rats. The lowest value for the male rats was for the group receiving the low-fat stock diet, but the highest value for the female rats was for the group receiving the low-fat purified diet, so liver fat did not reflect level of dietary fat. All other values ranged from 2.7 to 5.6%.

Average weights of the kidneys of the 4 groups of 400-day-old male rats receiving diets containing sucrose were higher than normal (table 5). However, eliminating one value of 11.7 gm from those for the animals receiving the low-fat diet, and one of 17.4 gm from the group receiving hydrogenated vegetable oil lowered the average weights from 4.3 to 3.6 gm and from 4.5 to 3.5 gm, which is within the normal range for a pair of kidneys of rats of this weight and age. Of 13 animals which received 15% lard all but 4 had normal kidneys. Of 14 animals which received 15% corn oil, only three had normal kidneys. The average adrenal weight (table 5) was in the normal range.

The male rats killed at 400 days of age had significantly higher serum cholesterol values than those 200 days old (table 3). The average cholesterol values (table 3) of the various groups of male rats 200 days old ranged from 105 to 155 mg%

TABLE 5

Organ weights and liver composition of male and female rats fed various fat-containing or low-fat diets

Diet[1]	200-day-old rats				400-day-old rats					
	Liver (wet wt.)	Liver protein	Liver/body wt. × 100	Kidney wt. (pair)	Liver (wet wt.)	Liver protein	Liver fat	Liver/body wt. × 100	Kidney wt. (pair)	Adrenal wt.
	gm	*%*	*%*	*gm*	*gm*	*%*	*%*	*%*	*gm*	*mg*
					Male					
Stock (pelleted)	12.8	22.2	3.1	3.0	20.3	21.0	—	3.7	4.5	22.8
Stock (ground)	12.7	21.9	3.2	2.9	16.6	20.7	1.9	3.4	3.7	17.9
Stock + corn oil	13.1	21.1	3.0	3.0	16.6	20.4	3.9	3.2	3.6	20.0
Stock + HVO	12.3	21.5	3.0	2.7	18.3	20.2	4.0	3.5	4.0	18.6
Stock + lard	13.2	21.1	3.1	2.7	16.0	20.6	3.9	3.1	3.5	20.6
PS 3% corn oil	14.1	20.6	3.1	2.7	19.4	18.8	5.8	3.4	4.3	21.4
PS 15% corn oil	15.1	20.3	3.0	3.0	23.0	18.9	5.8	3.5	6.2	23.2
PS 15% HVO	14.4	20.6	3.0	2.9	17.1	19.4	4.6	3.1	4.5	17.9
PS 15% lard	14.1	20.7	3.0	2.8	18.5	19.2	4.7	3.2	4.3	24.3
PC 3% corn oil					15.6	19.8	4.1	3.3	3.2	21.7
PC 15% corn oil					17.0	19.5	5.6	3.1	3.5	21.6
PC 15% HVO					15.6	20.0	4.4	3.0	3.3	19.0
PC 15% lard					14.6	19.8	4.3	2.8	3.2	20.1
					Female					
Stock (ground)					12.1	20.8	2.7	3.7	2.5	37.7
Stock + corn oil					13.7	21.2	4.2	3.7	2.6	32.6
Stock + HVO					9.8	20.7	2.5	3.0	2.1	28.7
Stock + lard					10.4	20.7	3.2	3.0	2.2	38.9
PS 3% corn oil					15.6	19.1	7.0	3.6	3.2	37.9
PS 15% corn oil					15.4	20.5	4.5	3.4	[2]	31.2
PS 15% HVO					11.8	20.5	3.8	2.8	2.5	30.1
PS 15% lard					13.5	19.9	5.3	3.1	2.9	30.9

[1] See footnote 1, table 1.

[2] The 4 values 2.4, 2.7, 3.3 and 15.1 gm were not averaged.

(test difference, 25), and of those 400 days old from 146 to 356 mg%[10] (test difference, 79). Neither level nor kind of fat appeared to influence serum cholesterol levels in the 400-day-old male animals receiving the stock diets, or the purified diets containing cornstarch. There were no significant differences in serum cholesterol values of male animals fed the stock diet, and those fed the stock diet plus the various fats (175 to 237 mg%), nor did the values for the animals fed the various diets containing cornstarch differ with the several kinds of fat (146 to 172 mg%). But the serum cholesterol value of 356 mg% found for the 400-day-old animals fed 15% corn oil with sucrose was significantly higher than the values found for any other 400-day-old group.

Differences in serum cholesterol level of rats attributable to differences in dietary carbohydrate have been reported previously. Portman, Lawry and Bruno ('56) reported higher serum cholesterol for rats fed diets containing sucrose and 8% corn oil, with cholesterol and cholic acid added, than for those fed similar diets containing cornstarch for 28 days. In the present study, groups of male rats 400 days old fed cornstarch as the source of carbohydrate showed lower serum cholesterol values than corresponding groups of animals receiving sucrose, regardless of kind or level of fat. However, the difference between the groups was not significant when the fat was hydrogenated vegetable oil. Of the groups of females, those receiving the low-fat diet with sucrose as the carbohydrate had the highest average serum cholesterol value.

Serum cholesterol values have been shown to vary from one strain of animals to another (Kohn, '50). Some of the animals in the strain of rats used in the studies reported here developed kidney damage, and elevated serum cholesterol levels are known to be associated with some types of kidney damage. However, as pointed out by Stamler ('58) it is not necessary to attribute to species differences the apparently divergent results of experiments with unsaturated vegetable oils in man and in chick, rabbit and rat as there are also great differences in experimental design. Lower serum cholesterol values in human subjects have been found after replacing saturated fat with corn oil or other oils high in unsaturated fatty acids for relatively short periods of time. There are no data to show what would happen to human subjects were they fed these high levels of sucrose with corn oil for the proportion of the human life span that 400 days represents for the rat.

The group of male rats with the highest cholesterol values had the highest body fat and the highest digestible calorie intake. Nevertheless there was no correlation ($r = -0.27$) between cholesterol level and body fat or between digestible calorie intake and serum cholesterol level ($r = 0.23$) for animals of this group.

SUMMARY

Groups of male and female rats were fed stock or purified low-fat diets and similar diets with corn oil, hydrogenated vegetable oil, or lard added. Records of weight gains and food intakes were kept until the animals were killed when either 200 or 400 days old. Of the groups of male animals, those fed the low-fat stock diet and the low-fat purified diet with cornstarch as the carbohydrate had the lowest final body weights and those fed 15% corn oil, with sucrose as the carbohydrate had the highest final body weight. Weight gains were correlated with apparent digestible energy intake ($r = 0.91$) but not necessarily with fat intake; the average final weight of the group of animals receiving the low-fat diet with sucrose as the carbohydrate was not significantly higher than the weights of the animals which received 15% hydrogenated vegetable oil or lard. The addition of any of the fats improved digestibility of the energy supply of the stock diet but had no consistent effect on digestibility of the purified diets. Digestibility of nitrogen

[10] In 4 of the groups a single cholesterol value was found that was more than twice as high as the next highest value; this was omitted from the averages. These groups, and the figures omitted were as follows: ♂, stock, 400 days old, 714 mg%; ♂, sucrose, 15% corn oil, 400 days old, 1725 mg%; ♂, sucrose, 15% lard, 855 mg%; and ♀, sucrose, 15% lard, 400 days old, 609 mg%. Two other groups with 4 values each were considered to be too variable to average. These groups were ♀, stock plus corn oil, 400 days old, 138, 171, 396 and 1044 mg% and ♀, sucrose plus corn oil, 171, 201, 350 and 809 mg%.

supply and nitrogen retention expressed as per cent of nitrogen intake did not appear to be related to kind or amount of fat in the diet.

The animals receiving the purified diets containing sucrose and either three or 15% corn oil had significantly more body fat than those receiving corresponding diets containing cornstarch, or the stock diets. Of the male animals 400 days old, those which received 15% corn oil and sucrose had the highest body fat, and had the highest level of serum cholesterol, but there was no correlation ($r = -0.27$) between per cent body fat and serum cholesterol for animals of this group. The per cent of the digestible calorie intake stored as fat was significantly higher for the groups of animals fed the diets containing sucrose and three or 15% corn oil than for animals receiving comparable diets containing cornstarch, or the stock diets. When animals with the same calorie intake were compared, regardless of kind or level of fat, those which received sucrose stored a higher per cent of their caloric intake as fat than those which received cornstarch.

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The Excretion of Nitrogen by the Rat in Vitamin B_6 Deficiency[1]

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In vitamin B_6 deficiency in the rat the concentration of urea in the blood is high both when the animal is in the fasting state (Hawkins et al., '46; Beaton et al., '53a), and after the administration of test loads of amino acids (Hawkins et al., '46; Beaton et al., '50; Beaton et al., '53b). This is apparently not the result of impaired excretion (Beaton et al., '53a), and seems likely to be the reflection of a disturbance in amino acid metabolism. This interpretation is supported by the findings that in vitamin B_6-deficient rats there is an increased excretion of nitrogen (Beaton et al., '53a), and that in liver slices from them there is an increased production of urea (Caldwell and McHenry, '53).

It seems probable that in vitamin B_6 deficiency the rat excretes increased amounts of urea. The present experiments were undertaken in order to ascertain this, and to obtain details concerning the partition of urinary nitrogen in this condition. Comparison was made with control animals fed the same amount of food, and with others on restricted food intakes, which would be expected to excrete more urea as a result of the oxidation of a higher proportion of amino acids for energy production.

EXPERIMENTAL PROCEDURES

In two experiments Wistar rats of both sexes were used, at starting weights of 80 to 100 gm. In each case 60 animals were divided into three sub-groups with the same average body weights. Those in sub-group C were deprived of vitamin B_6. Those in sub-group A were given the vitamin, and were fed the same amounts of food as those voluntarily eaten by the vitamin-deficient animals. Those in sub-group B were also given the vitamin, but were fed smaller amounts of food, to keep their weights comparable to those in sub-group C.

The diet was high in protein because that was considered an advantage for studying the nitrogen excretion. Its percentage composition was Vitamin-Test casein 40, sucrose 45, cellulose flour 4, corn oil 7. The following supplements were added per 100 gm: cod liver oil concentrate 0.2 mg (40 I.U. vitamin A and 10 I.U. vitamin D), α-tocopherol acetate 3 mg, 2-methyl naphthoquinone 0.2 mg, choline chloride 100 mg, inositol 50 mg. The diet contained 5.5 to 5.7% nitrogen as determined by the Kjeldahl method.

Each rat was given by subcutaneous injection per day: thiamine hydrochloride 12.5 µg, riboflavin 25 µg, calcium pantothenate 100 µg, nicotinic acid 20 µg, *p*-aminobenzoic acid 20 µg, biotin 2 µg, folic acid 2 µg, vitamin B_{12} 0.01 µg. Control animals were given in addition 10 µg of pyridoxine hydrochloride.

After 67 to 83 days differences in weight of about 15% were shown between the animals of sub-group A and those of B and C. For the rest of the experimental time they were all fed the same amount of food per unit of body weight, this being 4.5 to 6.0 gm per 100 gm, depending upon the appetite of the animals in sub-group C. On the 5th to the 8th day of this regimen urine, and in one experiment feces, were collected from the animals of sub-group C, and on the following two days from those of B and A respectively.

The urine was collected in dilute acid and toluene. Total nitrogen, urea (Archibald, '45), allantoin (Young and Conway, '42), uric acid (Folin, '33; '34), ammonia

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TABLE 1

The excretion of nitrogen in rats as affected by vitamin B_6 deficiency[1]

	Total	Fecal	Urinary								
			Total*	Urea		Ammonia		Allantoin	Uric acid	Creatinine	Amino acid
Group	I	I	I	I	II	I	II	I	I	I	I
Sub-group[2]											
A	740–970	40–80	691–908	628–819	501–641	51–55	44–64	8.8–12.3	0.6–0.8	4.3–4.8	6.7–7.8
	890	*60*	*821 ± 62*	*728 ± 51*	*582 ± 50*	*53*	*55 ± 6.4*	*10.6*	*0.7*	*4.5*	*7.1*
B	690–1200	20–110	652–1087	583–920	469–728	55–63	44–65	8.8–12.7	0.5–0.8	4.0–4.3	6.8–8.2
	950	*60*	*902 ± 99*	*771 ± 79*	*645 ± 63*	*59*	*60 ± 5.5*	*10.2*	*0.7*	*4.1*	*7.6*
C	840–1030	20–80	812–975	671–868	547–674	58–70	47–65	8.1–14.1	0.6–0.7	4.1–4.4	5.4–6.3
	920	*50*	*877 ± 47*	*766 ± 48*	*617 ± 40*	*64*	*55 ± 4.8*	*10.6*	*0.7*	*4.3*	*5.8*

[1] Values are expressed as micrograms of excreted nitrogen per milligram of ingested nitrogen. Ranges, averages (in italic) and some standard deviations are shown. The average values for sub-group A differed sufficiently from those of both B and C to give a P value of <0.01 for total urinary nitrogen in group 1 and of < 0.05 for urea nitrogen in both groups. In group I the sums of the urinary nitrogen fractions were 95 to 98% of the total* urinary nitrogens as determined by Kjeldahl.

[2] A, food controls; B, weight controls; C, vitamin B_6-deprived.

(Delory, '49), creatinine (Folin and Wu, '19), and amino acid nitrogen (Frame et al., '43; Russell, '44) were determined in one experiment (group I), and urea and ammonia in the other (group II). Modifications were made in some of the methods. Those in which visual colorimetry was originally used were adapted for use with the Coleman Model 14 Universal Spectrophotometer. The total nitrogen of urine, feces, and diets was determined by a titrimetric microKjeldahl procedure (Hiller et al., '48; Sobel et al., '44).

RESULTS

The results are shown in table 1, in which the excreted nitrogen is expressed in relation to the ingested nitrogen. Since the rats were fed on the basis of body weight during the time of the metabolic tests, the excreted nitrogen is therefore also expressed in relation to the body weight.

It is apparent that when there is an increased excretion of nitrogen in vitamin B_6 deficiency in the rat, it is a reflection of an increased output of urea. In this respect the animals resemble those whose growth has been impaired to the same extent by simple food restriction.

DISCUSSION

The results of these experiments are in harmony wtih those which have shown in rats high blood urea (Hawkins et al., '46; Beaton et al., '53a) and increased production of urea by the livers (Caldwell and McHenry, '53) in vitamin B_6 deficiency. There is apparently an increased oxidation of amino acids, which would explain the increased output of urea in the inanition control animals, with which the vitamin-deficient animals were comparable in this regard.

One possible explanation for this is that vitamin B_6 deficiency affects the oxidation of amino acids the least of any of the major metabolic processes by which they are utilized, and that consequently more of them are channeled into the oxidative systems.

SUMMARY

The excretion of nitrogen and its urinary partition were studied in rats which showed impairment of growth from deprivation of vitamin B_6 on a diet containing 40% of protein. Comparison was made with two control groups: animals which had received the same amount of food, and those whose growth had been restricted by feeding them smaller amounts of food. For a short period preceding, and during the metabolic tests the animals were fed on the basis of body weight.

The vitamin B_6-deficient animals excreted more urea in relation to the ingested nitrogen than the controls which had previously been maintained on the same level of food intake. In this respect their nitrogen metabolism was similar to that of the controls whose growth had been restricted to the same extent by limitation of food intake.

The results are in harmony with other findings regarding the state of nitrogen metabolism in vitamin B_6-deficient rats, and they point to an increased oxidation of amino acids.

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The Amino Acid Composition and the Nutritive Value of Proteins

I. ESSENTIAL AMINO ACID REQUIREMENTS OF THE GROWING RAT[1]

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Attempts have been made to evaluate the nutritive value of proteins from their amino acid content. Mitchell and Block ('46) used the amino acid composition of whole egg protein as the standard for computing the "chemical score" of protein based on the most limiting amino acid and obtained a correlation of 0.86. Oser ('51), instead of using the most limiting essential amino acid alone, calculated an amino acid index for proteins by a summation of the differences between the concentration of all the essential amino acids in the protein and their concentration in whole egg. Mitchell ('54) improved on Oser's method by omitting arginine as an essential amino acid, and including tyrosine with phenylalanine and cystine with methionine, retaining egg as the primary standard. However, the essential amino acid and total protein requirements of the rat should serve as a better standard for calculation of amino acid indices. Thus, these requirements have been redetermined for this purpose. A preliminary account of this work was reported earlier by our group (Rama Rao et al., '57) and is detailed in this paper.

METHODS AND MATERIALS

Male weanling rats[2] (40 to 50 gm) were used in 21-day growth tests on casein-amino acid diets. The composition of the basal diet is given in table 1. Casein[3] at a 5% level in the diet provided part of each of the essential amino acids (table 2). The amino acid composition of the batch of casein used was determined by chromatography on Dowex 50 resin according to Moore and Stein ('51). Duplicate values for each of the amino acids agreed closely and the error was not more than 2% of the mean. Tryptophan was determined by the method of Spies and Chambers ('48). Six determinations of tryptophan by this

TABLE 1
Composition of the basal diet

Constituent	Amount
	%
Casein	5.0
Starch	51.0[1]
Cerelose	10.0
Sucrose	10.0
Lard	5.0
Corn oil	5.0
Wheat germ oil	0.5
Cod liver oil	1.5
Vitamized cerelose[2]	5.0
Mineral mix 446[3]	4.0
Sodium chloride	1.0
Woodflock[4]	2.0
Total	100.0

[1] Free essential L-amino acids and glycine were added as required at the expense of starch in the diet. All the amino acids were purchased from the California Foundation for Biochemical Research, Los Angeles, California.

[2] The percentage composition of vitamized cerelose is as follows: Thiamine hydrochloride and riboflavin 20 mg each; calcium pantothenate 100 mg; pyridoxine hydrochloride 10 mg; nicotinic acid 40 mg; folic acid 4 mg; *p*-aminobenzoic acid 100 mg; menadione 0.2 mg; biotin 0.2 mg; vitamin B_{12} 2 mg; inositol 660 mg; choline chloride 1.32 gm; and cerelose to 100 gm.

[3] Spector ('48).

[4] Brown Company, Chicago, Illinois.

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[2] Sprague-Dawley.

[3] Labco.

TABLE 2

Casein-amino acid diets

Amino acid	Amino acid of diet provided by 5% casein (0.8 gm N)		Total amino acid in diet tested
	%		%
L-Lysine	0.42		0.8, 0.9, 1.0
L-Tryptophan	0.07		0.07, 0.11, 0.16, 0.21
L-Isoleucine	0.38		0.45, 0.5, 0.55, 0.6, 0.65, 0.70
L-Valine	0.39		0.39, 0.51, 0.56, 0.61, 0.71
L-Methionine	0.171	0.19	0.19, 0.29, 0.39, 0.49, 0.59
L-Cystine	0.019		
L-Threonine	0.23		0.41, 0.46, 0.51, 0.56, 0.61, 0.66
L-Leucine	0.49		0.49, 0.59, 0.69, 0.79
L-Phenylalanine	0.32	0.58	0.58, 0.72, 0.82, 0.92
L-Tyrosine	0.26		
L-Histidine	0.17		0.17, 0.21, 0.26, 0.31, 0.36, 0.41

method yielded an average value of 1.4% with an error of less than 3%. Free essential L-amino acids were added to the diet to increase each of them in the diet to the minimum requirement according to Rose et al. ('48), with the exception of the one that was under test at different levels. All the diets were adjusted to 10% protein (N × 6.25) in the diet by adding calculated quantities of glycine. The calculated nitrogen contents of the diets were confirmed by the Kjeldahl method. The rats were fed ad libitum. Daily food intake and growth data were obtained for a period of three weeks.

Preliminary growth tests indicated that supplements of isoleucine added to a diet containing the essential amino acids at 80% of the requirement at the 10% protein level given by Rose et al. ('48) produced a striking improvement in growth of from 1.6 to 3.7 gm per day, indicating that this amino acid mixture is limiting in isoleucine.

RESULTS

Lysine. It is apparent from the data in table 3 that lowering lysine from 0.9 to 0.8% in the diet significantly decreases the growth of the rat. Also, no significant difference in growth is noticed when the lysine content is increased from 0.9 to 1.0% of the diet.

Tryptophan. The data in table 3 show the requirement of tryptophan to be 0.11% of the diet. A decrease of tryptophan level from 0.21 to 0.11% does not result in any significant depression of growth or of efficiency of protein utilization. Williams et al. ('54) reported a requirement of 0.1% in the diet estimated on the basis of amino acid composition of the dry, fat-free rat carcass.

Isoleucine. The data in table 3 clearly show that a level of 0.55% isoleucine in the diet is needed for maximum growth. No improvement in growth or efficiency of protein utilization is obtained by increased supplements of isoleucine beyond the 0.55% level. Growth and protein utilization are lower both at the 0.5% level ($P < 0.05$) and at the 0.45% level ($P < 0.01$) as compared with that obtained at 0.55% level in the diet.

Valine. The data on valine requirement (table 3) show that the generally accepted level of 0.7% in the diet (Rose et al., '48, '49) is an overestimate. It is seen that decreasing it to the 0.55 (0.56) level does not lower either growth or efficiency of protein utilization.

Threonine. The data on threonine requirement (table 3) are in agreement with the value of 0.5% given by Rose et al. ('48). At 0.4 and 0.45% of threonine in the diet, growth is significantly less at the 1% ($P < 0.01$) and 5% ($P < 0.05$) levels, respectively. The efficiency of protein utilization is also significantly lowered ($P < 0.02$) at these levels of threonine in the diet. No significant increase in weight gain or protein utilization is evident at higher levels.

Methionine + cystine. The data presented in table 3 for the methionine +

TABLE 3

Minimum amino acid requirements for maximum growth for the rat (21-day growth test)

Amino acid	Protein (N × 6.25)	Weight gain	Protein efficiency ratio[1]
	%	%	*gm/day*
Lysine			
0.8	10.1	3.5 ± 0.21[3]	3.9 ± 0.13
0.9(8)[2]	9.8	4.4 ± 0.25	4.1 ± 0.11
1.0	9.9	4.4 ± 0.20	4.2 ± 0.13
Tryptophan			
0.07(5)	10.0	0.9 ± 0.20	2.2 ± 0.29
0.11(5)	9.8	4.7 ± 0.17	4.2 ± 0.18
0.16	9.9	4.3 ± 0.17	4.1 ± 0.17
0.21	9.9	4.4 ± 0.20	4.2 ± 0.13
Isoleucine			
0.45	9.7	2.6 ± 0.30	3.5 ± 0.41
0.50(8)	9.6	3.8 ± 0.18	4.0 ± 0.34
0.55(8)	9.6	4.4 ± 0.14	4.8 ± 0.10
0.60	9.8	4.3 ± 0.20	4.7 ± 0.15
0.65	9.8	4.2 ± 0.24	4.5 ± 0.30
0.70	9.7	4.2 ± 0.19	4.9 ± 0.25
Valine			
0.39(5)	9.8	1.3 ± 0.22	2.9 ± 0.30
0.51(5)	9.7	3.5 ± 0.21	3.6 ± 0.18
0.56	9.8	4.3 ± 0.15	4.2 ± 0.13
0.61	9.9	4.4 ± 0.20	4.3 ± 0.16
0.71	10.0	4.4 ± 0.20	4.2 ± 0.19
Threonine			
0.41(5)	10.0	3.6 ± 0.18	3.6 ± 0.17
0.46	10.1	3.7 ± 0.22	3.5 ± 0.21
0.51	10.1	4.4 ± 0.20	4.2 ± 0.13
0.56(8)	10.0	4.7 ± 0.22	4.3 ± 0.14
0.61	10.0	4.6 ± 0.21	4.4 ± 0.16
0.66	10.0	4.7 ± 0.22	4.5 ± 0.18
Leucine			
0.49(5)	10.0	1.9 ± 0.21	2.9 ± 0.30
0.59	10.0	3.7 ± 0.12	4.0 ± 0.21
0.69	10.1	4.3 ± 0.19	3.9 ± 0.14
0.79	9.8	4.4 ± 0.20	4.2 ± 0.13
Histidine			
0.17(5)	10.0	1.8 ± 0.19	2.8 ± 0.29
0.21	10.0	4.2 ± 0.23	4.1 ± 0.13
0.26	10.0	4.3 ± 0.28	4.0 ± 0.16
0.31	9.8	4.5 ± 0.17	4.2 ± 0.16
0.36	10.0	4.2 ± 0.16	4.0 ± 0.10
0.41	9.8	4.4 ± 0.20	4.2 ± 0.13
Methionine + cystine			
0.19	10.0	0.9 ± 0.19	2.2 + 0.21
0.29	10.0	2.5 ± 0.24	3.1 ± 0.14
0.39	10.0	3.6 ± 0.22	3.7 ± 0.19
0.49	9.7	4.2 ± 0.16	3.9 ± 0.15
0.59	9.8	4.4 ± 0.20	4.2 ± 0.13
Phenylalanine + tyrosine			
0.58	10.0	2.0 ± 0.17	2.2 ± 0.28
0.72	10.1	4.2 ± 0.13	3.9 ± 0.15
0.82	10.1	4.0 ± 0.12	3.9 ± 0.11
0.92	9.8	4.4 ± 0.20	4.2 ± 0.13

[1] Protein efficiency ratio is the weight gain (in grams) per gram protein intake.
[2] Figures within parentheses indicate number of rats used in that group. Six rats were used in all other groups.
[3] Standard deviation of the mean.

cystine requirement show that maximum growth is obtained at a total of 0.5% (0.49) of methionine + cystine in the diet. No significant increase is obtained in either growth or protein utilization by increasing the methionine supplement to 0.6%. A significant decrease in growth rate is noticed at levels below 0.5%. Williams et al. ('54) calculated a requirement of 0.43% based on rat carcass assay.

Histidine. The requirement of histidine reported by Rose et al. ('48) is 0.4% of the diet. The data in table 3 show that even at the 0.20% (0.21) level no significant decrease in growth or protein utilization is obtained. At the 0.17% level, however, a significant drop in the growth rate and protein utilization results. This value of 0.2% (0.21) is close to the value of 0.24% provided by 10% of whole egg.

Leucine. The data in table 3 show that equal growth and protein utilization are obtained with 0.7% (0.69) or 0.8% (0.79) of leucine in the diet, and no deleterious effect is produced on growth of rats on diets containing 0.7% (0.69) of leucine and nutritionally adequate otherwise. At the 0.5% level, decrease in growth is highly significant and at 0.6% in the diet the decrease is significant at the 5% level ($P < 0.05$; < 0.01).

Phenylalanine + tyrosine. The data on the requirement of phenylalanine + tyrosine show that maximum growth is obtained at 0.72% in the diet. Further supplementation with phenylalanine up to a total of 0.9%, the requirement reported by Rose ('48), does not improve growth or protein utilization. The total was made up of 0.26% of tyrosine from casein and 0.32% of phenylalanine from casein and the added L-phenylalanine.

TABLE 4

Effect of increasing all the essential amino acids above the minimum requirement level (21-day growth test)

Diet	Protein	Weight gain
	%	*gm/day*
A. Casein-amino acid diet	11.0	4.6
B. Diet A + 10% more of each of the essential amino acids	12.0	4.6

Growth on the minimal diet. It was considered necessary to obtain growth data for this casein-amino acid ration containing the concentrations of essential amino acids which had been shown to meet minimum requirements. Also, to find out if any of these amino acids would turn out to be limiting, each of the essential amino acids added was increased by another 10%, and comparative growth data obtained. The results are reported in table 4.

DISCUSSION

It is clear from data in table 4 that a diet providing the essential amino acids at these minimum levels supports maximum growth of the rat. The lack of any increase in growth with additional supplements of the essential amino acids supports this statement.

The minimum requirements for the essential amino acids arrived at are lower than those reported by Rose et al. ('48, '49) for all of the essential amino acids except threonine and isoleucine. The requirement value, 0.5%, obtained for threonine is the same value as that reported by Rose et al., whereas the requirement for isoleucine is 0.55% as compared with 0.5% given by Rose et al. ('48). It must be emphasized, however, that Rose et al. used pure amino acid diets, whereas we used casein-amino acid diets. Hence the difference in the rate of availability of amino acids due to their enzymatic release from a protein during digestion may be significant. Also in the studies by Rose and associates the minimum requirement for any one essential amino acid was determined with the use of diets containing the other essential amino acids far in excess of their requirement levels. In our studies all the essential amino acids were at their requirement levels except the one under test. The individual variations in rats tends to become noticeable at near-marginal levels. The total protein ($N \times 6.25$) level in the diets in our studies was maintained at 10% of the diet and only the L forms of essential amino acids were incorporated into the diets, whereas the diets as used by Rose et al. ('48) contained DL forms of some of the essential amino acids in a total of 12.5 to 15% of protein ($N \times 6.25$).

Rose et al. ('54) have pointed out that the human subject, at least, seems to require more energy to maintain nitrogen equilibrium on an amino acid mixture. However, Metta, Firth and Johnson ('57) reported that the energy requirement of the adult rat does not increase or decrease if the protein of the diet is replaced by its natural forms of the amino acids.

SUMMARY

The minimum requirements for the essential amino acids by the weanling rat for maximum growth have been determined, using casein-amino acid diets at the 10% protein level. Growth tests were of 21 days' duration.

The requirements for lysine, histidine, tryptophan, isoleucine, leucine, valine, threonine, methionine + cystine, and phenylalanine + tyrosine are: 0.9, 0.21, 0.11, 0.55, 0.69, 0.56, 0.51, 0.49 and 0.72% of the diet, respectively.

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Urolithiasis in the Rat[1,2]

I. THE INFLUENCE OF DIET ON THE FORMATION AND PREVENTION OF CALCIUM CITRATE CALCULI

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Numerous studies have been reported in the literature on the effect of the mineral content of diets on the severity of dental caries in rats. In the course of similar investigations on the caries-susceptible Osborne-Mendel rat of the NMRI-D strain (Losee and Gerende, '57), it was observed that numerous rats died on some diets which appeared to be adequate. Autopsies revealed extreme distention of the bladder, accompanied by extensive stone formation in the urinary apparatus (Van Reen, Lyon and Losee, '58). Further studies were performed to elucidate the role of the diet in the production of the syndrome. This has led to the conclusion that the NMRI-D strain of rat may be more sensitive to the mineral level of the diet than other strains and that the casein level of the diet plays a part in the prevention of citrate urolithiasis.

Other investigations of urinary calculi in experimental animals have led to valuable information concerning the factors which are predisposing to this condition. Osborne, Mendel and Ferry ('17) demonstrated the influence of vitamin A deficiency on stone formation while Rosenow and Meisser ('22) clearly illustrated the effect of chronic infection. Keyser ('45) and Wilson, Benjamin and Leahy ('45) directed attention to high dietary minerals in causing urinary calculi while Schneider and Steenbock ('40) indicated that diets extremely low in phosphates would lead to urolithiasis. The various factors which may be involved in urolithiasis in man along with a review of the initiating lesion-nidus or nucleus of stones are discussed in a recent publication by Burkland ('54).

EXPERIMENTAL

Weanling, albino rats of the NMRI-D strain were used in most experiments and, except where indicated, males were employed. At 21 days of age, rats weighing between 25 and 45 gm were randomly distributed into the desired groups and placed in individual stainless steel cages with screen bottoms. Food and water were provided ad libitum. External conditions were controlled so that the range of temperature was from 22 to 25°C and the humidity from 50 to 70%.

All experimental animals were weighed weekly and the average body weights in each group calculated. Any animal which succumbed during the course of the experiment was eliminated from the group means.

Phosphate contents of the diets were determined by calculation and included that contributed by the salt mixture and casein. As a check, the phosphate content was determined chemically by the following procedure: 200 to 250 mg of diet were digested with 2.0 ml of concentrated H_2SO_4 in the presence of 0.3 gm K_2SO_4 and a selenium chip. After digestion the clear solution was analyzed for phosphate by the method of Fiske and Subbarow ('25). Twenty determinations of phosphorus checked the calculated values within 1%. The calcium contents of the diets were determined also by calculation and by chemical analysis using the method of Clark and Collip ('25). For these analyses 1.0 to 2.0 gm of diet were ashed by heating overnight at 500°C, the ash taken up in acid

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and determined by the titrametric technic. Similar tests were used for the calculi. Eight calcium determinations on the diets checked the calculated values within 1.5%.

The calculi which were found were washed thoroughly, dried, and analyzed according to standard methods (Hawk, Oser and Summerson, '47). When it was found that the stones would decolorize $KMnO_4$ but were not oxalate, determinations of citrate were performed by the pentabromoacetone method of Pucher as modified by Hess and White ('55).

Autopsies were performed in all cases where death occurred. Experiments were terminated after 35 days, all animals sacrificed, and examined for kidney, ureteral, bladder and urethral calculi.

The composition of the various diets used in the experiments are presented along with the statistics regarding the occurrence of .calculi. For simplicity in discussing the results, the diets are labelled using the protein and mineral contents, i.e., 20P2 equals 20 gm of casein and 2 gm of Hubbell, Mendel and Wakeman ('37) salt mixture and 30P4 equals 30 gm of casein and 4 gm of the same salt mixture. An asterisk indicates that the diet has been modified in some manner from the basic formula.

RESULTS

Urinary calculi. The occurrence of urinary calculi with various diets in the first series of experiments is summarized in table 1. It was readily seen that while stones occurred with the 20% casein diet and 4% Hubbell, Mendel and Wakeman salt mixture, none occurred at the 30 or 40% casein levels. Urolithiasis did not appear in rats fed the 20P2 diet, however numerous stones were observed in rats fed the 15P2 and 15P4 diets. Diet 20P2* was formulated to contain the calcium concentration found in the 20P4 diet by the inclusion of $CaCO_3$. This addition of $CaCO_3$ resulted in the formation of numerous stones throughout the urinary tract. This suggests that it is this salt of the Hubbell, Mendel and Wakeman salt mixture which is important in the etiology of calculi formation in the present situation. A group of 20 female NMRI-D strain rats was also fed the 15P4 diet, although not reported in the table. One of the female rats died during the course of the 35 days with severe calculi formation. Ten additional rats had calculi at the end of the experiment. It thus seems probable that the etiology of the calculus formation is similar in the two sexes but the male is more likely to develop blockage of the urinary tract and subsequent uremia and death.

It should be noted that in the diets listed in table 1 the increased amounts of casein were added at the expense of the sucrose. Thus, the high-casein diets on which the rats did not develop calculi contained the

TABLE 1

The composition of diets and occurrence of urinary calculi

Ingredient	Diets						
	20P4	30P4	40P4	20P2	15P2	15P4	20P2*
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Vitamin-test casein	20.0	30.0	40.0	20.0	15.0	15.0	20.0
Sucrose	71.8	61.8	51.8	71.8	76.8	76.8	71.8
Salt mixture[1]	4.0	4.0	4.0	2.0	2.0	4.0	2.0
Vitamin mixture[2]	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Linoleic acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Corn oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$CaCO_3$							1.0
Ca content	0.86	0.86	0.86	0.43	0.43	0.86	0.86
P content	0.31	0.37	0.43	0.21	0.19	0.28	0.21
No. of rats	18	18	18	20	20	20	20
Deaths with calculi	6	0	0	0	3	5	5
Total with calculi	16	0	0	0	14	17	14
Wt. gain (35 days), *gm*	126	148	162	122	95	73	98

[1] Formulated per Hubbell, Mendel and Wakeman ('37).

[2] Complete Vitamin Diet Fortification Mixture (Van Reen, Lyon and Losee, '58).

least sucrose and it was not certain whether casein tended to prevent lithiasis or whether sucrose predisposed rats to the condition. To resolve this question several diets were formulated as shown in table 2. The diets differed from those previously used in that, as the casein level was decreased, a corresponding amount of non-nutritive cellulose was added to adjust for the decrease. In this way, all the diets had the same amount of sucrose. It was anticipated that if sucrose predisposed rats to urolithiasis, all groups would have the same proportional occurrence of calculi. It can be seen, however, that 60% of the rats fed the 15P2* diet developed calculi whereas only 15% of the animals fed 20P2 did so. These findings suggest that the casein/mineral ratio of the diet is of prime importance in the etiology of stone formation under our conditions. Further intimation of this is found in the very similar proportion of rats with calculi on diets 15P4 (table 1) and 15P4* (table 2), although the latter had 5% less sucrose than the former.

The results in this experiment differed from those reported in table 1 in that some rats fed the 20P2 diet developed stones. This suggests that for the NMRI-D rat this proportion of casein and mineral is not quite adequate for complete protection from lithiasis.

Emmel ('57) and Moore et al. ('58) have shown that the unsaturated fatty acids can bring about post-mortem histological changes in kidneys of rats. It was thought that possibly the linoleic acid provided the NMRI-D rats was having an influence on the occurrence of calculi through some kidney change. Therefore, groups of rats were fed the 15P4 diet modified by removal of the linoleic acid or by including 2 or 4% of the fatty acid. None of the rats died during the course of the experiment; however, after 35 days when the rats were sacrificed and examined for lithiasis, an equal number of stones occurred in all groups and therefore this does not appear to play a role in the current studies.

Composition of the urinary calculi. A variety of tests were made on calculi removed from rats. Negative tests were obtained for uric acid, xanthine, cystine, and carbonate. A trace of phosphate was found to be present. A strong positive test was obtained for oxalate by the decolorization of $KMnO_4$. The stones also appeared to be oxalate according to the test which involves heating the stone to convert oxalate to carbonate and then testing for effervescence with HCl. A quantitative titration with $KMnO_4$, however, revealed that the calculi could not be oxalate; therefore further tests were conducted. Testing for oxalate with resorcinol and with MnO_2 gave negative results. Since citrate has many of the properties of oxalate, as far as decolorization of $KMnO_4$ and conversion to carbonate on heating are concerned, specific tests for citrate were made. The pentabromoacetone test for citrate was positive and quantitative determinations for citrate demonstrated that the calculi were mainly

TABLE 2

The composition of diets equalized in sucrose content and the occurrence of urinary calculi

Ingredient	Diets				
	15P2*	17P2*	19P2*	20P2	15P4*
	gm	*gm*	*gm*	*gm*	*gm*
Vitamin test casein	15.0	17.0	19.0	20.0	15.0
Sucrose	71.8	71.8	71.8	71.8	71.8
Salt mixture[1]	2.0	2.0	2.0	2.0	4.0
Vitamin mixture	2.2	2.2	2.2	2.2	2.2
Linoleic acid	1.0	1.0	1.0	1.0	1.0
Corn oil	1.0	1.0	1.0	1.0	1.0
Non-nutritive cellulose	5.0	3.0	1.0	0.0	5.0
No. of rats	20	20	20	20	20
Deaths with calculi	2	0	2	0	5
Total with calculi	12(60%)	10(50%)	4(20%)	3(15%)	17(85%)

[1] Hubbell, Mendel and Wakeman ('37).

citrate. The presence of citrate was substantiated by the microdiffusion assay of Bessman and Anderson ('57). Quantitative tests by this method indicated that the stones contained 71% citric acid. Calcium determinations on 8 stones indicated a mean concentration of 19.3% of calcium compared with 21.1% in pure tricalcium citrate. These data made it certain that the calculi formed under our conditions had the composition of calcium citrate with minor contaminations.

Additional information on the renal calculi was obtained from the pyrolysis curves recorded on a thermo-recording balance. This balance allows the sample to be heated, weighed and recorded simultaneously. Table 3 shows the changes in weight sustained by samples of calculi and $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$ on being heated from room temperature to 1050°C, the temperature being raised uniformly at the rate of 400°C per hour. The citrate sample and the calculi were held at the plateaus indicated in the table until the loss of weight was less than 1 mg per hour.

Further tests on the calculi indicated the presence of small amounts of protein and amino acids. It is not certain whether the protein fraction was the nidus or nucleus for calculi formation or whether it was incorporated during the development of the concretions. The amino acids and phosphate are constituents of urine and can probably be considered as contaminants.

Comparison of NMRI-D, Sprague-Dawley, and Long-Evans strains of rats. The diets which were used in the present study were not markedly different from those used without deleterious effects by other investigators in innumerable experiments which have been reported in the literature. It was desirable, therefore, to determine whether the NMRI-D strain was more susceptible to stone formation than other strains. Groups of 20 male rats between 20 and 22 days of age and of the NMRI-D, Sprague-Dawley, and Long-Evans strains were maintained under the same environmental conditions and fed diet 15P2 (table 1). Within the 5-week experimental period three deaths occurred in the NMRI-D group of rats, whereas none occurred with the other strains. Severe calculi formation was obtained with the NMRI-D strain (35% of the rats), only two of the Sprague-Dawley rats showed small concretions and none of the Long-Evans rats showed any indications of calculi on autopsy. While further data must be obtained using a variety of diets, these observations suggest that the NMRI-D rat is somewhat more susceptible to calculi formation on this particular diet.

TABLE 3

Pyrolysis data on renal calculi

Conditions Loss from	$Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$	Calculi
	%	%
23° to 195°C	12.36	18.33
195° to 365°C[1]	12.73	13.63
365° to 550°C[1]	26.84	26.68
550° to 1050°C[1]	26.73	24.95
195° to 1050°C[1]	66.30	65.26

[1] Calculated from 195°C weight.

DISCUSSION

The occurrence of calcium citrate calculi appears to be a relatively unusual situation. Most review articles concerning the composition or assay of calculi do not mention citrate except its use in the dissolution of stones. A review of the literature revealed, however, that calcium citrate stones have been reported when rats were fed diets low in phosphate. Schneider and Steenbock ('40) demonstrated the formation of such calculi when the diet contained 0.57% of calcium and only 0.04% of phosphorus. They also quote the work of Verkoren who produced stones with a diet containing 3% of $CaCO_3$. In further studies by Morris and Steenbock ('51) similar results were obtained, however, with 0.48% of calcium and 0.035% of phosphorus none of the young rats developed stones in 19 weeks, presumably because of the slightly lower calcium. Sager and Spargo ('55) worked with adult Sprague-Dawley rats and were able to produce calcium citrate calculi with diets containing only 0.03% of phosphorus.

The results of the present experiment indicate that the extremely low phosphorus diets used in the above experiments may not be necessary for calculus formation in the NMRI-D rat. The calcium/phosphorus ratio of the 15P2 diet (0.43/0.19) can be considered within a normal range and yet the young, NMRI-D rats fed this ration

developed uroliths. This observation and the suggestion of a difference in susceptibility among strains indicates that other factors must be involved. It is of interest in this regard that Day and McCollum ('39) fed rats with a diet having a calcium/phosphorus ratio of 0.40/0.017 but did not observe the occurrence of urinary calculi.

The possibility that the protein level of the ration plays some role in preventing the formation of calculi deserves attention. A comparison of diet 30P4 with 15P2 indicates that the calcium/phosphorus ratios of the two diets are the same; the former contains twice the total mineral content, but the latter produces calculi. A similar comparison can be made between the 40P4 and 20P2 diets; however, the latter diet appears borderline, one time giving calculi and another time not giving them. The protein level of the diet may be a significant factor in view of the observations of Sager and Spargo ('55) who showed that their protein-depleted animals had an early and high incidence of calcium citrate calculi.

SUMMARY

In the course of studies on the influence of dietary minerals on the severity of caries in the NMRI-D, caries-susceptible, strain of rat, it was observed that numerous animals died when fed what appeared to be an adequate diet. Autopsies revealed extensive stone formation in the urinary tract.

The casein-mineral ratio of the diet appears to be the determining factor in the formation or prevention of the uroliths. Thus, a high casein level or a low concentration of Hubbell, Mendel and Wakeman salt mixture in the diet, or both resulted in normal animals without the appearance of calculi.

Rats of the NMRI-D strain formed numerous calculi on a diet containing 15% of casein and 2% of Hubbell, Mendel and Wakeman salt mixture, whereas only a few of the Sprague-Dawley rats showed small concretions and none of the Long-Evans rats showed any sign of calculi on autopsy. This suggests that the NMRI-D strain is the most sensitive of the three strains to dietary alterations.

The stones formed in the present experiment consisted almost entirely of calcium citrate. This type of concretion has been reported previously in rats only under conditions of extremely low phosphorus intake.

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Urolithiasis in the Rat[1]

II. STUDIES ON THE EFFECT OF DIET ON THE EXCRETION OF CALCIUM, CITRIC ACID AND PHOSPHATE

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During studies on the influence of dietary minerals on the severity of dental caries in the NMRI-D strain of rat, it was observed that numerous animals died on some dietary regimens but not on others (Van Reen, Lyon and Losee, '58). Autopsies revealed that the deaths were due to blockage of the urinary tract by numerous calculi. Subsequent studies indicated that the casein-mineral ratio of the diet was of considerable significance in the prevention of the uroliths (Van Reen et al., '59). Thus, few or no stones were formed when rats were fed a diet containing 20% of casein and 2% of Hubbell, Mendel and Wakeman salt mixture, whereas, if the salt mixture was increased to 4% numerous uroliths were observed. Likewise, if the salt mixture was held at 2% and the casein level lowered to 15%, again numerous stones formed. The NMRI-D strain of rat appeared much more prone to calculi formation under these dietary alterations than either the Long-Evans or Sprague-Dawley strains.

Further investigations revealed that the calculi formed under our conditions were calcium citrate rather than the other more common forms, such as oxalate, phosphate, carbonate, etc. The presence of stones indicated that, at some point in the urinary tract, there existed a condition in which the solubility product of calcium citrate was exceeded and a nidus or nucleus was available to initiate the calcification. Nothing is known of the excretion of calcium and citric acid by the NMRI-D rat under the dietary conditions employed in the various experiments. Therefore, it was decided to obtain some information concerning the excretion of these materials when rats were fed either diets which result in urolithiasis or which prevent stone formation. Phosphate excretion was also investigated since this anion has been shown to be intimately related to calcium metabolism. When it was found that there was a marked difference in the excretion of citric acid by rats fed the various diets, it was considered important to determine whether the enzymatic capacity of tissues to form or degrade citric acid was being affected. To accomplish this, citrogenase, aconitase, and isocitric dehydrogenase activities of kidneys were determined since this tissue has an active tricarboxylic acid cycle.

MATERIALS AND METHODS

Excretion studies. Male, weanling, albino rats of the NMRI-D strain were used in the experiments. At 21 to 23 days of age, rats weighing between 34 and 51 gm were placed in individual, stainless steel metabolism cages and were provided food and water ad libitum. Spill-proof cups were employed to prevent contamination of the excreta. Four experimental diets were used (table 1). The diets are labelled according to the casein and salt mixture contents, i.e., 15P2 represents 15% of casein and 2% of Hubbell, Mendel and Wakeman ('37) salt mixture. Groups 15P2, 30P2, and 30P4 consisted of 4 rats per group

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[2] Supported in part by a contract with the Department of the Navy.

TABLE 1

Composition of diets

Ingredient	Calculi forming		Calculi preventing	
	15P2	15P4	30P2	30P4
	gm	*gm*	*gm*	*gm*
Vitamin-test casein	15.0	15.0	30.0	30.0
Sucrose	78.8	76.8	63.8	61.8
Salt mixture[1]	2.0	4.0	2.0	4.0
Vitamin mixture[2]	2.2	2.2	2.2	2.2
Linoleic acid	1.0	1.0	1.0	1.0
Corn oil	1.0	1.0	1.0	1.0
Calcium content[3]	0.43	0.86	0.43	0.86
Phosphorus content[3]	0.19	0.28	0.28	0.37

[1] Formulated after Hubbell, Mendel and Wakeman ('37).
[2] Provided a complete mixture of the vitamins.
[3] By calculation but verified by chemical analysis. Phosphorus content includes that contributed by the casein.

while 15P4 contained 6 rats. Urine samples were collected under toluene for three-day periods. The samples were brought to constant volume with water and aliquots taken to determine calcium, citric acid and phosphate.

Calcium was determined by the method of Shohl and Pedley ('22) in which the urine sample is oxidized with ammonium persulfate before oxalate precipitation and permanganate titration. Citric acid was determined by the microdiffusion technic of Bessman and Anderson ('57). For these determinations the samples were evaporated to dryness to remove volatile ketones, then brought to volume with water. Phosphate was determined by the standard method of Fiske and Subbarow ('25).

Collections of urine were made 6 times during the 31-day experimental period. During the second week of the experiment, two collections were made because previous studies had indicated that stone formation could first be observed at this period.

Metabolic studies. NMRI-D rats as described above were used for these studies. The rats were maintained in individual stainless steel cages and fed the 4 diets given in table 1 for a period of two weeks. Ten animals were used for each group.

The animals were anesthetized and the kidneys removed rapidly and immediately placed on ice. Homogenates were prepared for enzyme analyses by grinding the tissue in a glass homogenizer with 5 times its weight of tris(hydroxymethyl)aminomethane buffer, pH 7.4 and then centrifuging out the debris for 15 min. at 1,100 g. All operations were carried out in the cold.

Citrogenase or the condensing enzyme was determined by the procedure of Ochoa ('55) which is based on the conversion of a mixture of acetyl phosphate and oxalacetate to citrate in the presence of transacetylase. The citrate formed was determined by the procedure of Bessman and Anderson ('57). Aconitase activity of the kidney homogenates was determined by the spectrophotometric method of Racker ('50) in which the appearance of *cis*-aconitic acid from citric acid is measured. Isocitric dehydrogenase activity was assayed by the procedure of Grafflin and Ochoa ('50) in which the reduction of triphosphopyridine nucleotide (TPN) is measured by observing the optical density change at 340 mμ. The procedure was altered by the addition of nicotinamide to prevent the breakdown of the coenzyme and of KCN to prevent the reoxidation of the reduced coenzyme. Values are expressed as activities per gram of tissue and also per milligram of protein. The protein content of the homogenates was determined by the method of Lowry et al. ('51).

RESULTS

Body growth. Marked differences in weight responses were apparent between the groups receiving 15% of casein and the groups receiving 30% of casein. These results were to be expected since in previous investigations (Losee et al., '57), it was shown that the protein requirement of

the NMRI-D strain of rat is quite high and is not met by the inclusion of even 20% of casein in the diet.

In contrast to the effect of the protein level, the level of Hubbell, Mendel and Wakeman salt mixture in the present experiment had little influence on the total body weights of the animals either on the low- or high-protein rations. The results suggest, therefore, that under our laboratory conditions, 2% of Hubbell, Mendel and Wakeman salt mixture is adequate for optimal body weight gains. The average weights of rats receiving 15% of casein were 44, 54, 69, 84 and 107 gm at the start, first, second, third and 4th weeks, respectively. At comparable times, the rats receiving 30% of casein weighed 43, 76, 105, 135 and 168 gm.

Calcium excretion. The excretion of calcium was inversely related to the casein level of the diet (fig. 1). The greatest excretion was observed in rats fed the 15P4 diet and the least excretion of calcium in rats receiving the 30P2 diet. It should be noted that the excretion values are reported on the basis of the total calcium excreted during the three-day period, thus, the smaller animals were excreting the higher level of calcium. The observation of high-calcium excretion on the low-protein diet is even more impressive when the excretion values are expressed on the basis of 100 gm of body weight. On this basis, the rats receiving diet 15P4 excreted 0.22 mg of calcium/3 days/100 gm body weight vs. 0.05 mg of calcium for the rats fed diet 30P4.

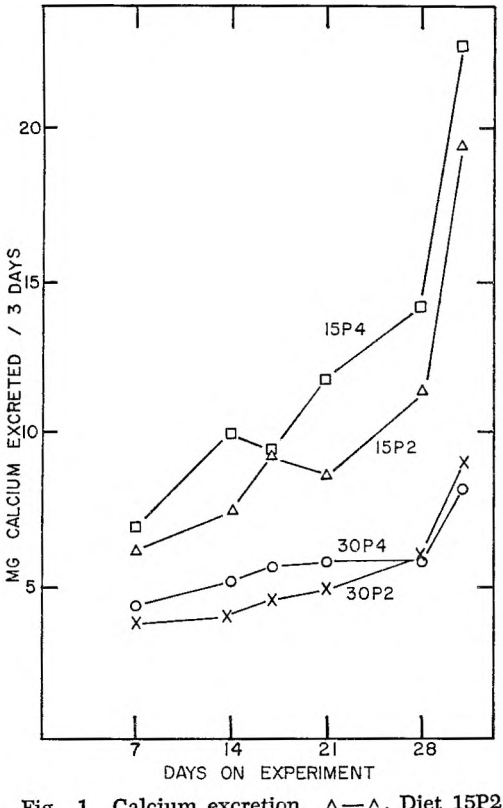

Fig. 1 Calcium excretion. △—△, Diet 15P2. □—□, Diet 15P4. ×—×, Diet 30P2. ○—○, Diet 30P4.

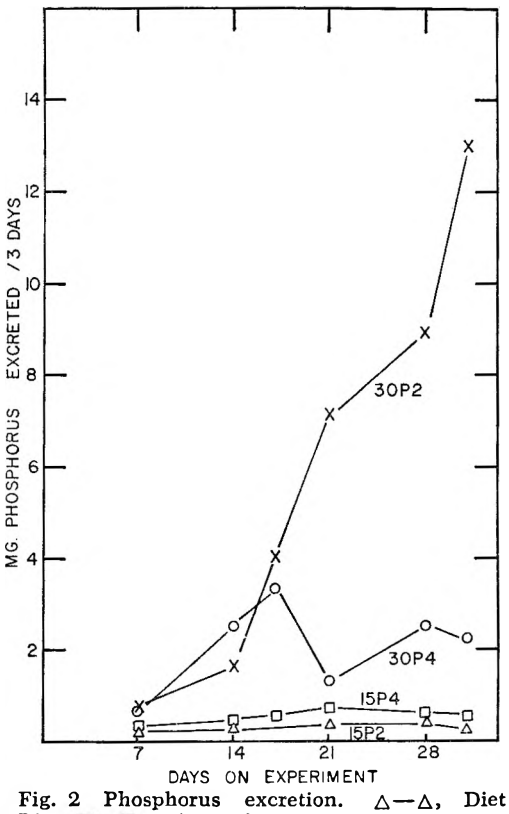

Fig. 2 Phosphorus excretion. △—△, Diet 15P2. □—□, Diet 15P4. ×—×, Diet 30P2. ○—○, Diet 30P4.

Phosphate excretion. The data concerning the excretion of phosphate by rats fed the 4 different experimental diets are presented in figure 2. The most striking observation was the high level of phosphate excretion by the rats receiving the 30P2 diet in comparison with the other rations. Very little phosphate excretion was ob-

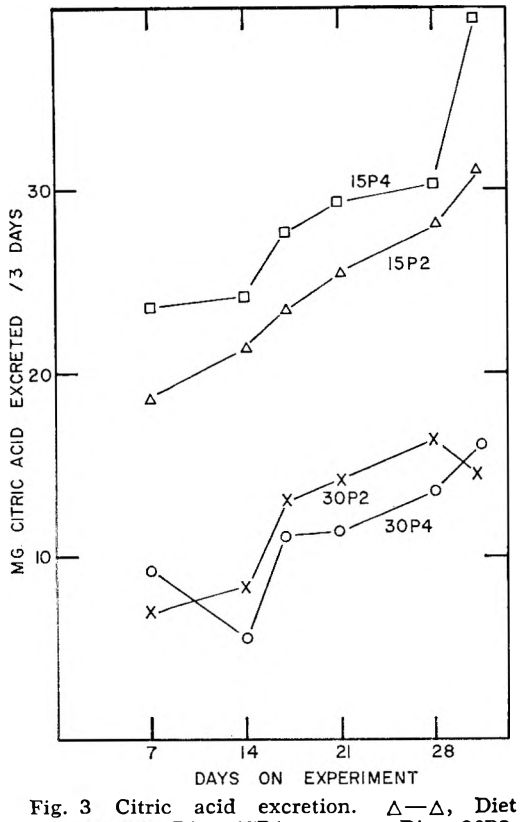

Fig. 3 Citric acid excretion. △—△, Diet 15P2. □—□, Diet 15P4. ×—×, Diet 30P2. ○—○, Diet 30P4.

served in the groups receiving either the 15P2 or 15P4 diets. Since the groups receiving the higher protein level also grew at a faster rate, the differences in the phosphate excretion are not as pronounced if expressed on the basis of 100 gm of body weight. Even with this mode of expression, however, the rats receiving the 30P2 diet excreted more phosphate.

Citric acid excretion. Citric acid excretion for the experimental groups is recorded in figure 3. It can be seen that there was a clear distinction between the rats receiving the high- and low-protein rations. On the 15% casein diets, there was considerably more citric acid excreted than on the 30% casein diets. Much smaller differences were observed with changes in the mineral contents, although there was a consistent trend toward higher citric acid excretion by the rats receiving the 15P4 ration in comparison to the rats on 15P2. As in the case of calcium excretion, if the values are expressed on the basis of 100 gm of body weight, the rats on the low-protein diet excreted considerably more citric acid than those on the 30% casein ration.

Metabolic studies. The activities of rat kidney citrogenase, aconitase and isocitric dehydrogenase were determined; however, there was very little difference in the pattern of these three enzymes in the kidneys from rats fed the 4 experimental diets. The average activities were: citrogenase, 4 μmoles of citric acid formed per hour per milligram of protein; aconitase, 4 μmoles citric acid metabolized per hour per milligram of protein; and isocitric dehydrogenase, 14 μmoles isocitric acid metabolized per hour per milligram of protein. While the enzyme data do not show significant differences in activities, it should be noted that the activities were measured under optimal conditions of pH, substrate concentration, etc., and, therefore, may not be a true measure of *in vivo* conditions. For example, a condition in which there was limited TPN could result in restricted isocitric oxidation and accumulation of citric acid because of the equilibrium among isocitric, *cis*-aconitic, and citric acids, an equilibrium which by far favors citric acid (Krebs and Eggleston, '43).

DISCUSSION

The factors which may affect the absorption and excretion of calcium and phosphate have been discussed in many publications. Similarly, many investigations have been reported on the excretion of citric acid and its possible relationship to calcium excretion. From these studies it is clear that calcium has an effect on the absorption of phosphate; however, the reverse relationship has been questioned (Nicolaysen et al., '53). Thus, with a normal level of phosphate in the diet, a high level of calcium will reduce its absorption, presumably through the formation of an insoluble calcium phosphate salt. The changes in absorption are eventually reflected in alterations in the excretion of these ions.

In regard to citric acid excretion, it is clear that this organic component of the urine arises from the metabolic processes within the body and on the whole is related

to the excretion of calcium. The quantity of citric acid in the urine is markedly affected by the diet showing a tendency to be increased by factors which raise the pH of the urine and decreased by factors which lower the pH.

It is understandable in view of the above comments that the range of values for the excretion of calcium, phosphate and citric acid which appear in the literature is extremely large. Variations due to diet, strain of animal, age, sex and environmental conditions make it necessary to determine the excretion values for individual sets of conditions. The present results are discussed in terms of the general principles given above.

Diet 15P4. The highest excretion of calcium and citric acid was observed in this group, whereas very little phosphate was found in the urine. These results are particularly interesting since the most severe calcium citrate stone formation has been observed in rats fed this ration. The calcium/phosphorus ratio of this diet was 3:1, the highest of the 4 diets used. The form in which the calcium was excreted was not determined, although a soluble calcium citrate complex is likely in view of the reports of Shear and Kramer ('28) and Hastings et al. ('34). The local factors in the kidney tubules which lead to the formation of the insoluble tricalcium citrate uroliths are still unknown.

Diet 15P2. The excretion of both calcium and citric acid by rats fed this ration was less but almost equivalent to that of animals fed 15P4. The lower quantities were to be expected in view of the lower concentration of salt mixture in the ration. Phosphate excretion was very low, amounting to only 0.5 mg/three days. Again, the excretion of high levels of calcium and citric acid is compatible with the observation of a high incidence of citrate uroliths in NMRI-D strain rats maintained on this diet.

Diet 30P4. This diet contained the same concentration of calcium as diet 15P4; however the phosphate content was higher and subsequently a poorer absorption of calcium might have occurred. In any case, the excretion of calcium by rats fed this ration was considerably less than by rats receiving the 15% casein diets. A second factor to be considered is the greater growth rate with this diet than with the 15% casein rations, thus requiring more calcium for bone formation. Both factors would be expected to keep the urinary calcium low.

The excretion of citric acid followed the pattern of calcium with a gradual tendency to increase during the experimental period. This observation is further substantiation of the relationship which appears to exist between calcium and citrate excretion.

One of the striking differences in the excretion patterns on the various diets was observed in regard to phosphate. The rats fed the 30P4 diet excreted considerably less phosphate than those fed 30P2. It is believed that the higher calcium content of the 30P4 diet was responsible for this effect. As stated above, calcium salts can prevent the absorption of phosphate by the formation of an insoluble calcium phosphate. Absolute proof of this was not obtained in the present study since fecal calcium and phosphate were not determined.

Diet 30P2. Calcium and citric acid excretion by rats fed this ration followed a pattern very similar to that of rats fed the 30P4 diet, that is, low calcium and citric acid in the urine. These observations are in agreement with the fact that rats fed these rations do not develop calcium citrate urinary calculi and show normal urogenital tracts. The excretion of phosphate with this diet was higher than in any of the other experimental groups, whereas the excretion of calcium was the lowest. This is undoubtedly a reflection of the fact that this diet had the lowest calcium/phosphorus ratio of the 4 groups (1.5:1).

The comments concerning the excretion of calcium, citric acid and phosphate have been mainly in terms of the mineral content of the diets and, more particularly, the calcium/phosphorus ratios of the diets. However, the influence of protein should be taken into account. In the previous report (Van Reen et al., '59) the influence of the casein/mineral ratio in the formation and prevention of urinary calculi was discussed. It was pointed out that a comparison of the 15P2 and 30P4 diets indicates that both have the same calcium/

phosphorus ratio; the latter has twice the mineral content, but rats fed the former diet develop the urinary calculi. Thus, protein appears to have an effect. Likewise, the present studies indicate clearly that less calcium and citric acid are excreted on the high-protein diets. Nevertheless, it was shown by McCance et al. ('42), Hall and Lehmann ('44) and by subsequent investigators that the protein level of the diet can increase the absorption of calcium. In view of the known factors, it is tentatively suggested that on our high-protein diets considerable calcium was absorbed but was retained for at least two reasons: (1) greater growth on the high-protein diets, and (2) an effect of the protein in reducing citrate excretion by means of changing the urinary pH and thus reducing calcium excretion. Complete balance studies will be necessary to clarify these points.

Regardless of the mechanism by which the casein/mineral ratio of the diet is important in the prevention of citrate urolithiasis, the present studies clearly indicate that the rats receiving a diet which frequently leads to stone formation excrete more calcium and citric acid and less phosphate than the animals fed diets which do not lead to the formation of stones. Also, the differences in citric acid excretion cannot be explained on the basis of altered capacity in the synthesis or degradation of citric acid by kidney homogenates.

SUMMARY

1. Groups of NMRI-D rats were fed 4 different diets, two of which contained 15% of casein and lead to urinary calculus formation, and two of which contained 30% of casein and prevent calculus formation.

2. The growth of rats and the excretion of calcium, citric acid and phosphate was studied over a 5-week period.

3. The rats fed the diets resulting in lithiasis excreted large quantities of calcium and citric acid but small amounts of phosphate.

4. The rats fed diets preventing lithiasis excreted small quantities of calcium and citric acid but high levels of phosphate.

5. The results are discussed in relation to the formation of calcium citrate calculi.

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Effects of Essential Fatty Acids, Inositol, Vitamin B_{12} and Hydrolyzed Glucose-Cyclo-Acetoacetate on Blood Coagulation Factors in Rabbits Exhibiting Hyperlipemia Induced by Feeding Saturated Fat

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Atherosclerosis induced by fatty meals has often been thought to be associated with coronary thrombosis resulting from shortening of coagulation time and other coagulation factors (Fullerton et al., '53). Although the effects of different dietary fats on blood coagulation have been studied by a number of workers, the results of some of those studies have been found to be contradictory. Duncan and Waldron ('49) demonstrated shortening of clotting time in dogs and in human beings after ingestion of cream. Fullerton et al. ('53) observed a significant reduction of clotting time in human volunteers who were fed animal fat. Keys et al. ('57) observed that after all fatty meals, irrespective of degree of saturation or source of the fat, whole blood clotting time was significantly shortened. Maclagan and Billimoria ('56) also reported that the ingestion of butter shortened the postprandial whole blood clotting time. On the other hand, Hall ('56) and Mersky and Nossel ('57) could not agree with the view that animal and vegetable fats affect the clotting time. The reduction of hemoglobin content of the blood in atherosclerotic subjects has also been suggested by Joslin and Root ('48), Best ('52) and many others.

That the essential fatty acids present in oils are the important factors in lowering the serum cholesterol level in hypercholesteremia and atherosclerosis has recently been claimed by Kinsell et al. ('58), Hegsted and associates ('57) and Rustein et al. ('58). Beveridge et al. ('57) and Jones and collaborators ('56) held, however, that there is some factor other than the essential fatty acids which occurs in the unsaturated oil and which has a significant effect in lowering the level of serum cholesterol. Recently Grande and associates ('58) have reported that the unsaponifiable fraction of corn oil is more effective than the essential fatty acids in lowering the serum cholesterol level in atherosclerosis. It has also been reported by us ('58, '59a, '59b) recently that glucose-cyclo-acetoacetate (GCA), which is known to yield 1,2-dienol glucose on acid hydrolysis with 2N HCl (Nath and Bhattathiry, '56), not only prevents the onset of various types of experimental diabetes (Nath and associates, '56a, '56b, '57, '58) but also interferes significantly with the development of experimental atherosclerosis of various kinds by lowering the increased value of C/P(total cholesterol/lipid phosphorus) ratios and by maintaining almost normal cholesterol and phospholipid levels in the blood and tissues. This compound has been found to have much more effective anticholesterogenic action than vitamin B_{12} or inositol (Nath and Saikia, '59). Recently Nath et al. ('59c) also reported that hydrolyzed GCA has a pronounced effect in maintaining the normal pattern of the serum protein fractions of saturated fat-induced atherosclerotic animals even to a better extent than essential fatty acids, vitamin B_{12} and inositol.

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It seemed desirable, therefore, to investigate whether experimental atherosclerosis induced by a diet containing highly saturated fat is associated with reduction in coagulation time, prothrombin time and the hemoglobin and platelet content of blood. The present paper reports the effects of essential fatty acids, vitamin B_{12}, inositol and hydrolyzed GCA on the same factors in saturated fat-induced atherosclerotic animals. The C/P ratios of the serum and tissues which indicate the degree of atherosclerosis have also been recorded in all groups of animals.

EXPERIMENTAL

Forty-two male albino rabbits, 1.2 kg in body weight, were distributed among 7 groups of 6 each and caged individually. The animals of group I were kept as normal controls receiving a laboratory stock diet, consisting of casein 20 gm, sucrose 30 gm, wheat flour 39 gm, groundnut[3] oil 6 gm, and Hawk-Oser salt mixture 5 gm. The vitamin mixture consisted in micrograms of thiamine·HCl, 3; riboflavin 3; nicotinic acid, 30; calcium pantothenate, 17; pyridoxine·HCl 3; *p*-aminobenzoic acid, 3; inositol, 200; choline chloride, 200; biotin, 0.2; folic acid, 0.0002; and vitamin K (menadione), 0.3 per gram of food; two or three drops of a vitamin A and D preparation[4] were given per rabbit per day. Alpha-tocopherol acetate was given daily in an oral dose that furnished 10 mg/rabbit/week. The remaining groups were given 20% of saturated fat[5] in place of groundnut oil; the diet was made isocaloric with the control diet at the expense of carbohydrate. The animals of group II were kept as experimental controls and groups III, IV and V were given 1% of linoleic acid, linolenic acid,[6] or inositol respectively. The animals of groups VI and VII were injected daily with a dose of 15 μg/kg and 300 mg/kg body weight of vitamin B_{12}[7] and hydrolyzed GCA respectively. All groups except I were fed the saturated fat.

The experiment was continued for 12 weeks after which the bleeding time, coagulation time, prothrombin time, blood platelet content and hemoglobin content of the blood were determined in the fasting animal.

1. *Bleeding time.* This was determined according to the method of Dacie ('51a) by pricking the ear of the rabbit with a capillary pricker to the depth of 3 mm in an area where no superficial venules were evident. The blood was soaked at intervals with a piece of blotting paper until the bleeding stopped and the time from the pricking of the ear until oozing stopped was noted.

2. *Coagulation time.* The capillary tube method of Wright and Colebrook ('21) was used. The rabbit ear lobe was pricked, the time being noted. When there was a free flow of blood, a small column of blood was allowed to flow by capillary attraction into each of a dozen capillary tubes of equal caliber. The tubes were then sealed with plasticine and immersed in a water bath at 37°C. After wating for three to 4 minutes the first tube was removed from the water bath, the end filed off and the open end immersed in a dish containing water at 37°C and the blood expelled into the water. Thereafter, at intervals of 30 seconds the remaining tubes were similarly treated. When the expelled blood had the form of a worm-like clot, the end point was considered to have been reached and the time was noted.

3. *Prothrombin time.* This was estimated according to the method modified by Quick et al. ('35) using thrombokinase "Geigy." One tenth milliliter of citrated plasma was taken in the test tube and warmed for three to 5 minutes in a water bath at 37°C. The thrombokinase suspension was also warmed at this temperature and 0.2 ml of the suspension added to the plasma and the chronometer set in motion simultaneously. The time which elapsed before the plasma showed clotting was

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[3] Peanut.

[4] Adexoline, Glaxo.

[5] Dalda, a hydrogenated fat prepared from groundnuts (peanuts). The iodine value was 68. Product of Hindusthan Lever India (Private) Ltd., Bombay, India.

[6] The linoleic acid was supplied by the British Drug Houses, Ltd. Its iodine value was 235. The linolenic acid (iodine value 245) was supplied by the Regional Research Laboratory, Deccan, Hyderabad, India.

[7] Macrabin.

measured. The mean value of duplicate determinations was recorded.

4. *Blood platelet content.* The direct method of Kristension as modified by Lampert (Dacie, '51b) was used.

5. *Hemoglobin content* of the blood was determined by the method of Wong ('28) on blood taken from the marginal ear vein.

The animals were then sacrificed and the biochemical determinations made.

I. Free, total and ester cholesterol of the serum, liver and heart were measured by the method of Schoenheimer and Sperry ('34) as modified by Mookerjea and Sadhu ('55).

II. Lipid phosphorus of the serum and tissues was estimated by the modified method of Youngburg and Youngburg ('30) using the phosphate procedure of Fiske and Subbarow ('25). The phospholipid levels were determined by multiplying lipid phosphorus by the factor 25. The C/P (total cholesterol/lipid phosphorus) ratios were calculated from the experimental results.

III. The *liver fat* from fresh liver was extracted according to the method of Ferguson ('54). The entire liver, except a small portion left for cholesterol and phospholipid determinations, was blotted to remove the blood and other tissue fluid. The liver was weighed and then homogenized carefully with 95% ethanol and then transferred to a weighed Soxhlet thimble quantitatively. This was then extracted with 95% ethanol for 4 hours. As, according to the method of Ferguson ('54), the extraction of fat from fresh liver is still not complete, further extraction of the residue with an ether-alcohol (1:1) mixture for a period of 12 hours in the Soxhlet apparatus was necessary. The extract was filtered and evaporated to dryness in a weighed crucible. The residue was washed with petrcleum ether several times, evaporated to dryness and then kept in a desiccator for 24 hours, for constant weight. Liver fat is expressed in terms of percentage of liver tissue (wet basis).

RESULTS

In table 1 it will be seen that on prolonged feeding of saturated vegetable fat to the rabbits, the blood coagulation factors, namely, bleeding time, coagulation time and prothrombin time, are shortened to a greater extent (group II) than those of the normal animals (group I). The diet of saturated fat also has been found to increase the platelet count and to reduce the percentage of hemoglobin in the blood significantly.

These values could, however, be restored more or less to those for the normal state when the highly saturated fat diet of the animals was supplemented with the essential fatty acids, linoleic and linolenic, or inositol, or by injecting the animals with vitamin B_{12} or hydrolyzed GCA (groups III, IV, V, VI and VII) respectively.

It is to be noted from the results obtained that the substances here studied are not equally effective in bringing these values to within normal limits. The effect of linoleic acid (group III) is very insignificant in changing the coagulation time; the difference between the means of groups II and III is not significant. The "t" value required for significance here is 2.228, whereas the value actually obtained is only 1.6.

Other groups (IV, V, VI and VII), when compared with the normal (I) show significant differences with respect to shortening the coagulation time but the differences between these groups themselves are insignificant; linolenic acid (group IV) and hydrolyzed GCA (VII) had about the same effect in this respect.

It will be noted in table 1 that in all of the experimental groups the bleeding time was shortened, the greatest difference being between the group fed the saturated diet unsupplemented with test substances (group II) and the normal control (I) and that in all groups receiving the test supplements the bleeding time was lengthened somewhat over that for the unsupplemented group (II). However, the differences between most of these groups are insignificant. In the case of groups III (linoleic acid) and VII (hydrolyzed GCA) the difference is somewhat significant, the "t" value being 3 as compared with 2.228 required for significance.

With respect to the prothrombin time, the greatest effect occurred in the group fed the saturated fat unsupplemented with

TABLE 1

Blood coagulation factors and hemoglobin content of normal rabbits and rabbits fed for 12 weeks on a saturated-fat diet with and without various test supplements. Means ± S.E. Six animals in each group.

Factor of interest				Saturated-fat diet together with				
		Normal laboratory diet	Saturated fat diet only	Linoleic acid	Linolenic acid	Inositol	Vitamin B_{12} injected	Hydrolyzed GCA injected
	Group:	I	II	III	IV	V	VI	VII
Coagulation time, min.		4.8 ± 0.1	3.4 ± 0.2	3.7 ± 0.1	4.3 ± 0.3	4.2 ± 0.25	4.0 ± 0.2	4.3 ± 0.3
Bleeding time, min.		2.6 ± 0.2	1.8 ± 0.1	2.15 ± 0.1	2.1 ± 0.2	2.1 ± 0.1	2.2 ± 0.2	2.4 ± 0.2
Prothrombin time, sec.		21	10	13	13	14	17	18
Platelets, M/mm³		377 ± 14	625 ± 16	555 ± 20	545 ± 20	530 ± 15	435 ± 31	425 ± 22
Hemoglobin, gm%		13 ± 1	7.6 ± 0.5	9.0 ± 0.5	10.5 ± 0.4	9.6 ± 0.8	9.8 ± 0.5	10.9 ± 0.6

Significance of differences

Coagulation time			Bleeding time			Hemoglobin		
Groups compared	"t" found	"t" needed	Groups compared	"t" found	"t" needed	Groups compared	"t" found	"t" needed
III with II	1.6	2.228	IV with III and V	1.0	2.228	III with V	1.0	2.228
IV with VII	0		III with VII	3.0		VI with VII	2.5	
IV with V	1.0		IV with VII	1.6		IV with VII	1.4	
IV with VI	1.4							

the substances under investigation (II) compared with the normal controls (I). The effect of the test substances was only moderate, to be regarded as significant except with groups VI (vitamin B_{12}-injected) and VII (injected with hydrolyzed GCA) where the prothrombin times were brought back almost to that characteristic of the normal controls (I).

The reduced hemoglobin content of the blood brought about by feeding a diet of saturated fat (II) was raised appreciably by all of the supplements, the maximum effects being seen in groups IV (linolenic acid) and VII (hydrolyzed GCA). The differences between the various groups, however are not significant.

With respect to the platelet counts, it is evident that the highest count was obtained with the animals fed the saturated diet unsupplemented with test substances (group II). The count was reduced in every group, the greatest reduction being seen in the animals receiving hydrolyzed GCA (VII).

Looking at all of the coagulation factors as a group, it is evident that linolenic acid and hydrolyzed GCA were more effective than linoleic acid, inositol and vitamin B_{12} in restoring the various values to their normal limits. With respect to raising the prothrombin time and reducing the blood platelet count, hydrolyzed GCA had a greater effect than any of the other substances.

It will be observed in table 2 that in all groups receiving saturated fat (all except I) there were increases in the serum cholesterol, phospholipid and C/P ratio. The levels of total and ester cholesterol and phospholipid, as well as values for the C/P ratio decreased significantly in all groups that received test supplements (III, IV, V, VI and VII). With respect to free cholesterol, the differences between groups V and VI and between these groups and the unsupplemented group (II) were not significant. This means that supplementation with inositol (V) and vitamin B_{12} (VI) had no effect in lowering the free cholesterol level. Hydrolyzed GCA (group VII) was most effective in this respect but was only slightly more effective than linoleic (III) and linolenic (IV) acids.

Concerning the levels of *total cholesterol* (table 2), hydrolyzed GCA (VII) was much more effective in lowering the level than any of the other substances tested. Compared with group IV, the other three groups (III, V and VI) lowered the level to about the same extent. Inositol had very little effect.

With respect also to *ester cholesterol,* hydrolyzed GCA (VII) was effective in lowering the levels in animals fed saturated fat, but the differences between the test substances were not as marked; inositol was least effective of them all.

In table 2 it is evident that in the case of *lipid phosphorus,* the values were reduced by linoleic and linolenic acids (groups III and IV) and the difference between them is without significance; vitamin B_{12} has about the same effect, the "t" value for this group (VI) being only 1.8 as compared with the required value of 2.228 for significance. Hydrolyzed GCA (VII) gave the maximum effect in reducing the level of lipid phosphorus.

It is thus evident that hydrolyzed GCA was more effective in lowering serum total and ester cholesterol and phospholipid levels in animals fed saturated fat, resulting in lower C/P ratios, than the other substances tested.

From the analysis of lipids in the liver and heart, including cholesterol and phospholipid, it is observed in table 2 that the feeding of saturated fat raised the cholesterol in these tissues (groups II) to levels much higher than those in normal animals (I) fed a laboratory stock diet. The C/P ratio of all tissues so far studied were found to be elevated. From this table it can also be seen that linoleic and linolenic acids and hydrolyzed GCA (groups III, IV and VII) reduced total cholesterol to almost the same extent but the effect of hydrolyzed GCA and vitamin B_{12} (groups VII and VI) in reducing the ester cholesterol was more pronounced than that of the other test substances.

Linoleic acid, vitamin B_{12} and hydrolyzed GCA (table 2: III, VI and VII) were found to reduce the level of ester cholesterol in the heart to an extent greater than that observed with linolenic acid (IV) and inositol (V). Concerning phospholipid in the heart, although vitamin B_{12}

TABLE 2

Cholesterol, phospholipid, lipid phosphorus and cholesterol/phosphorus ratio (C/P) of blood serum and of liver and heart tissues of rabbits fed a normal diet and rabbits fed 12 weeks on a saturated-fat diet with and without various test supplements. Means ± S.E.

Factor of interest	Normal laboratory diet	Saturated fat diet only	Saturated-fat diet together with: Linoleic acid	Linolenic acid	Inositol	Vitamin B_{12} injected	Hydrolyzed GCA injected
Group:	I	II	III	IV	V	VI	VII
Blood serum[1]							
Cholesterol:							
Total	133 ± 10	340 ± 15	200 ± 8	195 ± 7	240 ± 10	200 ± 12	175 ± 5
Free	59 ± 8	100 ± 10	80 ± 6	85 ± 6	100 ± 10	100 ± 12	75 ± 5
Ester	74 ± 3	240 ± 12	120 ± 6	110 ± 5	140 ± 8	100 ± 3	100 ± 3
Phospholipid	175 ± 12.5	250 ± 12.5	200 ± 12.5	200 ± 12.5	225 ± 25	212.5 ± 12.5	187.5 ± 10
Lipid P	8 ± 0.5	10 ± 0.5	8 ± 0.5	8 ± 0.5	9 ± 1	8.5 ± 0.5	7.5 ± 0.4
C/P Ratio	19	34	25	24.3	26.6	23.5	23.3
Significance—Lipid phosphorus: VII with III and IV, "t" is 4; 2.179 needed							
Liver tissue[2]							
Cholesterol:							
Total	220 ± 12	440 ± 10	300 ± 15	280 ± 10	350 ± 12	300 ± 15	280 ± 10
Free	162 ± 10	212 ± 12	163 ± 6	160 ± 4	200 ± 10	200 ± 10	180 ± 6
Ester	58 ± 5	228 ± 6	137 ± 8	120 ± 3	150 ± 6	100 ± 8	100 ± 6
Phospholipid	1500 ± 50	750 ± 50	1250 ± 50	1375 ± 75	1000 ± 75	1125 ± 50	1350 ± 75
Lipid P	60 ± 2	30 ± 2	50 ± 2	55 ± 3	40 ± 3	45 ± 2	54 ± 3
C/P Ratio	3.6	14.6	6.0	4.7	8.7	6.6	5.1
Liver fat: total	3.8 ± 0.3	8.0 ± 0.5	4.8 ± 0.2	4.8 ± 0.2	6.0 ± 0.5	5.0 ± 0.3	4.5 ± 0.2
Iodine number	94 ± 2	45 ± 1	70 ± 2	72 ± 2	60 ± 3	74 ± 1	76 ± 2
Significance—Liver fat: III with IV and VI—"t" is 1.5; 2.5 needed VII with III and IV—"t" is 2.8; 2.5 needed							
Heart tissue[2]							
Cholesterol:							
Total	210 ± 9	300 ± 8	220 ± 6	230 ± 6	260 ± 8	220 ± 5	220 ± 5
Free	175 ± 3	200 ± 6	180 ± 3	180 ± 6	170 ± 5	180 ± 4	180 ± 2
Ester	35 ± 2	100 ± 4	40 ± 5	50 ± 4	90 ± 8	40 ± 4	40 ± 4
Phospholipid	2000 ± 25	625 ± 25	1250 ± 25	1375 ± 50	1000 ± 25	1325 ± 50	1500 ± 75
Lipid P	80 ± 1	25 ± 1	50 ± 1	55 ± 2	40 ± 1	55 ± 2	60 ± 3
C/P Ratio	2.6	12	4.4	4.1	6.5	3.4	3.3

[1] All values except for C/P are mg/100 ml. Six rabbits in each group.
[2] All values except for C/P are mg/100 gm wet weight basis. Five rabbits in each group.

(VI) and linoleic (III) and linolenic acids (IV) produced higher levels than inositol, none of these substances was as effective as hydrolyzed GCA (VII). The C/P ratio in group VII was also lower than in the other groups.

It is evident from table 2 that linoleic and linolenic acids and vitamin B_{12} have similar effects in maintaining the tissue total and ester cholesterol, phospholipid levels and C/P ratio more or less within normal limits, but the effect of hydrolyzed GCA is much more pronounced than these other substances. Inositol had very little effect in lowering the tissue total and ester cholesterol levels and in raising the phospholipid level.

Table 2 presents data on liver fat and iodine values of that fat. It is seen that the feeding of saturated fat without the test supplements (II) increased the percentage of liver fat to a great extent. This higher level of liver fat was reduced when the animals were given linoleic (III) or linolenic (IV) acids, inositol (V), or injected with vitamin B_{12} (VI) or hydrolyzed GCA (VII). Examination of the "t" values showed that the difference between the essential fatty acids (III and IV) and vitamin B_{12} (VI) was not significant. Of the comparisons made, only the difference between hydrolyzed GCA (group VII) and the essential fatty acids (III and IV) was significant.

The administration of saturated fat reduced the iodine number of the liver fat. It might be argued that this was due to deficiency of essential fatty acids in the diet, but this is contraindicated by the results with groups III and IV which received these acids. The effect of vitamin B_{12} and hydrolyzed GCA were again more pronounced than those of the other substances tested. It is interesting to note that hydrolyzed GCA raised the iodine value of the liver fat to a greater extent than all of the other substances studied.

DISCUSSION

Although Fullerton et al. ('53) and Waldron and Duncan ('53) observed a shortening of clotting time after a fatty meal, Manning and Walford ('54) and Merskey and Nossell ('57) could not find any such change. Further, it was recently reported by O'Brien ('58) that patients with coronary thrombosis did not show any greater increase in blood coagulability immediately after the ingestion of a selected fatty meal than did healthy persons. The increase in the coagulation factors observed in our experiment may, therefore, have been due to some other factor (or factors?) arising out of the prolonged use of meals containing saturated fats.

Robinson et al. ('56, '57) have reported that phosphatide ethanolamine of the blood platelets is a constituent essential for the initiation of the coagulation process and O'Brien ('57) is of the opinion that phospholipids in platelets become available for *in vitro* coagulability after the platelets have been "activated" during the initial stages of coagulation. Any meal, therefore, which increases the blood platelets may be held responsible for shortening of the coagulation time. According to Yeager ('56), there are three factors to be considered in the pathogenesis of venous thrombosis one of which is the increased coagulability or shortening of the coagulation time of the blood.

It was observed by Warren and LeCompte ('52) that there are evidences of swelling and loosening of the inter-cellular substance of the intima in atherosclerosis along with the deposits of lipid droplets which are mainly cholesterol esters. It was further observed by Bevans ('49) that there is deposition of calcium in the plaques, causing stretching and injury to the superficial intima layers sufficient to produce ulceration, which, it is felt, may be responsible in some way or other for the "activation of the platelets."

It will be noticed from the results in table 1 that prolonged use of saturated fat increases the number of blood platelets considerably. It is also evident from table 2 that, although continued use of saturated fat causes a decrease in the *tissue* phospholipids, their concentration in the *serum* is very much elevated which may be responsible for activating the platelets and thus increasing the blood coagulability.

Essential fatty acids, inositol, vitamin B_{12} and hydrolyzed GCA, when administered along with the saturated fat diet,

were found to increase tissue phospholipid and to decrease its release in the serum as well as to check the rise in blood platelets. Hydrolyzed GCA, like vitamin B_{12}, was previously reported to aid in the biosynthesis of methionine in the liver (Nath and Saikia, '59a, '59b) which may be helpful in the long run for more and more accumulation of phospholipid in the tissue and for checking its drainage into the blood stream.

Such an effect has been found to be more pronounced in the animals treated with hydrolyzed GCA which has recently been shown by us ('58, '59a, '59b) to prevent various kinds of experimental atherosclerosis. Grande et al. ('58) have shown recently that the unsaponifiable fraction of corn oil (Factor X) is a better antilipemic agent than the essential fatty acids. GCA, on hydrolysis, gives rise to 1,2-dienol glucose (Nath and Bhattathiry, '56) which forms molybdenum blue with a molybdenum reagent in the acid medium. A similar test has recently been found to be positive with the unsaponifiable fraction of the crude oil (Nath and Saikia, '59d). It is suggested, therefore, that 1,2-dienol glucose or any other factor which may prevent the development of atherosclerosis and decrease the C/P ratios in blood and tissues, may aid in preventing the lowering of coagulation factors and help towards the prevention of thrombosis.

SUMMARY

Prolonged feeding of a diet containing 20% of highly saturated fat to rabbits and which leads to experimental atherosclerosis has been found to shorten the coagulation factors (bleeding time, coagulation time and prothrombin time), as well as to increase the liver fat and number of blood platelets, and to increase ester cholesterol in blood and liver and heart tissues.

It is suggested that the rise of tissue ester cholesterol especially of the superficial intima layers may activate blood platelets thus leading to the increased coagulability of blood and thrombosis. The administration of essential fatty acids (linoleic and linolenic), inositol, vitamin B_{12} and hydrolyzed GCA was again shown to counteract such shortening of coagulation factors. A possible mechanism of such action is discussed.

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The Antithyrotoxic Factor of Liver

III. COMPARATIVE ACTIVITY OF LIVER RESIDUE AND OTHER PROTEINS

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Previous publications (Overby et al., '59a, b) described methods used for routine testing of materials for antithyrotoxic activity in rats. Liver residue was consistently active and the activity was found to be independent of known nutrients. In addition to liver residue, fats were found to have specific antithyrotoxic effects which appeared to be directly related to the amount of linoleic acid supplied. The fat fraction from liver residue had high antithyrotoxic activity, but the major portion of the activity remained in the predominantly-protein portion of liver residue.

Ershoff ('50), Tappan et al. ('53), Graham et al. ('53) and Stevens and Henderson ('58) have tested various natural products of both animal and vegetable origin for antithyrotoxic activity. Several semi-purified materials, rich in proteins, including liver residue, were found to promote growth and survival of thyrotoxic rats, usually on a purified sucrose-casein basal ration. The implication was that an unidentified organic factor was being supplied by these crude materials.

Because most highly active materials are high in protein, we felt it necessary to rule out a non-specific protein balance or specific amino acid effect before attempting isolation procedures for an unidentified entity. We report here comparative effects of 6 proteins of high biologic value, including casein and liver residue, and a mixture of pure L-amino acids simulating liver residue.

EXPERIMENTAL

Animals

Weanling, male Sprague-Dawley rats, in groups of 10, were used according to the experimental procedure previously described (Overby et al., '59a). Weight gains and survivals were measured at weekly intervals. In two series, the animals were decapitated at the end of 4 weeks and the adrenals, spleen, thymus and kidneys were removed and weighed immediately.

Materials

Liver residue. The commercial product[1] was extracted with petroleum ether (b.p. 60 to 80°) in a Soxhlet type apparatus for 16 hours. The insoluble portion was dried in air and autoclaved at 12 p.s.i. for one hour after suspension in 4 times its weight of distilled water. The excess water was removed by filtration through canvas while the mixture was still hot. The insoluble residue was dried in air to constant weight. Yield of the defatted, washed product was about 70%. Total nitrogen was 12.84%, moisture, 4.37% and ash 6.22%.

Casein-Vitamin Test,[2] supplemented with 1% of L-cystine; total nitrogen, 13.60%.

Egg albumin, powdered;[3] total nitrogen, 12.47%.

Whole egg, defatted;[4] total nitrogen, 12.18%.

Blood fibrin, purified, dry beef fibrin;[5] total nitrogen, 14.97%.

Lactalbumin,[6] total nitrogen, 12.55%.

L-*Amino acids*, purchased commercially as indicated in footnotes: L-alanine, L-aspartic acid, L-methionine, L-threonine, L-

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[1] Abbott Laboratories, North Chicago, Illinois.
[2] Nutritional Biochemicals Corp., Cleveland.
[3] Kuriaka, Fletcher-Eichman and Co., Chicago.
[4] Rutgers University, Bureau of Biological Research, New Brunswick, New Jersey.
[5] Armour and Co., Chicago.
[6] Crest Foods Co., Ashton, Illinois.

tryptophan, L-proline, L-valine, L-tyrosine and L-serine;[7] L-arginine hydrochloride, L-histidine hydrochloride, L-leucine and L-lysine monohydrochloride;[8] L-isoleucine and glycine;[9] L-phenylalanine;[10] L-glutamic acid;[11] and L-cystine.[12]

Diets

Basal diet 14, previously described (Overby et al., '59a) was used. The gross percentage composition was sucrose, 57.5; cottonseed oil, 5; salt mixture (Jones and Foster, '42) 4; inert ingredients, 3.25; choline chloride, 0.1; vitamin mixture 0.15; and casein, 30. In the experimental variations, the casein was replaced, partially or entirely, by other proteins on an isonitrogenous basis. In each case the level of sucrose was varied to balance the diet to 100%. Twenty dietary variations in 4 experimental series were used as shown in table 1. One-half the

[7] Nutritional Biochemicals Corporation, Cleveland.
[8] Merck and Company, Rahway, New Jersey.
[9] Dow Chemical Company, Midland, Michigan.
[10] Mann Fine Chemicals, Inc., New York.
[11] Interchemical Corporation, Union, New Jersey.
[12] Keratene Company, Inc., Winsted, Connecticut.

TABLE 1

Growth and survival responses of rats receiving variations of dietary proteins

Diet	Protein composition			Gain		Survival
	Casein	Other protein				
	%	*Type*	%	*gm*	S.E.[1]	%
		Experimental series 1 (5-week results)				
Ia[2]	30	None	—	203 ±	7.5	100
Ib[2]	20	None	—	209 ±	7.2	100
Ic[2]	10	None	—	122 ±	5.0	100
Id[2]	40	None	—	188 ±	7.2	100
Ie	30	None	—	112	—	10
If	20	None	—	101	—	10
Ig	10	None	—	86 ±	7.1	50
Ih	40	None	—	120 ±	14.0	20
Ii	20	Liver residue	10	180 ±	9.1	100
		Experimental series 2 (4-week results)				
IIa	30	None	—	92 ±	7.4	30
IIb	15	Egg albumin	16.3	93 ±	5.7	70
IIc	15	Whole egg	17.1	122 ±	13.9	30
IId	15	Liver residue	15.9	128 ±	7.1	100
IIe	15	Blood fibrin	13.6	110 ±	7.7	60
IIf	15	Lactalbumin	16.3	118 ±	12.0	50
		Experimental series 3 (4-week results)				
IIIa	30	None	—	99 ±	6.7	70
IIIb	0	Egg albumin	33.5	73 ±	8.1	70
IIIc	0	Whole egg	33.2	139 ±	4.8	100
IIId	0	Liver residue	31.5	131 ±	4.9	100
IIIe	0	Blood fibrin	26.9	123 ±	5.1	100
IIIf	0	Lactalbumin	32.1	125 ±	9.2	90
		Experimental series 4 (5-week results)				
IVa[2]	30	None	—	190 ±	6.0	100
IVb	30	None	—	139 ±	9.1	50
IVc	20	Liver residue	10	191 ±	8.4	100
IVd	20	Amino acids[3]	7.6	121 ±	5.0	70

[1] Standard error $= \sqrt{\Sigma d^2/n(n-1)}$.

[2] Basal without iodinated casein. All other diets contained 0.35% of iodinated casein (Protamone, Cerophyl Laboratories, Kansas City, Missouri).

[3] A mixture of the following composition, as per cent of the final diet: L-alanine 0.81, L-arginine·HCl 0.54, L-aspartic acid 0.38, L-cystine 0.12, L-glutamic acid 0.87, glycine 0.40, L-histidine·HCl 0.31, L-isoleucine 0.44, L-leucine 0.84, L-lysine·HCl 0.54, L-methionine 0.17, L-phenylalanine 0.42, L-proline 0.40, L-threonine 0.36, L-tryptophan 0.11, L-tyrosine 0.29, L-valine 0.51, L-serine 0.41. This mixture simulates the balance of amino acids provided by liver residue as 10% of the diet.

animals in each group receiving diets IIb, IIc, IIIb and IIIc were injected intraperitoneally three times weekly with a solution supplying 30 μg of biotin. This was to prevent biotin deficiency from feeding avidin in the egg albumin.[13]

Amino acid analyses

Five-gram samples of each protein were hydrolyzed with 50 ml of 8 N hydrochloric acid by heating under reflux for 8 hours. After appropriate dilution, the hydrolysate was analyzed microbiologically for amino acids (Stokes et al., '45; Hac and Snell, '45; Henderson et al., '48). Chemical procedures were used for estimating tryptophan (Graham et al., '47).

RESULTS

Growth and survival results at the 4- and 5-week intervals are shown in table 1. Four experimental series were run with appropriate controls in each series.

Series 1. Variations in levels of dietary casein

Figure 1 shows the 5-week weight gains and survival responses of rats on the basal and thyrotoxic rations containing 4 levels of casein. Maximum growth was obtained on the basal ration without thyroxine with 20% casein. The 30% ration was almost as active. However, the 40% level of casein was significantly less active than the 20% level (188 gm gain vs. 209 gm).

In the thyrotoxic rations slight improvement of growth and survival was found with increasing levels of casein up to 40%. The improved survivals were most evident at the 4-week period. At 5 weeks, almost all the animals had died. Survival was somewhat better in the 10% casein group, but these animals failed to eat at rates comparable to the other groups. They received less of the diet, resulting in poorer growth, but less iodinated casein, resulting in better survival.

For comparison with the different amounts of casein, diet Ii contained 20% of casein and 10% of liver residue. This supported a growth of 180 gm and 100% survival, in the thyrotoxic rat. Diet Ie (30% of casein) gave only 10% survival.

Series 2. Partial substitution of casein by other proteins

Growth and survival results at the 4-week period are shown in table 1, series 2.

[13] The injection of biotin did not affect the growth or survival in any group compared to the animals in the same group not given biotin. The subsequent experimental results, therefore, include both injected and non-injected animals.

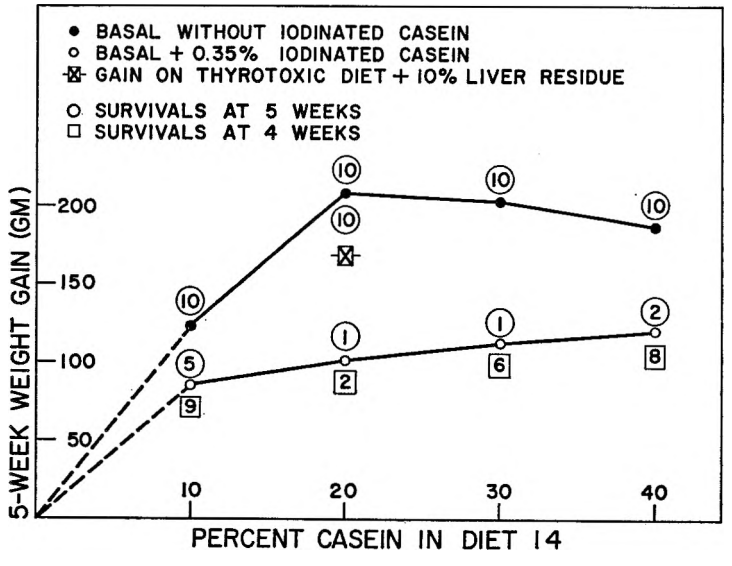

Fig. 1 Response of rats to varying amounts of casein in diet ATF no. 14 with and without iodinated casein.

Liver residue was most effective in promoting 100% survival and a weight gain of 128 gm. Comparable weight gains were given by diets containing whole egg and lactalbumin. However, the poor survival of only 30 and 50% indicated an activity lower than liver residue. Egg albumin appeared not much better than casein, although survival was 70%. Blood fibrin appeared to be intermediate in promoting both growth and survival.

To assess more fully the protective action of the different proteins, we removed and weighed the adrenals, spleen, thymus and kidneys. The results are shown in table 2, diets IIa to IIf. For comparison, the control, non-thyrotoxic group from series 1 (diet Ia) was similarly examined. The adrenals were hypertrophied in the thyrotoxic rats. Liver residue inhibited the enlargement most. Casein, egg albumin and whole egg were least effective and fibrin and lactalbumin were intermediate. There appeared to be no orderly pattern in the weights of the other glands. The thymus appeared to be atrophied in those animals receiving casein only. The spleen and kidneys were uniformly enlarged in all thyrotoxic groups.

Series 3. Complete substitution of casein with other proteins

Table 1, series 3, shows the weight gains and survivals when various proteins were used at levels corresponding to 30% of casein. Survivals were uniformly better than in the series 2 experiments; however, weight gains were comparable in the two experiments. As the sole source of protein, casein and egg albumin were least effective. Whole egg and liver residue were equally active and most effective. Fibrin and lactalbumin were slightly less active.

The weights of the adrenals and kidneys (table 2, diets IIIa to IIIf) indicated liver residue most active in lessening the enlargement of these tissues in thyrotoxic rats. Egg albumin and casein were least effective. Whole egg, fibrin and lactalbumin were intermediate. Again, no consistent pattern was obvious from the weights of the thymus and spleen.

Series 4. Partial substitution of casein with pure L-*amino acids simulating liver protein*

Amino acid composition of the proteins. The amino acid spectrum of each protein was estimated to search for a correlation with the general order of antithyrotoxic activity observed in experiments 2 and 3. The values are shown in table 3. In addition, the amount of protein in the various products was calculated from the relative proportions of amino acids found. This was compared with the "rule of thumb" of total $N \times 6.25$ for total protein. The amount of non-protein nitrogen was high for whole egg and liver residue. About

TABLE 2

Comparative organ weights of thyrotoxic rats receiving casein or other proteins

Diet	Body weight		Organ weights/100 gm body weight							
			Adrenals		Thymus		Spleen		Kidneys	
(*Table 1*)	*gm*	*S.E.*[1]	*mg*	*S.E.*	*mg*	*S.E.*	*mg*	*S.E.*	*mg*	*S.E.*
Ia	228	± 5.7	16	± 1.1	334	± 14	249	± 7	871	± 18
IIa	131	± 6.6	49	± 4.4	245	± 16	390	± 38	1850	± 23
IIb	134	± 5.5	54	± 3.4	298	± 7	430	± 19	2020	± 93
IIc	164	± 18.6	53	± 1.0	385	± 25	510	± 32	1790	± 46
IId	168	± 7.2	35	± 1.6	367	± 17	420	± 12	1600	± 22
IIe	150	± 9.1	44	± 3.1	282	± 21	400	± 19	1640	± 42
IIf	160	± 12.5	40	± 2.3	313	± 42	400	± 23	1640	± 187
IIIa	142	± 7.8	43	± 5.2	327	± 18	374	± 42	1765	± 20
IIIb	118	± 8.2	48	± 2.2	262	± 14	362	± 12	2110	± 63
IIIc	182	± 5.9	35	± 1.1	405	± 19	438	± 17	1550	± 43
IIId	175	± 5.1	25	± 1.1	413	± 20	417	± 11	1416	± 23
IIIe	167	± 5.7	33	± 2.1	327	± 20	422	± 21	1542	± 35
IIIf	169	± 6.8	32	± 1.1	314	± 13	415	± 20	1544	± 41

[1] Standard error $= \sqrt{\Sigma d^2/n(n-1)}$.

20% of liver residue was not accounted for by protein, moisture and ash. There appeared to be no trend in amino acid patterns paralleling the observed antithyrotoxic activity.

Comparative activity of liver residue and a pure L-*amino acid mixture simulating liver protein.* The preceding experiments suggested some protective action for certain materials, rich in protein, especially liver residue. It was not clear whether the protein supplied additional amino acids essential for the hyperthyroid rat, or served simply as a carrier for some unidentified required nutrient. The amino acid analyses did not suggest special activity for any individual amino acid, but the possibility of favorable balance of amino acids was not eliminated. For experiment 2, the dietary amino acid balance was the mean of that for casein and the test protein. For experiment 3, it was that recorded in table 3 for the test protein.

Table 1, series 4, gives the comparative growth and survival responses of rats receiving 10% of liver residue in the diet and those receiving a mixture of 18 L-amino acids identical to that supplied by 10% of liver residue. The amino acid mixture had no antithyrotoxic activity and appeared to inhibit growth slightly when compared to the 30% casein control group (diet IVd vs. IVb). Thus it appeared that the protective effect of liver residue was not duplicated with a comparable mixture of natural L-amino acids.

DISCUSSION

The study of the effects of dietary variations on thyrotoxic animals is beset with difficulties because of the abnormal metabolism and hormonal imbalances. It is, however, a challenging problem because of similarities between thyrotoxicosis and other chronic environmental and biochemical stresses. The known nutrients in many combinations and unusual balances have been tested to promote growth and prolong survival of hyperthyroid and thyrotoxic animals.

The most consistently active antithyrotoxic materials are liver residue and unsaturated fats (Overby et al., '59a, b). Boldt et al. ('58) tested limited amino acid mixtures and individual amino acids and reported that methionine increased survival of hyperthyroid rats. O'Dell et al. ('55), however, reported that a mixture of

TABLE 3

Amino acid spectrum of 6 proteins

L-Amino acid	Mg/100 mg of total nitrogen					
	Casein	Egg albumin	Whole egg	Fibrin	Lactalbumin	Liver residue
Alanine	21	46	44	25	41	63
Arginine	24	37	39	49	24	32
Aspartic acid	46	69	67	41	74	32
Cystine	2.5	19	14	13	34	9
Glutamic acid	140	81	79	86	100	79
Glycine	13	22	20	34	22	32
Histidine	19	15	13	17	17	19
Isoleucine	40	40	39	31	42	38
Leucine	63	55	56	87	72	71
Lysine	51	45	38	55	60	34
Methionine	20	26	20	19	16	15
Phenylalanine	34	37	35	37	34	35
Proline	82	51	24	33	24	24
Serine	43	53	45	60	30	32
Threonine	28	31	31	49	42	30
Tryptophan	6	7	7	21	16	8
Tyrosine	32	21	22	36	33	22
Valine	46	47	44	37	40	43
Total N × 6.25	85.0	77.9	76.1	93.6	78.4	80.3
% Protein calc. from amino acids	85.8	77.5	68.8	96.2	80.2	69.9

glycine, methionine and arginine was inactive. Ershoff ('47) also reported methionine inactive.[14]

The persistent failure in clear-cut fractionation of an active principle from liver residue suggests non-specific activity of the crude material. Reasons for this failure were sought in terms of amino acid nutrition. A change in amino acid balance obviously results when one-third or more of casein in a diet is replaced by liver residue or other material rich in protein. Likewise, the beneficial effects of fats for thyrotoxic rats could represent a sparing action, allowing improved protein nutrition. Some clinical features of thyroid disease and studies with hyperthyroid animals suggest a major role of the thyroid hormone in protein metabolism (Sokoloff and Kaufman, '59).

The present experiments suggest an increasing requirement for protein by the thyrotoxic rat. Maximum growth of normal animals was realized with a diet containing 20% of casein. Improvement in survival and slight growth increments were obtained in thyrotoxic rats with increasing levels up to 40% casein. However, the major deficiencies were not corrected with casein.

Replacing one-half the casein with other good quality proteins (experiment 2) gave increased growth except with egg albumin. Liver residue, in this experiment, was better than any other protein tested in supporting both growth and survival. Interestingly, 30% of liver residue (diet IIId) promoted growth no better than 15% of liver residue (diet IId), but adrenal enlargement was inhibited to a greater extent with the higher level of liver residue (25 vs. 35 mg). Likewise, the high levels of whole egg, fibrin and lactalbumin appeared more protective than the low levels of the same proteins.

To rate objectively the relative activity of various materials one must consider growth, survival and tissue damage. We found that a usable "antithyrotoxic index" could be calculated from the product of the weight gain and per cent survival divided by the adrenals weight (mg/100 gm body weight). In a total of 6 experiments, the control non-thyrotoxic groups gave values of 1000 to 1150 at the 4-week interval. The mean value for the various dietary treatments in experiments 2 and 3 were as follows:

Dietary protein	ATF index
Control (non-thyrotoxic)	1000
Liver residue	445
Fibrin	261
Lactalbumin	250
Whole egg	238
Casein	109
Egg albumin	104

Thus, when all three factors were considered, liver residue was considerably more active than the other proteins. Fibrin, lactalbumin and whole egg showed definite activity.

The absence of any favorable effect from the mixture of L-amino acids (experiment 4) suggests that something other than the known amino acids is responsible for the pronounced antithyrotoxic activity of liver residue. The amino acid balance required for optimum nutrition of the hyperthyroid rat is, of course, still not known. These experiments, likewise, do not rule out the possibility that nutritional adequacy is provided better by intact protein than by equal amounts of amino acids. Nevertheless, it appears that liver residue has antithyrotoxic properties much greater than other proteins of high biologic value. This activity is not accounted for by the readily extractable lipids, the amino acids or the ash.

SUMMARY

Improvement in survival and slight growth increments were obtained in thyrotoxic rats with increasing levels of dietary casein up to 40%. Partial or complete replacement of casein with liver residue gave good growth and survival. Egg albumin was inactive. Intermediate activity was found with fibrin, whole egg and lactalbumin. A mixture of L-amino acids simulating that supplied by liver residue was inactive. The antithyrotoxic activity appeared to be distinct from the protein portion of liver.

[14] We also have found a lack of activity of methionine. Specific results will be reported subsequently along with tests of lipotropic substances, such as choline and betaine.

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Effect of Normal and High Intakes of Orthophosphate and Metaphosphate in Rats

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The increased use of phosphates in foods and beverages raises the question of what are the long-term effects of feeding various types of phosphorus-containing compounds. Examination of the literature reveals a number of, often conflicting, reports on this subject.

Mellanby ('49), who experimented with vitamin D-deficient puppies, was one of the first to claim that phosphates may interfere with calcium absorption. However, no adverse effects of high dietary levels of phosphates on calcium absorption were noted in rats by Hansard and Plumlee ('54) or in chicks by Gershoff and Hegsted ('56). Essentially similar results were obtained from limited studies with humans by Baylor et al. ('50) and Malm ('53). These results do not agree with the findings of Leichsenning et al. ('51) who reported that dibasic phosphate reduced calcium absorption in women.

A reduction in iron absorption from the addition of phosphates to rat diets has been reported by Day and Stein ('38) and Hegsted et al. ('49). Working with dogs, Schweitzer ('56) found that sodium hexametaphosphate decreased iron absorption. On the other hand, Chapman and Campbell ('57) concluded that disodium phosphate and sodium hexametaphosphate had no effect on iron utilization by rats. It might also be mentioned that Elvehjem et al. ('33) and others have shown that certain iron phosphates, commonly used in enrichment programs, are available sources of iron.

Both physiologically beneficial and harmful effects have been attributed to dietary phosphates. House and Hogan ('55) and Maynard et al. ('58) reported that excessive phosphate intake caused stiff joints or resulted in calcium phosphate deposition in guinea pig tissues. Selye and Bois ('56) showed that an excess of phosphates resulted in extensive renal calcification in rats. Nevertheless, Care and Wilson ('56) found that sodium hexametaphosphate helped to prevent the formation of calculi in rat bladders.

Previous studies on the effect of feeding sodium hexametaphosphate, one of the phosphates used in the experiment to be reported, have not been extensive. Initial studies by Schwartz et al. ('40) led to the conclusion that sodium hexametaphosphate was relatively nontoxic. Further investigations by Borenstein and Schwartz ('48) showed that high levels of sodium hexametaphosphate had a favorable effect on calcium retention. Using labeled Ca^{45} in rats, Mattern and Schreier ('56) found that large doses of polyphosphate reduced calcium absorption. However, small doses of sodium hexametaphosphate or the addition of sodium citrate were without effect on the amount of calcium absorbed.

EXPERIMENTAL

Five groups of 12 rats each were used in this study. The rats were Wistar Strain males weighing from 50 to 60 gm each. All animals were kept in conventional type wire-bottom individual cages in an air-conditioned room. Diets and distilled water were fed ad libitum.

Semi-synthetic diets containing casein as the protein were used. All diets were nutritionally complete for the rat and contained ample vitamin D. The amount and composition of the mineral salts were the main variables. The diet serving as the control contained 4% of U.S.P. Salts XIV, which resulted in levels of approximately

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TABLE 1

Composition of diets

Constituent	Control	Normal ortho-phosphate	High ortho-phosphate	Normal meta-phosphate	High meta-phosphate
	%	%	%	%	%
Dietary ingredients					
Casein	22.2	22.2	22.2	22.2	22.2
Cornstarch	65.5	60.1	59.4	59.9	58.8
Cellulose	2.0	2.0	2.0	2.0	2.0
Corn oil	6.0	6.0	6.0	6.0	6.0
Vitamin mix[1]	0.3	0.3	0.3	0.3	0.3
Mineral salts[2]	4.0	9.4	10.1	9.6	10.7
Phosphorus sources					
Dibasic potassium phosphate	0.87	0.87	5.1	—	—
Sodium hexameta-phosphate	—	—	—	0.93	3.5
Analytical values					
Moisture	8.5	7.6	8.0	7.4	7.4
Protein	20.5	21.0	21.2	20.5	20.9
Fat	6.0	6.1	6.0	6.1	6.1
Calcium	0.56	0.47	0.50	0.47	0.50
Phosphorus	0.42	0.43	1.30	0.46	1.20
Calculated values					
Sodium	0.12	0.86	0.86	0.86	0.86
Potassium	0.65	2.5	2.5	2.5	2.5
Magensium	0.05	0.05	0.05	0.05	0.05
Chlorine	0.42	3.2	1.6	3.3	2.5
Calcium citrate	1.2	1.2	1.2	1.6	1.6

[1] The standard vitamin mix contained all the vitamins known to be required by the rat.

[2] Different amounts of salts were added at the expense of cornstarch in order to equalize levels of major minerals.

0.5% of calcium and 0.4% of phosphorus from orthophosphate. The composition of all diets is given in table 1.

The mineral mixtures in the 4 experimental diets were modifications of U.S.P. Salts XIV in which the phosphorus was entirely supplied by either orthophosphate or metaphosphate. Dibasic potassium phosphate served as the main source of orthophosphate. However, the calcium biphosphate normally present in U.S.P. Salts XIV was allowed to remain in the orthophosphate mineral mixtures. A food grade of sodium hexametaphosphate was used as the metaphosphate source.

As shown in table 1, the experimental diets designated as "normal orthophosphate" or "normal metaphosphate" contained adequate levels of 0.43 and 0.46% of phosphorus, respectively. The diets referred to as "high orthophosphate" and "high metaphosphate" contained levels of 1.3 and 1.2 % of phosphorus, respectively. To attain these levels, potassium orthophosphate was added at a 0.87% level to the normal-orthophosphate diet and at a 5.1% level in the high-orthophosphate diet. The hexametaphosphate was used at levels of 0.93% in the normal level diet and at 3.5% in the high level diet.

The mineral mixtures for the experimental diets were adjusted to contain approximately the same amounts of calcium, potassium, sodium and magnesium. As a result, mineral components accounted for 9 to 11% of the experimental diets. Thus, total mineral levels of the 4 experimental diets were much higher than that of the control diet.

The experiment was conducted in three stages, with experimental observations made when animals had consumed the test diets for 50, 60 and 150 days. Table 2 shows the experimental design and gives the procedure followed during each phase of the experiment.

TABLE 2

Experimental design and procedure

Diet	Started	50 Days[1]	60 Days[2]		150 Days[3]	
		Calcium and phosphorus balance	Weight gain	Clinical tests	Weight gain Clinical tests	Histology
	no. rats	*no. rats*	*no. rats*	*no. rats*	*no. rats*	*no. rats*
Control	12	9	12	5	6	4
Experimental						
Normal orthophosphate	12	9	12	5	7	5
High orthophosphate	12	9	12	5	6	5
Normal metaphosphate	12	9	12	5	7	0
High metaphosphate	12	9	12	5	7	5
Total	60	45	60	25	33	19

[1] Measurement of "apparent" calcium and phosphorus retained by the body.

[2] Feeding data included weight gains, food and protein efficiency. The clinical tests included determination of hemoglobin and blood serum calcium and phosphorus content. Internal organ weights were taken as a part of the autopsy procedure.

[3] Feeding data same as given above. Clinical tests included red blood cell counts, hemoglobin, and blood serum calcium and phosphorus. Analyses were made for carcass ash, calcium and phosphorus. Length, density and ash and calcium content of femurs were determined. Histopathological examination was made of heart and kidney tissues.

During the 7-day calcium and phosphorus balance conducted after 50 days on test, amounts of calcium and phosphorus consumed were measured and compared to the amounts excreted in feces and urine. Since the technique used did not differentiate between the unabsorbed exogenous and the endogenous fractions, "apparent" retention by the body was measured.

All animals were fasted for 18 hours prior to autopsy examination and the performance of clinical tests. In the clinical tests, hemoglobin was determined on tail blood using a standard cyanmethemoglobin solution. Calcium and inorganic phosphorus in blood serum were determined according to methods given by Fister ('50).

After autopsy of animals kept for 150 days on test, internal organs and both right and left femurs were removed from the carcasses. The carcasses were then autoclaved for two hours, homogenized in a Waring Blendor, and dried in a shallow pan in a Proctor-Schwartz oven. Analyses for calcium and phosphorus were performed on the dried carcasses.

The two femurs which were dissected out of the carcasses were dried at 70°C in a vacuum oven for two days. After removal of adhering tissues, the bones were extracted first with hot 95% ethyl alcohol, and then with ethyl ether for 18-hour periods. After measurements for length were taken, analyses for calcium and phosphorus were made on the dry, fat-free bones.

Ash content of carcasses and femurs and the calcium and inorganic phosphorus contents of carcasses, femurs, and feces and urine from the balance study were determined by methods prescribed in the A.O.A.C. ('55).

RESULTS AND DISCUSSION

Sixty-day feeding results. As indicated in table 3, with the exception of better food and protein efficiency in the animals fed the control diet, there were no other outstanding differences among any of the diets. Thus, when the control diet is not considered, 60-day performance on the 4 experimental diets, designated as normal and high orthophosphate and normal and high metaphosphate, was about equal.

The lower food and protein efficiencies obtained with the 4 experimental diets in comparison with the control diets was probably an indication of a low-order dietary mineral imbalance. This may have resulted from the large amounts of salts added to the 4 experimental diets, but not to the control, in order to equalize calcium, sodium and potassium levels. Previous workers have shown that high levels of

TABLE 3

Summary of 60-day feeding and clinical results[1]

	Body weight gain	Consumption		Efficiency[2]		Clinical tests		
		Food	Protein	Food	Protein	Hemoglobin	Blood serum	
							Calcium	Phosphorus
	gm	*gm*	*gm*			*gm/100 ml*	*mg/100 ml*	*mg/100 ml*
Control	294 ± 12	1095 ± 58	225 ± 12	0.27 ± 0.01	1.32 ± 0.04	17 ± 0.2	7.1 ± 1.3	8.2 ± 0.4
Normal orthophosphate	271 ± 7	1108 ± 38	233 ± 8	0.25 ± 0.01	1.18 ± 0.04	18 ± 0.5	7.1 ± 1.3	8.3 ± 0.6
High orthophosphate	275 ± 5	1197 ± 45	254 ± 10	0.23 ± 0.01	1.09 ± 0.04	18 ± 0.2	6.8 ± 1.8	7.2 ± 0.6
Normal metaphosphate	275 ± 5	1172 ± 48	240 ± 3	0.24 ± 0.01	1.16 ± 0.04	17 ± 0.2	7.9 ± 1.4	8.7 ± 0.6
High metaphosphate	269 ± 7	1198 ± 33	250 ± 7	0.23 ± 0.01	1.08 ± 0.03	18 ± 0.2	6.7 ± 1.7	8.0 ± 0.6

[1] There was no statistical significance among any values for body weight gain, food consumption, protein consumption, hemoglobin, serum calcium and serum phosphorus.

[2] Food and protein efficiency were significantly better on the control diet than on the following diets:

	Food efficiency	Protein efficiency
	P	*P*
Control ~ Normal orthophosphate	—	0.05
Control ~ High orthophosphate	0.01	0.001
Control ~ Normal metaphosphate	0.02	0.02
Control ~ High metaphosphate	0.01	0.001

sodium chloride caused adverse effects on rat growths. The higher salt intakes from the 4 experimental diets in this test did not significantly retard growth. This may have been so because of the extra potassium added to these diets since it is known that the addition of potassium may moderate the toxic effects resulting from a high sodium intake.

The lack of any symptoms of iron-deficiency anemia, as indicated by normal hemoglobin values with all diets, showed that absorption of dietary iron was probably sufficient and not affected by the phosphates. It is realized, however, that all diets contained high levels of iron and that fetal iron stores may have influenced the hemoglobin values obtained at this age.

Calcium and phosphorus balance and excretory patterns. The results of a calcium and phosphorus balance conducted on actively growing rats after 50 days on test are presented in table 4. The values represent "apparent" calcium and phosphorus retention because endogenous fractions were not measured. It is shown that approximately 10% more calcium was retained by animals fed the control and the two metaphosphate diets than by those fed the two orthophosphate diets.

The type of phosphate had more effect than the level in the diet. Calcium retention with a particular type of phosphate remained approximately the same, regardless of whether the normal or high level was fed. In contrast to the indication of better calcium utilization with metaphosphate, both orthophosphate and metaphosphate proved to be equally good sources of available phosphate. There was not significant difference in phosphorus retention among any of the 5 diets.

With either type of phosphate studied, high levels did not adversely affect calcium retention. Retention values for normal and high levels of orthophosphate differed only by 1%, while those for the metaphosphate diets differed by 2%. Thus, the results obtained in the experiment agree with many reports in the literature that the high levels of phosphate do not seriously impair calcium absorption. In this experiment, both the dibasic potassium orthophosphate and the sodium hexametaphosphate appeared to be well-utilized sources of phosphorus.

Through a study of excretory patterns, the balance study provided some information on the absorption and metabolism of the dietary calcium and phosphorus. The calcium excretory picture showed that the unretained calcium fraction was principally eliminated by way of the feces. Almost all of the portion which was absorbed appeared to have been stored in the body, for urinary calcium represented only 1 to 2% of the total cal-

TABLE 4

Calcium and phosphorus balance in growing rats[1]

Diet	Calcium			Phosphorus		
	Apparent retention[2,3]	Excreted		Apparent retention[2,4]	Excreted	
		Feces	Urine		Feces	Urine
	%	%	%	%	%	%
Control	54 ± 3	45	1	44 ± 3	38	18
Normal orthophosphate	44 ± 4	54	2	39 ± 1	28	33
High orthophosphate	45 ± 4	54	1	41 ± 1	16	43
Normal metaphosphate	56 ± 4	42	2	43 ± 3	35	22
High metaphosphate	54 ± 4	45	1	38 ± 3	25	37

[1] Data from a 7-day balance, 9 animals per group after 50 days on test diets.

[2] Apparent retention $= \frac{\text{intake} - (\text{fecal} + \text{urinary output})}{\text{intake}} \times 100.$

[3] Significantly less calcium was retained on the normal orthophosphate diet than with the following diets:

	P
Normal orthophosphate ~ Control	0.05
Normal orthophosphate ~ Normal metaphosphate	0.05

[4] Phosphorus apparent retention values were not significantly different.

cium intake with any diet. In keeping with the finding that more dietary calcium was retained with the metaphosphate than the orthophosphate diets, there was less fecal elimination of calcium by animals fed the metaphosphate diets.

The excretory pattern for phosphorus varied according to type and level of phosphate fed. It was apparent, however, that a large portion of the unretained phosphorus had been absorbed and subsequently excreted in the urine. Phosphorus absorption must have been considerable with the high-orthophosphate and the high-metaphosphate diets, for the urinary route was a more important means of phosphorus elimination than the feces. Nevertheless, serum phosphorus values taken at the end of the balance at 60 days (table 3) on fasted animals showed no differences between the high and low levels of phosphate feeding. This suggests that high levels of dietary phosphate, when absorbed, must raise serum phosphorus only temporarily, and that the blood and kidneys can readily dispose of large amounts of absorbed phosphorus. Similar observations have been reported for high calcium intakes by many workers including Bronner and Harris ('56).

One-hundred-fifty-day feeding, clinical and autopsy results. A summary of the long-term (150-day) weight gains, food and protein intakes, food and protein efficiencies, hemoglobin values, red blood cell counts and serum calcium and phosphorus levels is presented in table 5. As with the data for the 60-day early growth, the superiority of the control animals in utilizing food and protein most efficiently was confirmed. Other comparisons, especially between the 4 experimental ortho- or metaphosphate diets, failed to reveal any advantage or deleterious effect for any particular type or level of phosphate.

During the 150-day test period, rats fed the high-orthophosphate diet consumed the most food and gained the most weight. Animals receiving the high-metaphosphate diet gained the least weight. Body weight gains for the high-orthophosphate diet were significantly greater ($P = 0.05$ to 0.001) than the weights obtained for all diets except the control. This result differs from the 60-day weight gains where there was no significance among any weight gains. However, in the absence of carcass composition analyses, it it difficult to interpret the importance of the weight response on the high-orthophosphate diet. In this respect, values for femur length, used as a measure of body development, were similar for all of the diets.

Protein and food efficiency values were very sensitive to the experimental treatments. Following the pattern of the 60-day results, rats receiving the control diet had a significantly better food efficiency ($P = 0.01$) than animals fed the high-metaphosphate diet which supported the poorest growth. Protein efficiency on the control diet was significantly better ($P = 0.05$ to 0.01) than the values obtained with any of the 4 experimental diets. However, food and protein efficiencies among any of the 4 experimental orthophosphate and metaphosphate diets were not significantly different. These results indicate that, by the standards of the control diet, the 4 experimental diets were not optimal, probably because of excessive amounts of salts used to equalize some major mineral components. Nevertheless, when considered as a separate entity, statistical analysis showed that with the exception of weight gained, the 4 experimental diets produced generally identical feeding results.

The results of clinical tests for hemoglobin, red blood cell counts and blood serum calcium and phosphorus are given in table 5. Blood serum calcium values were statistically identical with all diets. Hemoglobin and red blood cell counts were not significantly different among any of the 5 diets. Thus, after 150 days of feeding, there was no clinical evidence of any impairment of calcium or iron absorption with either the normal or high levels of ortho- or metaphosphate.

All of the remaining animals on the long-term part of the test were sacrificed at the termination of the experiment at 150 days. Average weights of various internal organs per 100 gm of body weight are given in table 6. No abnormalities were observed in any of the organs. It was noted, however, that animals on the 4 experimental diets had heavier hearts, kidneys and testes per 100 gm of body weight than control

TABLE 5

Summary of 150-day feeding and clinical studies

	Body weight gain[1]	Femur bone length	Consumption		Efficiency		Clinical tests			
			Food[1]	Protein[1]	Food[1]	Protein[1]	Hemoglobin	R.B.C.	Serum calcium	Serum[1] phosphorus
	gm	*cm*	*gm*	*gm*			*gm/100 ml*	*10^6/mm^3*	*mg/100 ml*	*mg/100 ml*
Control	420±22	3.78±0.07	3002±180	617±37	0.14±0.01	0.69±0.04	17±0.9	7.8±0.6	9.2±1.3	6.4±0.6
Normal orthophosphate	385±11	3.68±0.04	3266±103	686±22	0.12±0.01	0.57±0.02	18±0.5	7.3±0.4	8.4±0.5	7.8±0.4
High orthophosphate	432±9	3.77±0.05	3521±83	748±18	0.12±0.01	0.58±0.02	16±0.8	7.5±0.2	8.8±0.7	6.0±0.2
Normal metaphosphate	403±9	3.73±0.05	3352±177	686±36	0.12±0.01	0.60±0.02	17±0.5	7.7±0.7	7.9±0.7	6.1±0.2
High metaphosphate	376±9	3.76±0.05	3473±149	725±31	0.11±0.01	0.52±0.02	17±0.3	7.6±0.1	7.9±0.5	6.3±0.4

[1] The following comparisons were found to be significantly different:

	Weight gain	Food consumption	Protein consumption	Food efficiency	Protein efficiency	Serum phosphorus[2]
	P	*P*	*P*	*P*	*P*	*P*
High orthophosphate ~ Normal orthophosphate	0.01	—	—	—	—	0.001
High orthophosphate ~ Normal metaphosphate	0.05	—	—	—	—	—
High orthophosphate ~ High metaphosphate	0.001	—	—	—	—	0.01
Control ~ Normal orthophosphate	—	—	—	—	0.02	—
Control ~ High orthophosphate	—	0.05	0.01	—	0.05	—
Control ~ Normal metaphosphate	—	—	—	—	0.05	—
Control ~ High metaphosphate	—	—	0.02	0.01	0.01	—
Normal orthophosphate ~ Normal metaphosphate	—	—	—	—	—	0.001

[2] Serum phosphorus level for the normal orthophosphate diet appears to be abnormally high and is responsible for the significant differences recorded.

TABLE 6

Internal organ weights at 150 days autopsy in grams per 100 gm of body weight

	Liver[1]	Heart[1]	Kidneys[2]	Spleen[1]	Testes[2]
Control	2.3 ± 0.03	0.28 ± 0.01	0.61 ± 0.03	0.27 ± 0.08	0.63 ± 0.06
Normal orthophosphate	2.3 ± 0.06	0.30 ± 0.01	0.71 ± 0.03	0.21 ± 0.03	0.79 ± 0.03
High orthophosphate	2.5 ± 0.21	0.30 ± 0.01	0.73 ± 0.04	0.20 ± 0.02	0.75 ± 0.19
Normal metaphosphate	2.3 ± 0.04	0.29 ± 0.01	0.68 ± 0.03	0.21 ± 0.03	0.72 ± 0.02
High metaphosphate	2.3 ± 0.08	0.31 ± 0.01	0.73 ± 0.04	0.18 ± 0.05	0.80 ± 0.01

[1] There were no significant differences in liver, heart and spleen weights among any of the diets.
[2] The following organ weight comparisons were found to be significantly different:

	Kidneys	Testes
	P	*P*
Control ~ Normal orthophosphate	—	0.05
Control ~ High orthophosphate	0.05	—
Control ~ High metaphosphate	0.05	0.02
Normal orthophosphate ~ Normal metaphosphate	—	0.05
Normal metaphosphate ~ High metaphosphate	—	0.01

animals. The kidneys of animals fed the high levels of either orthophosphate or metaphosphate were significantly heavier ($P = 0.05$) than those from the control rats. This was perhaps a manifestation of an increased "load" on the kidneys due to a high salt intake. Nevertheless, average kidney weights among the 4 experimental groups were not significantly different.

A total of 19 heart and kidney tissue sections representing samples taken from the control, normal-orthophosphate, high-orthophosphate and high-metaphosphate groups were examined by a pathologist. Despite reports that excess intake of phosphates may, under certain conditions, cause formation of calculi in the kidneys, the pathologist reported that there were no histological differences that could be attributed to the dietary phosphates.

Mineral content of carcasses and femurs. Table 7 shows the statistical similarity in carcass and femur ash values, the greater carcass calcium and phosphorus content in metaphosphate-fed rats, and that type of phosphate affected carcass mineral composition more than the dietary level of phosphate. Statistical analyses showed significantly less calcium ($P = 0.05$ to 0.01) was contained in the orthophosphate carcasses than in the metaphosphate carcasses.

Carcass phosphorus content was highest with the metaphosphate diets, intermediate with the orthophosphate diets and least with the control diet. Nevertheless, in spite of the differences in carcass calcium and phosphorus composition, values for femur ash and calcium content were not significantly different.

The results of the carcass analyses showing greater calcium storage with metaphosphate than with orthophosphate correspond somewhat with the 50-day calcium and phosphorus balance data. It will be recalled that the balance study showed a lower calcium retention in animals fed the orthorphosphate diets and more effect from type than level of dietary phosphate. However, neither the balance nor the carcass composition results revealed any deleterious effects of consequence.

It is of interest that the femur analyses showed no significant changes in ash or calcium with any of the dietary treatments. These results suggest that the increased amounts of calcium found in the metaphosphate carcasses and the increased amounts of phosphorus found in the orthophosphate and metaphosphate carcasses, as compared with the control, have been stored, not in the bones, but in other body tissues. Further studies are needed to confirm and interpret these results.

SUMMARY

Normal and high levels of phosphorus (0.4 and 1.2% phosphorus) in the form of orthophosphate and metaphosphate were fed to growing rats for 150 days. While the high-metaphosphate diet produced the poorest growth and food and protein effici-

TABLE 7

Effect of phosphates on carcass and femur mineral content[1]

	Carcasses			Femur bones	
	Ash[2]	Calcium[3]	Phosphorus[3]	Ash[2]	Calcium[2]
	% *dry basis*	% *dry basis*	% *dry basis*	% *dry basis*	% *dry basis*
Control	8.0 ± 0.5	2.6 ± 0.10	1.2 ± 0.03	67.8 ± 12	25.1 ± 4.5
Normal orthophosphate	8.4 ± 0.3	2.5 ± 0.06	1.3 ± 0.03	69.2 ± 10	25.8 ± 4.6
High orthophosphate	7.9 ± 0.6	2.4 ± 0.08	1.3 ± 0.03	69.7 ± 13	25.8 ± 4.6
Normal metaphosphate	8.8 ± 0.2	2.9 ± 0.11	1.5 ± 0.03	68.9 ± 10	25.5 ± 3.6
High metaphosphate	8.1 ± 0.4	2.8 ± 0.13	1.5 ± 0.06	68.4 ± 10	25.6 ± 3.6

[1] Carcass analyses do not include femurs or viscera. Values are averages of 6 to 7 animals.

[2] No significant differences existed among values for carcass ash, femur ash and femur calcium.

[3] The following comparisons are statistically different:

	Carcass calcium	Carcass phosphorus
	P	*P*
Control ~ Normal orthophosphate	—	0.05
Control ~ Normal metaphosphate	—	0.001
Control ~ High metaphosphate	—	0.001
Normal orthophosphate ~ Normal metaphosphate	0.01	0.02
Normal orthophosphate ~ High metaphosphate	0.05	0.02
High orthophosphate ~ Normal metaphosphate	0.01	0.001
High orthophosphate ~ High metaphosphate	0.05	0.01

ency, neither the types nor the levels of phosphates impaired growth conclusively as measured by weight gained and femur length.

Balance studies indicated that calcium retention was lower with orthophosphate, while phosphorus retention was similar with all diets. High levels of phosphate did not affect calcium retention significantly. The type of phosphate had more effect than the level fed. More calcium and phosphorus was stored in the carcasses of metaphosphate-fed animals, but femur analyses were similar for ash and calcium content.

No adverse physiological effects were observed from autopsy, histological and clinical studies. All of the data obtained from this study indicated that there was probably adequate absorption and utilization of calcium, phosphorus and iron with both high and normal levels of either orthophosphate or metaphosphate.

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Suppression of Rat Proteinuria by Dietary Glycine[1]

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The amount of protein in the urine of normal rats can be increased by feeding additional protein (Addis et al., '26; Linkswiler et al., '52; and Rumsfeld and Baumann, '55), urea (Finlayson and Baumann, '56) or a mixture of amino acids equivalent to those in casein (Finlayson and Baumann, '56). In general the degree of proteinuria seems to vary roughly with the amount of nitrogen ingested, but some differences between various sources of nitrogen have also been noted. In a preliminary experiment (Finlayson, '55), rats fed an amino acid mixture containing 61% of glycine not only failed to show an elevated proteinuria but their protein excretion was actually depressed somewhat. The present experiments show that glycine suppresses proteinuria at less extreme levels of administration, and include attempts at a possible explanation of the phenomenon.

EXPERIMENTAL

Adult male albino rats, approximately 300 to 400 gm in weight, of the Holtzman strain were housed in individual wire-bottom metabolism cages in an animal room maintained at 72 to 75°F and 40 to 50% relative humidity. Twenty-four-hour samples of urine were collected under toluene from experimental groups consisting of 5 animals each. The screens used to retain feces and the funnels were washed down into sample bottles with a fine spray of distilled water. When animals were fed during collection, 5 ml of 1 M acetate buffer pH 4.5 were added to the sample bottle and the washings performed with 0.02 M acetate buffer. The samples were filtered through Whatman no. 42 filter paper and the filtrates dialyzed in sausage casings[3] against 4 changes of distilled water for at least 48 hours in a cold room at 3°C. Trichloroacetic acid was added to a concentration of 5%, the samples heated on the steam bath to promote coagulation, and the precipitated protein centrifuged for 10 minutes at 2500 revolutions per minute. The supernatant was decanted and the precipitate dissolved in 1 ml of 1 N sodium hydroxide and transferred to a microKjeldahl flask for nitrogen determination by the method of Hiller, Plazin and Van Slyke ('48) as modified by Burris and Williams.[4] Glycine and urea were determined by the method of Alexander et al. ('45) and Archibald ('45) respectively. Oxalate was determined by a repeated precipitation of the filtered urine with an equal volume of 5% of calcium chloride, followed by titration of the sulfuric acid-treated solution with standard permanganate; this procedure yielded recoveries of 90 to 95% for oxalate added to urine. Urinary calcium was determined by the method of Shohl and Pedley ('22).

The basal diets contained Wesson salts 4%, corn oil 5%, vitamin mixture 0.1%, choline chloride 0.1%, α-tocopherol solution 0.09% and nitrogen sources and dextrin to 100%. The vitamin mixture consisted of the following in milligrams: inositol 100, calcium pantothenate 20, niacin 10, menadione 10, riboflavin 2, thiamine 2, pyridoxine 2.5, biotin 0.1, folic acid 0.2, vitamin B_{12} 0.02; and glucose to 1.0 gm. The vitamin E solution contained 50 mg of α-tocopherol per milliliter of corn oil. Each rat was also fed one drop of halibut liver oil weekly.

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RESULTS

Effect of certain nitrogen sources on proteinuria. In this experiment all diets were isonitrogenous with a control diet containing 20% of casein. Five per cent of glycine replaced an isonitrogenous amount of casein (6.6%) and the other substances tested were added in amounts isonitrogenous with 6.6% of casein. Thus the following levels were added to 13.4% of casein: 5% of glycine with high and low folic acid, 2% of urea, 9.8% of L-glutamic acid, 3.5% of L-arginine·HCl, 4.4% of L-asparagine, and 7.5% of diammonium citrate. Another diet contained 6.4% of citric acid added to the 20% casein diet. The amounts of diet offered were restricted to that consumed by the group that ate the least. Urine was collected after one week of adjustment to the diets.

Table 1 shows that the proteinuria produced by these diets decreased in the following order: 20% casein, 9.8% L-glutamic acid, 2.0% urea, 3.9% L-arginine, 7.5% diammonium citrate, 4.4% L-asparagine, 6.4% citric acid and 5.0% glycine. The decreases observed with diammonium citrate, citric acid and glycine were statistically significant ($P < 0.05$) when compared to protein excretion on the control diet containing 20% of casein. Although folic acid reduces the toxicity of high levels of glycine fed to rats (Pagé et al., '49, Dinning et al., '49) or chicks (Naber et al., '56), the addition of 2.0 mg of folic acid per kilogram of diet for a week or the omission of the vitamin from this diet did not appear to influence the ability of glycine to reduce proteinuria, the excretion of protein in both cases being 41% under that on the control diet. Citric acid added to a 20% casein diet caused a substantial decrease in urinary protein (36%) as did the replacement of 6.6% of casein with an isonitrogenous level of diammonium citrate (28%). Proteinuria due to urea was somewhat less than that due to casein, in agreement with Finlayson and Baumann ('56), while that due to L-glutamic acid or L-arginine was essentially equivalent to that due to casein.

Effect of supplemental glycine on proteinuria. Protein excretion was determined on a 12% casein diet prior to test and was found to be normal and uniform, 1.76 to 1.93 mg of nitrogen per day (table 2). Urinary protein was measured at inter-

TABLE 1

Effect of nitrogen source on proteinuria

Diet	Body weight	Food intake	Urinary protein
	gm	*gm/rat/day*	*mg N/rat/day*
13.4% Casein + 6.6% casein	325	12.3	1.92 ± 0.16[1]
13.4% Casein + 9.8% L-glutamic acid	313	12.3	1.69 ± 0.13
13.4% Casein + 2.0% urea	322	11.8	1.46 ± 0.13
13.4% Casein + 3.9% L-arginine·HCl	324	12.5	1.45 ± 0.18
13.4% Casein + 7.5% diammonium citrate	310	11.5	1.38 ± 0.12
13.4% Casein + 4.4% L-asparagine	314	11.5	1.29 ± 0.24
20% Casein + 6.4% citric acid	311	11.6	1.22 ± 0.18
13.4% Casein + 5.0% glycine (high folic acid)[2]	335	11.6	1.13 ± 0.15
13.4% Casein + 5.0% glycine (no folic acid)	332	11.7	1.13 ± 0.11

[1] Standard error of the mean.
[2] Supplied 2.0 mg/kg of folic acid.

TABLE 2

Proteinuria of rats fed supplemental glycine

Diet	0 Days	5 Days	16 Days[1]	32 Days	2 Days post treatment
	mg of urinary protein N/rat/day				
12% Casein	1.88 ± 0.09[2]	2.25 ± 0.16	1.99 ± 0.09	1.78 ± 0.06	1.67 ± 0.13
12% Casein + 2.5% glycine	1.93 ± 0.06	1.63 ± 0.06	1.73 ± 0.13	1.40 ± 0.09	1.56 ± 0.12
12% Casein + 5.0% glycine	1.86 ± 0.09	1.44 ± 0.10	1.48 ± 0.07	1.36 ± 0.07	1.70 ± 0.13
12% Casein + 7.5% glycine	1.76 ± 0.06	1.11 ± 0.09	1.78 ± 0.17	1.52 ± 0.10	1.57 ± 0.05

[1] Values after 13 or 19 days were very similar to those after 16 days.
[2] Standard error of the mean.

vals up to 32 days on test diets containing additional glycine at 2.5, 5.0 and 7.5% of the diet. Consumption was ad libitum and the rats were fed during collection. The average food consumption for the period directly preceding and including the collections varied from 21.6 to 23.6, 19.1 to 19.5 and 17.1 to 18.2 gm per rat per day for the 5th, 16th, and 32nd days respectively.

After 5 days on the test diets, protein excretion had decreased with increasing glycine content of the diet; rats receiving the 7.5% glycine diet excreted only one-half as much protein as those in the control group (table 2). The decrease in protein excretion of rats fed 2.5 and 5.0% of glycine was statistically significant ($P < 0.05$) in 9 of the 10 measurements made, the exception being for the 16th day for animals receiving the 2.5% glycine diet. The average protein excretion for all determinations made for rats receiving 2.5, 5.0 or 7.5% glycine diets was 79, 72 and 79% respectively of that produced by the control diet, in spite of the fact that nitrogen intake and urea excretion were increased by the additional glycine consumed. On continued feeding of the 7.5% glycine diet, proteinuria was lower than that in the control rats but not significantly so. Presumably the production of increased amounts of urea, which normally increases proteinuria (table 3) (Finlayson and Baumann, '56; Rumsfeld, '56), was counteracting the depressing effect of glycine under these conditions.

After the 32-day test period the rats were again fed the control diet containing 12% of casein, and proteinuria was measured after two days. Proteinuria was essentially similar for the test rats and those that had not received glycine (table 2). Thus the effect of high glycine diets on proteinuria appeared to be reversible.

Excretion of glycine, protein and urea by rats fed free glycine. Two and five-tenths to seven and five-tenths per cent of glycine were added to diets containing 10 or 20% of casein, and the diets were fed ad libitum for one week. The animals were fasted during the collection of urine, and the amounts of glycine, protein and urea excreted were determined. Daily glycine excretion was found to increase from 0.41 or 0.42 mg on 10 or 20% of casein to only 0.80 or 0.93 mg when 7.5% of glycine was added to these diets (table 3), but the addition of this level of glycine to the basal diets increased the excretion of urea by 50 to 60% (table 3). Thus it was evident that a considerable fraction of the added glycine was metabolized to urea.

Since there is a close parallelism between the hourly excretion of both urea and protein by rats injected with urea (Rumsfeld, '56), urinary protein/urea ratios provide a means of evaluating factors that alter proteinuria (Rumsfeld, '56; and Finlayson and Baumann, '56). In the present experiments the protein/urea ratio (urinary protein N $\times$ 10^2/urea N) decreased substantially when glycine was fed (table 3) e.g., from 2.08 to 0.90 when 7.5% of glycine was added to the 20% casein diet. Addition of the first 2.5% of glycine to 20% of casein produced a decrease in ratio from 2.08 to 1.52, while

TABLE 3

Excretion of glycine, urea, and protein by rats fed glycine

Diet	Glycine	Urea	Protein	Protein N $\times$ 10^2/ urea N
	mg/rat/day	*mg/rat/day*	*mg N/rat/day*	
20% Casein	0.42 ± 0.03[1]	232 ± 10	2.24 ± 0.20	2.08
20% Casein + 2.5% glycine	0.53 ± 0.02	285 ± 3	2.03 ± 0.09	1.52
20% Casein + 5.0% glycine	0.70 ± 0.05	313 ± 24	1.62 ± 0.12	1.11
20% Casein + 7.5% glycine	0.93 ± 0.10	376 ± 41	1.58 ± 0.18	0.90
10% Casein	0.41 ± 0.02	140 ± 7	1.30 ± 0.07	1.99
10% Casein + 2.5% glycine	0.56 ± 0.06	158 ± 6	1.07 ± 0.19	1.46
10% Casein + 5.0% glycine	0.72 ± 0.04	196 ± 11	1.04 ± 0.05	1.13
10% Casein + 7.5% glycine	0.80 ± 0.05	209 ± 28	1.04 ± 0.14	1.07

[1] Standard error of the mean.

the addition of the final 2.5% of glycine reduced the ratio merely from 1.11 to 0.90 although the glycine content of the urine increased at an approximately constant rate with increasing supplementation. Results for the 10% casein diets were similar. It would appear that the initial additions of glycine were the most effective and that additions in excess of 7.5% of glycine would have little effect on the ratio.

Effect of bound glycine on proteinuria. The control diet contained 20% of casein and the test diet 19.4% of gelatin and 0.6% of casein; these were fed to rats for one week prior to collection of urine. The test diet contained the equivalent of 5% of glycine. Average food consumption during the week was 12.3 and 11.8 gm per rat per day for the control and the test animals respectively. Although urinary protein averaged 0.98 mg of nitrogen per day for animals fed the gelatin diet compared to 1.20 for those fed the casein diet, the difference was not statistically significant. In another experiment rats fed a 10% casein-10% gelatin diet did not excrete significantly less protein than those fed a 20% casein diet. The data suggest that bound glycine does not reduce proteinuria to the same extent as does free glycine. Data by Rumsfeld ('56) on rats fed casein, egg albumin, or fibrin also suggest that the protein/urea ratio varies in an inverse way with the glycine content of the protein fed, though not to a significant degree.

Effect of glycine on oxalate excretion. It has been suggested that glycine toxicity in the chick may be due to oxalic acid arising from an enzymatic conversion of glycine to glyoxylic acid which is then oxidized further (Naber et al., '56); enzyme systems for such a conversion have been found in rat liver and kidney (Ratner et al., '44). In humans susceptible to hyperoxaluria, labeled glycine appears to be the major precursor of the oxalic acid in urine (Crawhall et al., '58). Table 4 illustrates the amounts of oxalate excreted by rats fed 2.5, 5.0 and 7.5% of glycine or 5% of potassium oxalate dihydrate. Urinary oxalate was increased from 12.2 to 31.8 mg by 7.5% of glycine. The increase by 7.5% of glycine over that produced by 5% of glycine was relatively small as was the depression in the protein/urea ratio (table 3), while the increase in oxalate excretions with the first 2.5% increment of glycine was marked, as was the depression in the protein/urea ratio. When 5.0% of potassium oxalate dihydrate was fed, oxalate excretion in the urine was intermediate between that produced by 2.5 and 5.0% of glycine. This represented an approximate 5% transmission of dietary oxalate into the urine, which is in agreement with the 2.3 to 4.5% conversion obtained for humans reported by Archer et al. ('57).

Effect of exogenous oxalate or calcium on proteinuria. Table 4 illustrates the effect of 1, 3 and 5% of potassium oxalate dihydrate on protein excretion and on the protein/urea ratio; 1.0% of potassium oxalate had no effect on either quantity while 5.0% produced a 42% depression in protein excretion and a 35% depression in protein/urea ratio.

The urinary calcium excretion of rats receiving 5% of either glycine or potassium oxalate dihydrate appeared to be

TABLE 4

Daily oxalate and protein excretion by rats fed supplemental glycine or oxalate

Diet	Urinary oxalate	Urinary protein	Protein N × 10²/ urea N
	mg/rat/day	*mg N/rat/day*	
20% Casein	12.2 ± 2.9[1]	1.88 ± 0.18	1.35 ± 0.07
20% Casein + 2.5% glycine	22.4 ± 2.9		
20% Casein + 5.0% glycine	29.4 ± 3.6		
20% Casein + 7.5% glycine	31.8 ± 2.6		
20% Casein + 1.0% $K_2C_2O_4 \cdot 2\,H_2O$		1.84 ± 0.19	1.36 ± 0.17
20% Casein + 3.0% $K_2C_2O_4 \cdot 2\,H_2O$		1.57 ± 0.09	1.20 ± 0.05
20% Casein + 5.0% $K_2C_2O_4 \cdot 2\,H_2O$	25.1 ± 2.1	1.10 ± 0.16	0.88 ± 0.01

[1] Standard error of the mean.

slightly below that of control rats on a diet containing 20% of casein. However, no direct relationship between protein excretion and calcium excretion seemed to exist, since 2% of exogenous calcium chloride dihydrate produced 300 to 400% elevations in calcium excretion without any significant alteration in the excretion of protein.

DISCUSSION

In contrast to the effects of most sources of dietary nitrogen, glycine has been found to reduce protein excretion substantially. It is more active in this respect than any other amino acid tested. The amount of glycine excreted in the urine is less than 0.1% of that consumed, while urea excretion is markedly increased by glycine feeding. Hence it does not appear that the glycine molecule is itself responsible for the critical alterations in kidney function that are involved. However these two facts do suggest that glycine is largely metabolized and that a metabolite could be responsible for the observed decrease in proteinuria. The increased production of urinary oxalate when glycine is fed and the parallelism between oxalate excretion and a depressed protein/urea ratio suggest that the proteinuria-suppressing action of dietary glycine may be due at least in part to oxalate that passes through the kidney.

Proteinuria results when plasma protein filters through the glomerulus and fails to be reabsorbed through the tubules; a significant change in either of these processes would be expected to alter proteinuria. Oxalate could conceivably decrease the filtration of protein by decreasing pore size; it might increase reabsorption by inhibiting the denaturation of filtered protein by urea. Simpson and Kauzmann, ('53) have shown that the denaturation of ovalbumin by urea is diminished somewhat by oxalate.

SUMMARY

Proteinuria in rats was measured as the nitrogen in the trichloroacetic acid precipitate of dialyzed urine. Most sources of dietary nitrogen increased urinary protein whereas supplemental glycine significantly reduced it. The ingestion of glycine failed to increase the excretion of glycine materially, but did increase the excretion of urea and of oxalate. The administration of oxalate depressed proteinuria somewhat. It is suggested that oxalate, a metabolite of glycine, may be in part responsible for the proteinuria-reducing action of the amino acid. Citrate also reduced protein excretion; calcium was without effect.

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The Effect of Certain Inorganic Salts upon Azo Dye Carcinogenesis[1]

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It has been shown that the addition of high levels of copper salts to the diet of rats fed carcinogenic azo dyes reduces the incidence of liver tumors and that this decreased incidence of tumors can be explained, in part at least, by the destruction of the dye catalyzed by the copper (King et al., '57). Manganese salts added to the diet have been reported to reduce the induction time of liver tumors produced by the azo dye 4-dimethylaminoazobenzene (Sharpless, '45).

This report presents the results of studies on the effect of high levels and of trace amounts of certain elements upon the incidence of liver tumors resulting from the feeding of the carcinogen, 3′-methyl-4-dimethylaminoazobenzene. The elements studied were manganese, zinc, nickel, fluorine, molybdenum, cadmium and cobalt. The effects of these elements upon liver riboflavin and total liver azo dye are also presented.

METHODS

Male rats[2] weighing approximately 200 gm were fed the carcinogen 3′-methyl-4-dimethylaminoazobenzene (3′me-DAB) for 8 weeks at a dietary level of 0.064% and then fed the basal diet dye-free for an additional 8 weeks after which time the animals were killed and the incidence of liver tumors was determined grossly or microscopically or both. The basal diet was similar to that used previously (Rusch et al., '45, King et al., '57) and had the following composition: glucose monohydrate[3] 79, vitamin-free casein 12, salts 4 (Wesson, '32) and corn oil 5%. Vitamins were added such that each kilogram of diet contained 3 mg of thiamine hydrochloride, 7.5 mg of calcium pantothenate, 2.5 mg of pyridoxine, 2 mg of riboflavin and 1 gm of choline chloride. The diets high in nickel, cobalt, manganese and zinc were prepared in two halves such that one half contained the salts and glucose and the other half contained the oil, dye, vitamins, casein and the balance of the glucose. Sufficient amounts of these two halves were mixed and placed in clean food cups daily. The high or low level of the element in the diet was only fed during the first 8 weeks along with the dye; during the final 8 weeks the basal diet was fed. Halibut liver oil was given every two weeks by dropper. Distilled drinking water was supplied when a low level of the element was under study. The Wesson's salts were compounded from analytical-grade reagents to minimize contamination. The amounts of the elements present in the diets are indicated with the results. The animals usually were housed 5 to a cage. Fifteen animals were fed each diet for the tumor experiments, and 5 animals were fed each diet for each time period for analytical studies. Stainless steel cages with no. 2-mesh wire bottoms were used where the possibility of contamination from galvanized cages existed.

Analyses were made of the livers of rats fed the diets with different levels of the elements, with and without the dietary azo dye, after varying periods of time. Riboflavin was determined fluorimetrically and total liver azo dye by the method of Spain and Clayton ('55).

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[2] Holtzman.

[3] Cerelose.

TABLE 1

The effect of inorganic salts upon the incidence of liver tumors induced by 3'methyl-4-dimethylaminoazobenzene (3'me-DAB)

Percentage of rats on control diet with tumors vs. percentage of rats on various levels of inorganic salt supplementation with tumors.[1]

(See text for levels of inorganic salts.)

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Control vs.:				
High Mn	57 vs. 53	91 vs. 86	100 vs. 82	
Low Mn	57 vs. 36	91 vs. 90	92 vs. 100	100 vs. 80
High Zn	92 vs. 100	100 vs. 100	100 vs. 86	
High Ni	91 vs. 64	92 vs. 100[2]	100 vs. 70	100 vs. 57
High F	91 vs. 92	93 vs. 100		
Low F	91 vs. 93	93 vs. 92		
High Mo	92 vs. 100	100 vs. 100	82 vs. 67	
High Cd	91 vs. 91	93 vs. 85		
High Co	91 vs. 89[2]	100 vs. 80	82 vs. 30	

[1] Analysis of results by the Chi-square method using Cochran's ('42) continuity correction indicated no significant effect of above salt levels except high Ni (P value less than 0.01) and high Co (P value less than 0.02) which were protective against formation of liver tumor.

[2] Most of the liver tumors on the high levels of salts of these groups were microscopic while the majority of the tumors in the control group were gross.

RESULTS

The results of all experiments are summarized in table 1. Measured food consumption and animal growth indicated that altered levels of salts did not influence food or dye intake sufficiently to be a factor in the results.

Manganese. No effect upon liver tumor incidence was observed in two experiments with a diet high in manganese ($MnCl_2$ equivalent to 750 mg of manganese per kilogram of diet) nor in three experiments in which this element was eliminated from the Wesson's salts (less than 0.01 mg of manganese per kilogram of diet[4]) when compared to the normal diet containing 3 mg of manganese per kilogram of diet. The dietary level of manganese did not alter the concentration of liver riboflavin nor of total liver azo dye after 6 weeks on the diets.

Zinc. Three experiments with zinc sulphate added to the diet so that the ration contained 3 gm of zinc per kilogram did not indicate any effect of this level of zinc upon azo dye carcinogenesis. The normal diet contained 2.5 mg of zinc[4] as a contaminant. The high levels of zinc added to the diet had no effect upon liver riboflavin concentration nor on total liver azo dye after three or 6 weeks on the regimen.

Nickel. The normal diet contained 1.25 mg of nickel per kilogram present as a contaminant.[4] When nickel chloride was added such that each kilogram of diet contained 450 mg of nickel there was evidence of a decreased incidence of liver tumors when compared to the normal diet. In 4 experiments the percentage of animals with liver tumors was altered from 91 to 64%, 92 to 100%, 100 to 70% and 100 to 57% in the presence of an increased amount of this element. The development of fewer liver tumors in the presence of the added nickel is similar to, but not as marked as, that found when copper salts were added to the diet (King et al., '57). Similarly both elements catalyze the destruction of the dye in the diet. When the complete diet was analyzed for dye after various periods of time at room temperature, and at 10°C, it was found that the rate of disappearance of dye was comparable to that found with added copper (King et al., '57). Although the diet fed to the animals was prepared fresh daily, it would appear that a resultant lower level of dye feeding could account for the reduced tumor incidence with the high dietary supplement of nickel. When liver ribo-

[4] Analysis of diets for elements by Crippen and Erlich Laboratories, Inc., Baltimore 2, Maryland.

flavin and total azo dye concentration were determined at two, three, 4 and 6 weeks it was found that there was no appreciable or consistent effect of the high level of nickel upon these liver components.

Fluoride. In two experiments, neither high levels of sodium fluoride (equivalent to 91 mg of fluorine per kilogram of diet) nor low levels of this halogen (0.37 mg of fluorine per kilogram of diet[4]) had any effect upon azo dye liver tumor induction when compared to the basal diet which contained 9 mg of this element per kilogram of ration.

Molybdenum. When sodium molybdate was added to the diet at a concentration of 300 mg molybdenum per kilogram of diet there was no effect upon tumor incidence in three experiments. The basal diet was reported to contain less than 0.01 mg of molybdenum per kilogram of diet.[4] Dietary addition of this element had no effect upon liver riboflavin nor upon liver total azo dye concentration at three or 6 weeks.

Cadmium. When 50 mg of cadmium chloride were added per kilogram of diet (equivalent to 33 mg of cadmium) there was no effect upon azo dye tumor induction in two experiments.

Cobalt. Cobaltus acetate incorporated into the diet such that each kilogram of diet had added 50 mg of cobalt inhibited liver tumor formation in three experiments, reducing the incidence from 91 to 89%, 100 to 80% and 82 to 30%. The control diet contained 2.5 mg of cobalt as a contaminant.[4] Cobalt also catalyzed the destruction of the dye in the diet in a manner similar to nickel (above) and copper (King et al., '57) and may be the reason for the decreased tumor formation. This element had no marked effect upon liver riboflavin and total azo dye concentration at two, three, 4 and 6 weeks. The rats on the high-cobalt diet were polycythemic as indicated by the hemoglobin which averaged 22 gm per 100 ml of blood compared to 16 gm for the non-cobalt-supplemented animals.

DISCUSSION

Of the inorganic salts here tested, none have been found by others to be carcinogenic *per se* when fed to rats, although certain of them will produce tumors when injected into the host animal (Hartwell, '51). From the present experiments it would seem that manganese, zinc, fluoride, molybdate, and cadmium also do not have any co-carcinogenic or any anti-carcinogenic effect upon azo dye-induced liver tumors. This is contrary to the finding of Sharpless ('45) who found that high levels of dietary manganese salts increased the rate of induction of azo dye liver tumors. He used, however, a weaker carcinogen and his method of measurement was different in that he fed the dye continuously and determined the average time needed to obtain palpable liver tumors.

Cobalt and nickel salts will warrant further investigation since they do tend to inhibit azo dye tumor formation. The effect of these salts is not nearly as marked as was that found with the copper salts (King et al., '57) on either tumor production or the effect upon liver riboflavin or total liver azo dye. The *in vitro* destruction of the dye by cobalt and nickel salts, however, was nearly as rapid as that produced by the copper salts. To further evaluate the anti-azo dye carcinogenic effect of these three salts will require a modified technique of administering the dye so that dye destruction will not be a factor.

SUMMARY

High dietary levels of the salts of manganese (750 mg per kg), zinc (3 gm per kg), fluoride (91 mg per kg), molybdenum (300 mg per kg), and cadmium (33 mg per kg) had no effect upon the incidence of liver tumors in rats fed the carcinogen 3′-methyl-4-dimethylaminoazobenzene.

Cobalt and nickel salts in the diet at levels of 50 mg and 450 mg per kg, respectively, inhibited the incidence of liver tumor formation but these salts also catalyzed destruction of the dye.

Low dietary levels of manganese (less than 0.01 mg per kg) and of fluoride (0.37 mg per kg) did not affect tumor incidence when compared to the controls.

The concentration of liver riboflavin and of liver total azo dye was not altered by the various levels of the salts under study.

[4] See footnote 4, p. 436.

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