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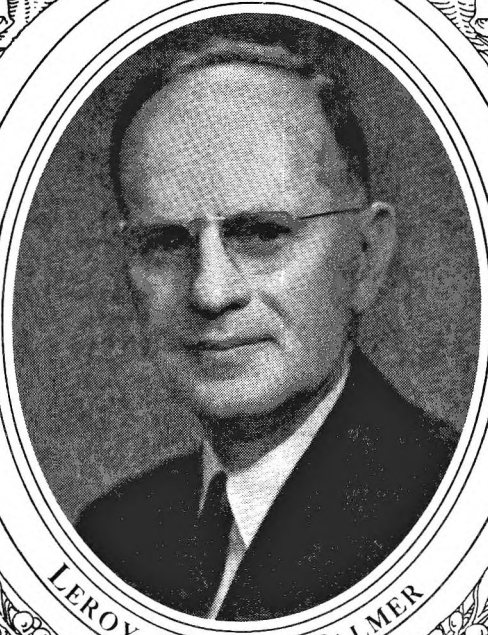

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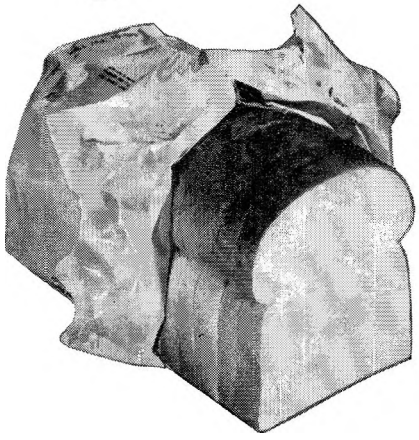

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INSIDE SCIENCE

The Vital Story of Vitamin B_1

(Thiamine)

by Science Writer

History. The discovery of vitamin B_1 resulted from research into the cause of beriberi. Almost 50 years passed between Eijkman's discovery of the relationship of the disease to diet and the famous work of Jansen and Donath who first isolated the crystalline vitamin from rice bran.

Within ten years of that first isolation the vitamin's chemical structure was determined and it was successfully synthesized.

Eijkman's work resulted in the development of a theory that beriberi was caused by a lack of some factor in the diet and not by a toxin or infectious agent. This idea was not readily accepted until the growth of dietary knowledge proved it correct.

Isolation and Synthesis. In 1926 Profs. Jansen and Donath accomplished the isolation of crystalline vitamin B_1 from rice bran. In 1931 Windaus and coworkers successfully isolated pure vitamin B_1 and established its empirical formula. In 1936 R. R. Williams, and independently R. Grewe, explained the vitamin's chemical structure. That year, R. R. Williams and J. K. Cline accomplished the synthesis of thiamine which is in wide use today. Andersag and Westphal also synthesized the vitamin in 1936. Another synthesis was described by Bergel and Todd in 1937.

Photomicrograph of B_1 crystals

Chemical and Physical Properties. Thiamine hydrochloride is white, water soluble, with a nut-like, salty taste and yeast-like odor. Its empirical formula is: $C_{12}H_{17}ClN_4OS \cdot HCl$. Thiamine produced by synthesis is identical chemically and in biological activity with that obtained in pure form from nature.

Deficiencies. A deficiency of thiamine is characterized by these symptoms: depression, irritability, fearfulness, lack of initiative and interest, loss of appetite. Symptoms vary since in usual practice deficiencies of other water-soluble vitamins occur. Medical treatment is simple: a sufficient amount of thiamine is administered to relieve symptoms quickly and the physician provides for a continuing adequate intake.

Beriberi victim

A severe deficiency of thiamine leads to beriberi, a serious and sometimes fatal disease. While beriberi is almost a medical curiosity in the United States, it is common in countries in which polished white rice is a staple of the diet.

Human Nutrition Requirements. Thiamine is one of the nutritive elements the human body needs daily and does not store in quantity. The minimum daily requirements established by the U. S. Food and Drug Administration for the prevention of symptoms of thiamine deficiency disease are:

Adults ....1.00 mg.	Children (1-5 incl.)..0.50 mg.
Infants ....0.25 mg.	Children (6-11 incl.).0.75 mg.

The Food and Nutrition Board of the National Research Council recommends the following dietary intake of thiamine for healthy persons in the U. S. A.

Recommended Daily Intake in Milligrams

Age	*Men*	*Women*
25	1.6	1.2
45	1.5	1.1
65	1.3	1.0
Pregnant (3rd trimester)		1.5
Lactating		1.5

The Council recommendations for infants and children vary below and above these figures, based on age and sex. Various illnesses and stress situations can exhaust vital reserves of thiamine. So, for the physician, vitamin B_1 is prepared in various dosage forms and potencies for therapeutic and prophylactic use.

How do human beings receive thiamine? It is widely distributed in foods of animal and vegetable origin, particularly cereal grains and dry legumes. Because of public demand for refined products which millers must meet for obvious economic reasons, a loss of thiamine and other factors occurs during processing. The thiamine loss is overcome through the use of *enrichment* in cereal grain products for which Federal Standards exist, or in other foods such as breakfast cereals, by *fortification* or *restoration*. When enriching, fortifying or restoring, the food processor adds the necessary amount of pure thiamine (and other vitamins and minerals) to the food so that the finished product meets Federal, state and territorial requirements or contributes to the consumer an amount of the vitamin which dietary experts believe significantly useful.

Thiamine is extensively used for the enrichment of cereal grain foods such as white flour, white bread and rolls, macaroni products, farina, corn grits and meal, milled white rice. The story of these uses is delightfully told in a separate brochure which is available on request for reference or educational purposes.

Production. Huge production facilities at the Hoffmann-La Roche plant in Nutley, New Jersey, deliver highest quality thiamine *by the tons*. Roche manufactures thiamine hydrochloride and thiamine mononitrate. These fine products, which equal or exceed U.S.P. specifications, are ideal for use by pharmaceutical makers and food processors. Years of experience in research and manufacture have made Roche the leader in vitamins.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better with essential, health-giving vitamin B_1. Reprints of this and others in the series are available on request. Write the Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon Street, St. Laurent, P. Q.

AMERICAN INSTITUTE OF NUTRITION AWARDS FOR 1958 INVITATION FOR NOMINATIONS

Nominations are invited for the 1958 annual awards administered by the American Institute of Nutrition. Nominations may be made by anyone, including all members of the Nominating Committees. The following information must be submitted: Name of the award for which the candidate is proposed and as convincing a statement as possible as to the basis for the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the appropriate nominating committee before January 1, 1958, to be considered for the 1958 award.

1958 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1,000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or any of its components.

The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another

administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are: 1944 — E. V. McCollum; 1945 — Harold H. Mitchell; 1946 — Philip C. Jeans and Genevieve Stearns; 1947 — Leonard A. Maynard; 1948 — Charles A. Cary; 1949 — Harry J. Deuel, Jr.; 1950 — Henry C. Sherman; 1951 — Paul György; 1952 — Max Kleiber; 1953 — Harold H. Williams; 1954 — Agnes Fay Morgan and Arthur H. Smith; 1955 — A. G. Hogan; 1956 — Frank M. Strong; 1957 — no award.

Chairman, Nominating Committee:
DR. W. C. ROSE
Department of Biochemistry
University of Illinois
Urbana, Illinois

1958 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 has been established by the Nutrition Foundation, Inc., for the recognition of outstanding basic research accomplishments in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Former recipients of this award are: 1949 — William C. Rose; 1950 — Conrad A. Elvehjem; 1951 — Esmond E. Snell; 1952 — Icie Macy Hoobler; 1953 — Vincent du Vigneaud; 1954 — L. A. Maynard; 1955 — E. V. McCollum; 1956 — A. G. Hogan; 1957 — G. R. Cowgill.

Chairman, Nominating Committee:
DR. ICIE MACY HOOBLER
Merrill-Palmer School
71 East Ferry Avenue
Detroit 2, Michigan

PERIODONTAL DISEASE IN THE RICE RAT[1]

III. SURVEY OF DIETARY INFLUENCES

AINA M. AUSKAPS, OM P. GUPTA[2] AND JAMES H. SHAW
Harvard School of Dental Medicine, Boston, Massachusetts

(Received for publication May 25, 1957)

The rice rat has been found to be unusually susceptible to the initiation and development of a form of periodontal disease (Gupta and Shaw, '56 a, b). Lesions of both soft and hard tissues of the periodontium were observed to occur in practically every young adult animal with widely varying degrees of severity. Thus the rice rat appears to offer a definite promise of becoming valuable in the study of periodontal disease. However, since the rice rat is a relatively new experimental subject, there is little available information about its adaptability to laboratory circumstances. A series of experiments was designed to test the ability of this species to survive on common laboratory diets, the effect of major variations in dietary vitamin and mineral concentrations, and the influence of housing conditions. In addition, among the rice rats in the preliminary surveys, subgingival hair impaction was frequently seen between adjacent maxillary teeth and between the maxillary teeth and the adjacent gingiva. The importance of this factor had to be evaluated experimentally to determine if it contributed to the initiation or progression of the periodontal syndrome in this species.

[1] This investigation was supported in part by research grants D-100 and D-322 from the National Institute of Dental Research, Public Health Service, and The Nutrition Foundation, Inc., New York, N. Y. We are indebted to Merck and Company, Inc., Rahway, N. J. for ample supplies of vitamin B complex.

[2] Now at the Murry and Leonie Guggenheim Foundation, Institute for Dental Research, New York University College of Dentistry, New York, N. Y.

EXPERIMENTAL

Four hundred and eighty rice rats were used in 6 different experiments. These subjects were obtained from a commercial source [3] where the stock colony was maintained during the early part of these studies on a mixed grain diet and during the latter part on a pelleted ration. The rice rats were tagged in the shipping crate according to litters. In all experiments, the rats were started on the appropriate experimental regimen as soon after weaning at 21 days of age as each shipment permitted. The distribution among the different groups within any experiment was arranged in order to provide comparability with respect to litter and sex. Each rice rat was individually housed in a screen-bottom cage, except where otherwise stated in the first experiment. The diets and drinking water were provided ad libitum.

After 21 experimental weeks, the animals were sacrificed and their heads fixed in 95% alcohol. Subsequently the evaluation of the periodontal lesions was made by the method of Gupta and Shaw ('56 b). Since a strong degree of bilateral symmetry in the distribution and severity of the periodontal lesions was demonstrated in this earlier study, only one randomly selected half of each upper and lower jaw was evaluated. Separate records were kept for lesions of the soft tissues of the periodontium and for the calcified tissues of the periodontium. For evaluation purposes, the periodontal tissues in each quadrant of the mouth were divided into 14 areas, each of which could have a disease severity score from 0 to 4 +, dependent upon the degree of abnormality. Thus, in the evaluation of one upper and one lower quadrant, the maximum severity of periodontal disease would have occurred when all 28 areas demonstrated soft tissues lesions and the total extent of these lesions was 112 +. Identical values would pertain for the lesions of the calcified tissues if maximal destruction had occurred during the experimental period.

[3] Tumblebrook Farm, Brant Lake, N. Y.

The following different diets and some modifications thereof were used: a natural mixed grain diet [4] that had been used originally as the stock colony diet by the commercial breeder, a laboratory chow, Keyes' cariogenic ration 4 (Keyes, '56), Harvard purified cariogenic ration 700 and Harvard sucrose-free ration 770. The latter two diets are identical with rations 100 and 170, respectively, as were described by Shaw ('54), except that the purified casein in the rations of the 100 series had been replaced by crude casein in the rations of the 700 series.

The first experiment was designed to test the influence of two different housing conditions when the natural mixed grain diet and Keyes' cariogenic ration 4 were used. Thirty-six rice rats were used, half of which were fed each ration. About half of the rice rats fed each diet were housed individually in screen-bottom cages; the other half were placed in small groups in breeding cages with wood shavings as the bedding material.

The second experiment with 52 rice rats was designed to establish the role of subgingival hair impaction in the initiation and progression of the periodontal disease syndrome. Three diets were utilized: Keyes' 4, Harvard 700 and the natural mixed grain diet. Two groups of animals were maintained on each of these rations: the experimental rice rats which were shaved weekly in order to eliminate as completely as possible the source of hair for subgingival impaction, and the unshaved littermate controls.

The third experiment was planned to test the effect of different dietary regimens upon the rice rat. One hundred and eight animals, in 7 different groups, were fed rations that varied with respect to both their dietary components and physical consistency. The rations for the 7 groups were Harvard 700, Harvard sucrose-free 770, Keyes' 4, Keyes' 4

[4] A mixture of cracked corn, wheat, oats, barley, buckwheat and a pelleted protein supplement that was composed of soy bean oil meal, linseed oil meal and powdered skim milk. The average protein content of this diet was 24%.

with added 20% lard by weight, laboratory chow in pellet form, ground laboratory chow [5] and the latter with 20% lard added.

The 4th experiment with 121 animals was designed to test the role of vitamin supplementation. To Keyes' 4 and Harvard 700, the following vitamin supplements were added: vitamin C, vitamin B complex, or vitamins A and D, as shown in table 3. Vitamin C was mixed in the ration in the proportion of 2 gm of ascorbic acid to 1 kg of ration. Vitamin B complex was also added to the ration 4, to provide about 10 times the amount used in ration 700. In order to achieve these levels, the following amounts of vitamins were added to the whole wheat flour used in 1 kg of ration: inositol, 10 gm; *p*-aminobenzoic acid, 3 gm; choline chloride, 10 gm; thiamine hydrochloride, 35 mg; riboflavin, 35 mg; pyridoxine hydrochloride, 35 gm; niacin, 250 mg; calcium pantothenate, 200 mg; biotin, 2 mg; folic acid, 20 mg; vitamin B_{12}, 10 mg. Vitamins A and D were given orally to the rats each day by dropper. A concentrate containing 65,000 units of vitamin A and 13,000 units of vitamin D per gm was diluted 1 : 9 with corn oil and 1 drop was given to each rat daily. Each drop of this mixture was calculated to contain about 185 units of vitamin A and 37 units of vitamin D.

Experiment 5 was composed of two parts that were conducted at different times. Four groups of rice rats were used in each part of the experiment with groups 1 to 4 in the first half and groups 5 to 8 in the second half. The control rice rats in the first and 5th groups were maintained on normal levels of calcium and phosphorus in approximately a 1 : 1 ratio. In the other 6 groups ration 700 had been modified to provide varying levels and ratios of calcium and phosphorus as shown in the 2nd and 3rd columns of table 4. In groups 2, 3 and 4, relatively little alteration was made in the calcium phosphorus ratios. The rice rats in groups 2 and 3 were provided both calcium and phosphorus at suboptimal levels, while the rice rats in group 4 were provided levels of calcium and phosphorus that were approximately double the normal require-

[5] Purina.

ments. In groups 6 to 8 in the second half of the experiment, the levels of calcium and phosphorus were provided at altered ratios. The rice rats in groups 6 and 7 received a low calcium intake with a low calcium phosphorus ratio, while the rice rats in group 8 received a low phosphorus intake along with a high calcium phosphorus ratio.

In experiment 6, the influence of varying levels of fluoride ingestion was tested, when rations 700 and 4 were used for groups 1 to 3 and 4 to 6, respectively. The control rats in groups 1 and 4 were supplied with tap water. The rice rats in groups 2 and 5 were provided with 15 p. p. m. of fluoride in tap water through the addition of an appropriate amount of sodium fluoride, while the rice rats in groups 3 and 6 received 30 p. p. m. of fluoride.

RESULTS

The rice rat proved to be able to adapt itself readily within the laboratory to a variety of dietary and housing conditions. During the three years that these studies have been in progress, no evidence was noted of any undue susceptibility to intercurrent infections, even though the rice rats were housed in the same rooms as other rodent species. A striking heterogeneity of all physical and disease characteristics was observed that made necessary the use of relatively large groups and littermate distribution. All laboratory handling was difficult and time-consuming because of the timidity and aggressiveness of these experimental subjects. Both the heterogeneity and handling difficulties gave clear evidence of the shortness of exposure that this species has had to laboratory circumstances.

The rice rats grew and developed normally on all different rations under the various experimental conditions imposed in these studies, except in the 5th experiment where calcium and phosphorus levels and ratios were altered. The male rice rat uniformly grew more rapidly and attained an appreciably larger adult body weight than the female from the same litter. Normal adult males in these experiments varied in weight from around 90 to 130 gm; normal adult females varied from around 50 to 85 gm. The most active period of weight gain occurred during the first 6 weeks on experiment.

Wide variations in susceptibility to periodontal lesions were observed in practically every experimental and control group of rice rats throughout these studies. Suggestions of an inherited constitutional difference in proneness to periodontal lesions are evident from our data. Ordinarily there was less variation in susceptibility among littermates and among rice rats from succeeding litters born to the same stock colony pair than there was among unrelated rice rats. The degree of variation in proneness to periodontal disease at present is sufficiently great to make littermate distribution among experimental and control groups mandatory. For the greatest usefulness of the rice rat in this area of investigation, reduction in the variation in susceptibility to disease will be necessary through the development of stock colonies where phenotypic selection is instituted while dietary practices are maintained constant.

A summary of the incidence and extent of periodontal lesions that were observed in the first experiment is presented in table 1. Housing conditions as tested in the comparison between single rats in screen-bottom cages and groups of rats in larger cages with wood shavings for bedding seemed to have no striking nor consistent relationship to the causation of periodontal lesions. Animals housed in groups in breeding cages on wood shavings and fed the mixed grain diet had somewhat fewer periodontally involved areas and much less extensive lesions in both soft and calcified periodontal tissues than animals kept under identical housing conditions but fed ration 4. These differences appear to have statistical significance.[6] Also the animals in screen-bottom cages on the mixed grain ration seemed to have a tendency towards somewhat fewer and less extensive periodontal lesions than animals on ration 4 under the same housing conditions. The latter differences were not statistically significant. While these comparisons between ration 4 and the mixed grain ration tend to suggest that ration 4 had a greater ability to cause the production of periodontal lesions

[6] N for determination of the standard error of the mean for both the number and the extent of the periodontal lesions was always the number of animals in the respective group.

TABLE 1

The effect of caging conditions and hair removal upon the production of periodontal lesions in the rice rat[1]

GROUP	RATION AND SPECIAL CONDITIONS	NO. OF RATS	SOFT TISSUES				CALCIFIED TISSUES			
			Number of periodontal areas		Extent of periodontal lesions		Number of periodontal areas		Extent of periodontal lesions	
			Av.	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]
	EXPERIMENT 1.									
1	4 (Screen-bottom cages)	7	11.4 (5.1)	1.0	23.7+ (11.7+)	0.2	25.6 (0.6)	0.1	44.7+ (7.7+)	0.2
2	4 (In shavings — breeding cages)	11	17.0 (2.1)	0.9	26.5+ (3.8+)	1.2	25.7 (0.6)	2.6	46.2+ (2.1+)	0.5
3	Grain (Screen-bottom cages)	11	6.7 (0.8)	0.6	10.1+ (0.7+)	0.6	23.4 (0.6)	0.9	40.0+ (4.3+)	0.7
4	Grain (In shavings — breeding cages)	7	7.6 (1.2)	3.9	11.9+ (2.8+)	3.1	22.4 (0.9)	3.0	36.6+ (1.6+)	3.7
	EXPERIMENT 2.									
1	4 (Control)	12	14.0 (0.6)	1.6	24.1+ (4.1+)	1.0	26.4 (0.8)	1.9	52.5+ (6.2+)	2.6
2	4 (Hair removed)	8	8.2 (3.6)		15.1+ (7.6+)		24.1 (1.0)		36.1+ (7.0+)	
3	700 (Control)	8	9.2 (3.9)	0.1	26.1+ (14.2+)	0.1	24.2 (1.4)	0.9	46.0+ (10.5+)	0.1
4	700 (Hair removed)	10	9.8 (3.0)		24.0+ (1.1+)		25.6 (0.7)		46.6+ (8.0+)	
5	Grain (Control)	10	12.7 (1.8)	1.7	17.1+ (2.4+)	2.8	21.9 (0.8)	0.9	33.7+ (2.1+)	2.2
6	Grain (Hair removed)	4	17.8 (2.4)		26.7+ (2.4+)		23.5 (1.5)		42.2+ (3.2+)	

[1] The value within parentheses represents the standard error of the mean.

[2] C.R. — Critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the ratio is 3.0 or higher, the difference is highly significant.

than the mixed grain diet, other experiments have not corroborated this finding.

It was interesting to note that rice rats fed ration 4, which has a very soft powdery consistency, had very little or no hair impaction between the gingiva and the molars or between the molars under both housing conditions. However, in the rice rats fed the mixed grain diet, there was a great deal of hair impaction under both housing conditions, especially in the maxillae on the lingual side, particularly in the third and second molar areas.

A high mortality was observed among the shaved rice rats in the early weeks of the second experiment. Part of this is attributable to the traumatic nature of the careful shaving procedure. The remaining deaths appeared to be due to difficulties in maintaining normal regulation of body temperature with a subsequent susceptibility to pulmonary infections. The data for the results of the evaluations on the initiation and progression of periodontal lesions in the second experiment are presented in table 1. Very little or no hair impaction was observed in either the shaved or the unshaved animals maintained on rations 4 and 700. This observation was in striking contrast to the findings in our early studies that had been conducted entirely with rice rats from a stock colony where the mixed grain ration had been fed throughout life (Gupta and Shaw, '56a, b). Morever, the rice rats on the mixed grain ration in groups 5 and 6 frequently showed extensive hair impaction lingually on the maxillary molar, whether they were shaved or not. Thus, the findings with the rice rats on the mixed grain diet in experiments 1 and 2 were identical with our observations in the earlier studies.

The shaved animals had similar numbers of periodontally involved areas and extent of periodontal lesions as their unshaved controls on the same diet. There seems to be a trend in the shaved animals on ration 4 to have somewhat fewer and less extensive periodontal lesions than their unshaved controls. The reverse picture was seen in the animals on the grain diet, where the shaved animals had more frequent and more exten-

sive lesions than their unshaved controls. However, these differences were either insignificant or were only borderline in statistical significance. The tremendous variation in susceptibility to periodontal lesions between unrelated rice rats and even the lesser variation between animals within the same litter probably accounts for such minor differences between groups.

The summary of results of the effect of different rations on the production of periodontal lesions in the rice rat is presented in table 2. The rice rats maintained on rations 4, 700 and laboratory chow pellets had relatively severe periodontal disease manifestations. The average incidence for the three groups was about the same. Rice rats maintained on noncariogenic, carbohydrate-free ration 770, on ground laboratory chow, or on the latter with 20% lard added had developed a much lower number of diseased periodontal areas and a much lower extent of lesions in the soft tissues of the periodontium. The differences between the incidence and extent of soft tissue lesions produced by rations 4, 700 and laboratory chow, and the incidence and extent of lesions caused by rations 770, ground laboratory chow and ground laboratory chow plus 20% lard were statistically highly significant. Reductions in the incidence and extent of lesions in the calcified tissues of the periodontium also resulted from the use of the latter three rations. However, these reductions were not as great and in some comparisons were not of statistical significance. Only carbohydrate-free ration 770 really caused striking and highly significant reductions in both soft and calcified tissue lesions. Yet even with ration 770 the degree of benefit to the calcified tissues was not as great as that to the soft tissues of the periodontium.

The addition of 20% lard to ration 4 caused small reductions in periodontal disease from that produced by ration 4, as can be seen by a comparison of the rice rats in group 4 with those in group 3. While these reductions in soft and calcified tissue lesions are small and not of statistical significance, it is interesting that the trend is in the same direction as the reduction caused by ration 770 in the complete isocalor-

TABLE 2

The effect of different rations on the production of periodontal lesions in the rice rat [1]

GROUP	RATION	NO. OF RATS	SOFT TISSUES				CALCIFIED TISSUES			
			Number of periodontal areas		Extent of periodontal lesions		Number of periodontal areas		Extent of periodontal lesions	
			Av.	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]
1	700	26	14.5 (1.0)	10.1	32.5+ (6.1+)	4.8	23.3 (0.9)	3.5	45.5+ (1.7+)	9.9
				1.8		0.9		0.9		0.4
2	770	18	2.7 (0.6)		3.1+ (0.7+)		18.9 (0.9)		21.8+ (1.7+)	
				1.7		2.6		0.7		1.1
3	4	19	19.1 (2.3)		39.9+ (6.2+)		24.6 (1.2)		47.3+ (4.8+)	
				0.4		1.6		0.4		1.1
4	4 + 20% fat	12	13.6 (2.0)		20.5+ (4.4+)		22.0 (3.5)		40.2+ (4.5+)	
				0.9		0.9		0.8		0.2
5	Laboratory Chow	8	17.3 (3.1)		26.1+ (3.5+)		24.6 (1.5)		44.8+ (4.1+)	
				3.8		4.9		1.8		3.5
6	Ground Laboratory Chow	12	4.5 (1.4)		5.5+ (2.4+)		20.4 (1.9)		26.1+ (3.4+)	
				5.9		4.2		1.4		5.1
7	Ground Laboratory Chow + 20% fat	13	3.6 (0.3)		4.2+ (1.5+)		22.4 (1.5)		26.2+ (2.3+)	
				10.3		4.5		0.5		6.7
								0.8		0.2

[1] The value within parentheses represents tho standard crror of thc mean.

[2] C.R. — Critical ratio.

ic elimination of carbohydrate from ration 700. The addition of 20% lard to ground laboratory chow appeared to cause a trivial but statistically insignificant reduction in soft tissue lesions, but no change in hard tissue lesions. The fact that the incidence of periodontal lesions on ground laboratory chow was already extremely low would tend to make any further reduction more difficult to detect.

The data from experiment 4 are presented in table 3. The addition of vitamin C or vitamins A and D to rations 700 or 4 did not influence the development of periodontal lesions in this species, as can be seen by a comparison of the rice rats in groups 2 and 3 with those in group 1, and the rice rats in groups 5 and 6 with those in group 4. The addition of the vitamin B complex to ration 4 appeared to produce small reductions in soft tissue lesions and less extensive hard tissue periodontal lesions, than the control animals maintained on standard ration 4. These reductions in the amount of soft tissue lesions and in the extent of calcified tissue lesions in group 8 when compared with group 7 were judged to be statistically highly significant. However, there was only a trend toward less extensive soft tissue lesions and fewer calcified tissue lesions in the same group of animals. Neither of the latter reductions were of statistical significance.

The summary of results observed in the 5th experiment is presented in table 4. All animals maintained on ration 700, but with varying amounts or ratios of calcium and phosphorus developed about an equal incidence and extent of periodontal disease in both the soft and calcified tissues. None of these variations in calcium or phosphorus concentration nor in calcium phosphorus ratio appeared to have altered the proneness to periodontal disease.

The rice rats in groups 1, 3, 5 and 8 developed equally well, gaining in weight and stature to the extent typical for their sex. Rice rats of both sexes grew somewhat more slowly and attained lower final body weights in groups 2, 6 and 7. Animals in group 4, with a high calcium and phosphorus intake, varied depending upon their sex. The male rats in

TABLE 3

The effect of vitamin supplementation upon periodontal lesions in the rice rat [1]

GROUP	RATION	NO. OF RATS	SOFT TISSUES				CALCIFIED TISSUES			
			Number of periodontal areas		Extent of periodontal lesions		Number of periodontal areas		Extent of periodontal lesions	
			Av.	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]
1	700	18	18.8 (2.3)		39.4+ (7.3+)		26.2 (0.9)		59.5+ (7.4+)	
				0.8		0.7		0.5		0.8
2	700 + vitamin C	17	21.6 (2.3)		46.5+ (8.0+)		26.8 (1.5)		66.9+ (8.0+)	
				0.5		0.1		0.4		0.2
3	700 + vitamins A, D	13	16.9 (3.4)		39.5+ (9.9+)		26.9 (1.5)		61.8+ (8.0+)	
4	4	11	11.7 (3.8)		29.1+ (11.2+)		22.9 (1.3)		41.6+ (7.8+)	
				0.9		1.2		0.7		0.6
5	4 + vitamin C	13	7.2 (3.2)		14.2+ (6.1+)		23.0 (0.7)		36.2+ (4.3+)	
				1.5		0.8		1.9		1.5
6	4 + vitamins A, D	11	19.5 (3.8)		40.7+ (8.3+)		25.7 (0.6)		55.5+ (5.1+)	
7	4	16	23.9 (0.9)		32.4+ (4.2+)		28.0 (0.0)		56.8+ (2.9+)	
				4.7		1.0		1.2		3.0
8	4 + vitamin B complex	22	12.2 (2.3)		25.9+ (5.5+)		25.8 (1.9)		43.3+ (3.5+)	

[1] The value within parentheses represents the standard error of the mean.

[2] C.R. — Critical ratio.

TABLE 4

The effect of varying levels and ratios of calcium and phosphorus upon the production of periodontal lesions in the rice rat [1]

GROUP	LEVELS OF CA AND P IN RATION 700		NO. OF RATS	SOFT TISSUES				CALCIFIED TISSUES			
				Number of periodontal areas		Extent of periodontal lesions		Number of periodontal areas		Extent of periodontal lesions	
	Ca	P		*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]
	%	%									
1	0.62	0.63	12	19.9 (3.8)	0.9	45.1 | (9.4+)	0.1	28.0 (0.0)	0.0	69.2+ (8.5+)	0.1
2	0.16	0.27	12	23.9 (2.3)	0.5	45.8+ (6.1+)	1.2	28.0 (0.0)	0.0	68.8+ (6.6+)	1.8
3	0.31	0.39	14	17.5 (3.2)	0.1	31.3+ (6.2+)	0.9	28.0 (0.0)	0.0	52.4+ (4.6+)	1.3
4	1.24	1.10	16	19.4 (2.4)		35.2+ (6.8+)		28.0 (0.0)		56.7+ (6.1+)	
5	0.62	0.63	12	17.2 (3.3)	1.3	35.7+ (8.6+)	0.7	28.0 (0.0)	1.0	60.1+ (8.9+)	0.4
6	0.11	0.23	14	22.2 (2.3)	1.3	43.4+ (6.6+)	0.1	27.8 (0.2)	1.7	64.5+ (7.3+)	0.8
7	0.11	0.68	11	22.1 (2.2)	1.1	37.1+ (6.0+)	0.2	27.5 (0.3)	0.6	51.7+ (5.8+)	0.2
8	0.62	0.28	11	21.7 (2.6)		37.4+ (6.5+)		27.7 (0.5)		58.2+ (6.1+)	

[1] The value within parentheses represents the standard error of the mean.

[2] C.R. — Critical ratio.

TABLE 5

The effect of varying levels of fluoride ingestion upon the production of periodontal lesions in the rice rat [1]

GROUP	RATION	NO. OF RATS	SOFT TISSUES				CALCIFIED TISSUES			
			Number of periodontal areas		Extent of periodontal lesions		Number of periodontal areas		Extent of periodontal lesions	
			Av.	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]	*Av.*	*C.R.*[2]
1	700	10	13.4 (3.6)	0.6	26.4+ (6.3+)	1.0	27.5 (0.3)	0.7	46.4+ (8.0+)	0.7
2	700 + 15 ppm. F in drinking water	12	10.6 (3.0)	1.0	17.3+ (6.4+)	0.6	27.2 (0.3)	0.3	39.8+ (5.9+)	0.8
3	700 + 30 ppm. F in drinking water	10	18.8 (3.8)		32.7+ (7.6+)		27.6 (0.2)		54.5+ (6.8+)	
4	4	8	22.5 (3.7)	0.6	34.0+ (4.5+)	0.8	27.8 (0.6)	1.0	53.0+ (4.8+)	0.6
5	4 + 15 ppm. F in drinking water	11	19.4 (3.3)	0.7	28.0+ (5.4+)	1.1	26.7 (0.8)	1.5	48.8+ (5.8+)	0.4
6	4 + 30 ppm. F in drinking water	10	18.9 (3.8)		26.1+ (5.7+)		25.8 (1.2)		49.5+ (7.5+)	

[1] The value within parentheses represents the standard error of the mean.

[2] C.R.—Critical ratio.

group 4 developed and gained weight equal to their littermate controls in group 1. However, the female animals in this group failed to gain in weight as rapidly and attained a smaller final weight than their controls in group 1.

The addition of sodium fluoride to the drinking water to provide 15 or 30 p. p. m. of fluoride did not alter the initiation and progression of periodontal disease in rice rats that were maintained on ration 700 or ration 4. From the data in table 5, it can be seen that the rice rats in groups 2 and 3 developed about equal amounts of periodontal disease as their littermate controls in group 1, maintained on the same ration, but drinking tap water with no added fluoride. The same holds true for animals in groups 5 and 6, when compared with those in group 4. Rice rats in all groups in this experiment grew and developed equally well, without penalty due to the added fluoride and attained the final weight typical for their sex.

DISCUSSION

The above experiments constitute a preliminary survey of how the rice rat can be expected to react to a variety of housing and dietary conditions. For an animal that has been maintained in a laboratory environment for a very limited number of generations, the adaptability to various regimens and the lack of epidemic infections have been surprisingly good. In addition, these preliminary experiments provide some suggestions about the complex nature of the periodontal disease syndrome in the rice rat and about the design of various potential experiments of a nutritional nature for further investigation of this syndrome.

The original concern that subgingival hair impaction might have been one of the primary etiologic factors in the initiation of periodontal lesions appears to have been unjustified. A significant local factor of this nature would have rendered the rice rat useless for periodontal studies. Housing conditions did not alter the impaction of hair nor the production of periodontal disease in the rice rat as might have been expected. Instead, rice rats fed the same diet but maintained

under two different housing conditions, developed an equal amount and an equal extent of lesions in their periodontal tissues. Subgingival hair impaction was only a problem on the mixed grain ration. When the latter diet was used, the hair impaction phenomenon occurred under both housing conditions; even when the rats were shaved weekly, hair impaction was not eliminated. Throughout the 6 experiments and among all control and experimental rice rats fed diets other than the mixed grain ration, subgingival hair impaction was not a problem. In addition, the areas where subgingival hair impaction occurred most frequently were in the maxillae while the highest incidence and greatest severity of periodontal lesions occurred in the mandibles.

Changes in the dietary regimen had profound influences on the initiation and progression of periodontal lesions in the rice rat. With variations in either the dietary components or the physical consistency of the diet, highly significant changes in disease manifestation occurred. Isocaloric replacement of sucrose in ration 700 with casein and lard (ration 770) caused the greatest improvement in the health of the periodontal tissues in the rice rat. Likewise, a trend toward a lesser number and less extensive lesions was observed in the rice rats fed the higher fat diets that were prepared by the addition of 20% lard to ration 4 or to the ground laboratory chow. These observations suggest that at least part of the influence of the dietary regimen may be mediated through a microbial agent that is less able to operate in a carbohydrate-free or carbohydrate-low oral environment.

The striking reduction in incidence and progression of periodontal lesions that occurred as a result of grinding laboratory chow pellets can not be satisfactorily explained with the present data. In view of the same distribution of nutrients in the pellets and in the ground chow, the physical difference between the two dietary regimens seems to be the only conspicuous factor upon which to base an explanation of the difference in extent of periodontal disease. Yet

the rice rat, like other rodents, uses its incisors to gnaw the pellets to produce particles of sufficiently small size to masticate readily with the molar teeth. This practice would seem to obviate any possibility of trauma to the periodontal tissues by reason of the necessity to grind extremely hard particles. Furthermore, the particles of the ingredients of rations 700 and 4 are as fine or finer than those of the ground chow; yet rations 700 and 4 have about the same ability to cause periodontal lesions as did the chow pellets. It is interesting to note that none of these diets are capable of causing the initiation of periodontal lesions in numerous commonly used strains of the Norway rat. The rice rat appears to have a unique constitutional proneness to this periodontal disease syndrome.

Supplementation of rations 700 or 4 with vitamin C or vitamins A and D did not alter the state of health of the periodontal structures in the rice rat. Supplements to ration 4 of high levels of the vitamin B complex seem to have been beneficial in some way. Further studies are needed to determine whether the finding is valid or whether the high variation in susceptibility to periodontal disease in this population of rice rats has led to a spurious result.

Neither reduced nor increased levels of calcium and phosphorus, nor disturbed calcium phosphorus ratios, nor elevated levels of fluoride altered the initiation and progression of periodontal lesions. Since these elements are closely related to bone metabolism, the lack of either positive or negative influence on the manifestations of periodontal disease in the mineralized tissues in particular, suggests the complex nature of the syndrome. Furthermore, those rations that did cause significant reductions in periodontal disease were much more effective in the prevention of soft tissue lesions than the mineralized tissue lesions. The latter observation suggests that the lesions of the supporting alveolar bone in this species can progress rapidly in almost the complete absence of preceding damage to the gingiva and the periodontal membrane. As yet we have no evidence to indicate that the re-

verse can occur under our experimental conditions. This ability to at least partially segregate the effects of various experimental regimens on the soft tissue components of the periodontium from the effects on the mineralized tissues of the periodontium may prove to be one of the more valuable assets of the rice rat in this area of investigation.

SUMMARY

Four hundred and eighty rice rats were used in a series of 6 experiments to test their ability to survive under various housing conditions and on different dietary regimens.

Housing the rice rats individually in screen-bottom cages or in groups in cages with wood shavings for bedding resulted in comparable initiation and progression of periodontal lesions.

Subgingival hair impaction did not occur on any other dietary regimen than a mixed grain diet. On this diet, even weekly shaving of the rice rats did not prevent some hair impaction. By choosing the appropriate diet, subgingival hair impaction can be eliminated as a possible etiologic factor in the incidence and progression of periodontal lesions.

Physical consistency of the diets appeared to be related in some way to the production of periodontal disease. Laboratory chow pellets caused severe manifestations of periodontal disease. When the chow was fed as a finely ground powder, a greatly reduced incidence of periodontal disease resulted. However, it is noteworthy that other finely divided diets such as rations 700 and 4 are capable of causing as much periodontal disease as chow pellets.

Complete elimination of carbohydrate from the diet caused major reductions in lesions of the soft tissues of the periodontium and moderate reductions in lesions of the mineralized tissues. Reduced dietary carbohydrate contents produced by addition of lard tended to cause similar reductions of lesser degree.

Supplementation with vitamin C and vitamins A and D had no influence on the initiation and progression of perio-

dontal lesions. There was a suggestion that high levels of vitamin B complex supplementation cause minor reductions in the periodontal disease syndrome.

Wide variations in the calcium and phosphorus levels and ratios in the diet did not influence the manifestations of the periodontal disease syndrome in the rice rat. Even where growth retardation occurred as a result of dietary restriction in calcium, there was no exacerbation in the periodontal lesions.

The addition of either 15 or 30 p. p. m. of fluoride to the drinking water of rice rats did not alter their rates of growth nor the initiation and progression of periodontal lesions.

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THE NUTRITIVE VALUE OF SEVERAL FOODS GROWN AT DIFFERENT LOCATIONS [1]

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Both the top environment (climate) and the root environment (soils) have been shown to affect the composition of plants significantly. Several excellent reviews (Beeson, '41; Hamner and Maynard, '42; Somers and Beeson, '48) and monographs (Proceedings, Specialist Conference in Agriculture, '51; Southern Cooperative Series Bulletins 36, '54, and 42, '55) have been written in which many of the studies of these environmental effects are discussed.

The importance to the animal of such environmentally-induced differences in the nutrient content of plants has not been demonstrated, however, except in the instance of the mineral deficiencies and toxicities which have been noted in grazing animals in certain areas. The study reported here is one which was planned to investigate the relative nutritive value of several human foods grown at widely different locations, as measured by the experimental animal.

EXPERIMENTAL PLAN

Crop production and preparation

Corn, cowpeas and turnip greens were selected for study since they are of considerable nutritional importance in many

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human diets in certain sections of the South. Personnel at 4 experiment stations — Blairsville and Experiment, Ga.; Raleigh, N. C.; and College Station, Texas — cooperated by producing the crops used in the animal studies. In 1950 and 1951, all crops were grown at two fertilizer levels — minimum and optimum — as selected by the agronomist at each location. At Blairsville and Experiment in 1953, only the optimum level was utilized. Seeds[3] from a common source were sent to each location, and the procedures used in crop production were standardized as much as possible. The corn and cowpeas were harvested at maturity and the turnip greens when full-grown but with no blooms.

The preparation of the crops after harvesting was also made as uniform as possible. The turnip greens were thoroughly washed to remove possible soil contamination and then dried in a forced-air hay-drier at 55 to 60°C. After shipment to this laboratory, they were de-ribbed, re-dried at about 55°C, and stored in air-tight containers at -4°C. The shelled corn and cowpeas were protected during storage against insect infestation.

Animal studies

Weanling rats from the laboratory colony, originally from the Sprague-Dawley strain, were used in all experiments with equal numbers of both sexes on all treatments. They were individually housed on wire screens in an air-conditioned animal room and given food and water ad libitum for the experimental period of 8 weeks. They were weighed weekly at which time the food consumed by the rats on each treatment was determined.

The corn, cowpeas and turnip greens were pulverized in a hammermill before combination into diets. A diet having the following percentage composition was selected for use in all experiments: corn 40, cowpeas 30, turnip greens 10, fat[4] 5, NaCl 1, and sucrose 14. An additional combination of corn,

[3] Dixie 11 corn, California 7 cowpeas, and Shogoin turnip greens.

[4] Primex – Procter and Gamble Co., Cincinnati, Ohio.

cowpeas and turnip greens (30:40:10) was used only in experiment 1. When dietary supplements were studied, they were added at the expense of sucrose. Viosterol[5] was given to each rat weekly by dropper. A control group, fed the laboratory stock diet[6], was included in each experiment.

Three animal experiments were conducted. In the first two, the effect of location on the nutritive value of all *three* foods was investigated; in experiment 3, only turnip greens grown at Blairsville and Experiment were studied, since the results of the first two experiments indicated that these foods were the only ones which differed in nutritive value for the rat.

Experiments 1 and 2. Crops grown in 1950 with minimum fertilization were compared in experiment 1 and those with optimum fertilization in experiment 2. Because of production difficulties (drought, pest infestation, etc.), no one location produced all three crops. It was therefore necessary to modify the original plan of combining all three foods from a location in a single diet and instead to study the effect of location on each of the foods separately. Both experiments were designed to permit statistical analyses of the data and thereby to compare corn from Texas and Experiment, cowpeas from Texas and Blairsville, and turnip greens from Blairsville and Experiment. Turnip greens grown at Raleigh and Experiment in 1951 with optimum and minimum fertilization were also studied in experiment 2. Crops from field replication 1 from all locations were arbitrarily combined in diets and those from field replication 2 were treated similarly. It was thus possible to study also the effect of field replication on all crops and of fertilizer levels on the 1951 turnip greens from Raleigh and Experiment.

Experiment 3. The objectives of this experiment were twofold: (1) to learn if the difference in the nutritive value of the 1950 Blairsville and Experiment turnip greens (noted in

[5] Viosterol – Parke, Davis and Co., Detroit, Mich.

[6] G. L. F. Dog Food – Cooperative G.L.F. Marketing Service, Inc., Canandiagua, N. Y.

experiments 1 and 2) would be observed when greens grown at these locations in another year were studied, and (2) to investigate possible reasons for their differing nutritive value.

Turnip greens, which were grown at one or the other of the two Georgia locations in 1953, were used in all diets (corn and cowpeas from a single source were used throughout). To learn possible reasons for the difference between the greens, 4 classes of nutrients were studied, i. e., (1) inorganic substances, (2) organic materials, (3) vitamins, and (4) protein. Methionine and vitamin B_{12} were also studied since there was evidence in preliminary work of their supplementary value for diets of similar composition. The supplements were investigated as follows:

(1) Inorganic substances. Ash equivalent to the amount of Blairsville (B) greens in the diets was added to a diet containing Experiment (E) greens, and vice versa. Thus, a mineral deficiency or toxicity, resulting from differences in the mineral contents of the two greens, would become evident.

(2) Organic materials. To study the possible presence of a toxic organic substance in the poorer greens, one group of animals was fed a diet containing 5% B + 5% E greens. The performance of these animals was compared with that of the animals on three other diets, i. e., 5% B, 10% B, and 10% E turnip greens.

(3) Vitamins. A complete vitamin mix[7] was added to both diets. Vitamin B_{12} was omitted to eliminate the possibility that its inclusion might mask differences due to other vitamins.

(4) Protein. Egg albumin[8] was selected as the protein supplement since it has been shown to be a protein of high biological value (Block and Mitchell, '46), and it was obtainable as a relatively pure protein having neither vitamin B_{12}

[7] Vitamin mix (mg/100 gm diet) – thiamine, 1.0; riboflavin, 2.0; pyridoxine, 1.0; calcium pantothenate, 10.0; nicotinamide, 10.0; biotin, 0.05; folic acid, 0.2; inositol, 5.0; *p*-aminobenzoic acid, 3.0; choline chloride, 100; menadione, 1.0; α-tocopherol acetate, 10.0. Vitamins A and D were administered to each rat weekly (A-D Percomorph Liver Oil).

[8] Nutritional Biochemicals Corp., Cleveland, Ohio.

nor unidentified growth factors associated with it. Levels of 2.5 and 5.0% were used.

(5) Methionine. A supplement of 0.6% DL-methionine was added to each diet.

(6) Vitamin B_{12}. Crystalline vitamin B_{12} was added at a level of 10 μg%.

Microbiological studies

The plant materials were assayed for vitamin B_{12} using two micro-organisms[9]. *Lactobacillus leichmannii* 4797 and *Ochromonas malhamensis* 11532. The plant samples were extracted by steaming them for 30 minutes in water (containing 10 mg% NaCN) which was adjusted to pH 5.0 with HCl before steaming. The method of Peeler et al. ('49) was used with *L. leichmannii,* and all values were corrected for desoxyriboside activity (Hoffman et al., '49). The *Ochromonas* assay procedure was that of Ford ('53).

RESULTS AND DISCUSSION

Location effects

The growth data for each animal experiment were submitted to an analysis of variance. No difference in nutritive value was found to be attributable to the source of corn or cowpeas, to the diet combinations used in experiment 1, to field replications, or to fertilization of the turnip greens at Experiment or Raleigh in 1951. Therefore, it was possible to combine appropriate data for a comparison of the nutritive value of the turnip greens from different locations. These results are presented in table 1. The superiority of the Blairsville greens over those from the other locations examined was clearly demonstrated. Blairsville greens were better than those grown at Experiment in two different years as shown by the results of experiments 1, 2 and 3 ($P < 0.01$). No difference between the greens grown at Experiment and Raleigh in 1951 was found, and the similarity between those

[9] Obtained from American Type Culture Collection, Washington, D. C.

grown at Experiment in three different years is apparent. Thus, it is evident that some set of factors at Blairsville, operative over a period of more than one year, significantly affected the nutritive value of the turnip greens grown there.

Any attempt at this time to explain the superior quality of the Blairsville greens in terms of differences in the climate or soils at the two Georgia locations is impossible. Before conclusions are warranted, experiments would have to be

TABLE 1

The nutritive value of turnip greens grown at different locations as shown by the growth response of rats

EXP. NO.	TURNIP GREENS		MEAN WEIGHT CHANGE OF RATS FED TURNIP GREEN DIETS			STOCK DIET
	Year grown	Fert. level	B [1]	E	N	
			gm/8 wk	*gm/8 wk*	*gm/8 wk*	*gm/8 wk*
1	1950	min. [2]	119 (16) [3]	90 (16)	—	201 (6)
2	1950	opt.	138 (16)	109 (16)	—	215 (6)
3	1953	opt.	143 (16)	113 (16)	—	199 (6)
2	1951	min.	—	97 (8)	102 (8)	215 (6)
2	1951	opt.	—	100 (8)	110 (8)	215 (6)

[1] Turnip greens grown at the following locations were compared: Blairsville, Ga. (B); Experiment, Ga. (E); Raleigh, N. C. (N). They were included in all diets at a level of 10%.

[2] Min. or opt. indicates minimum or optimum fertilization of the field plots on which the turnip greens were grown.

[3] Number of animals given within parentheses.

conducted in which the many variables in the plant environment are subjected to better control. However, a brief description of both locations, and some observations made at both sites during the growth period may answer some of the questions raised at this time. Blairsville is located in an inter-mountain valley in the southern Appalachian mountains at an altitude of 1938 ft., at lat. 34° 51 min. and long. 83° 56 min. Experiment is situated in the rolling area of the Piedmont upland at an altitude of 946 ft., at lat. 33° 16 min. and long. 84° 17 min. The soil at Blairsville has been characterized as State silt loam; it is relatively high in organic matter. That at Experiment is Lloyd sandy loam, and it is

quite low in organic matter. The weather data were reported for the three weeks prior to harvest at each location. These are summarized in table 2. It can be seen that higher temperatures prevailed generally at Experiment in both years, with slightly greater temperature ranges at Blairsville. The average precipitation was greater at Blairsville in 1950 (and spread over fewer days) than at Experiment; however, the opposite was true in 1953, with equal numbers of days with no precipitation at both sites.

TABLE 2

Summary of weather data recorded at Blairsville and Experiment during the latter part of the growing season [1]

LOCATION	1950					1953				
	TEMPERATURE (°F)			RAINFALL		TEMPERATURE (°F)			RAINFALL	
	Range	Mean	Mean daily range	Mean	No. days with none	Range	Mean	Mean daily range	Mean	No. days with none
				in/day					*in/day*	
B	39-84	64	51-77	0.22	18	40-88	66	54-79	0.14	14
E	54-92	71	60-83	0.16	10	46-89	70	60-80	0.31	14

[1] The data are for the last three weeks preceding harvest at Blairsville (B) and Experiment (E) in both years. The exact planting dates in 1950 were not recorded, but they are similar from year to year and the data for 1953 are given below:
Blairsville: Planted April 7; harvested May 25. Growth period = 48 days.
Experiment: Planted April 1; harvested May 18. Growth period = 47 days.

Supplementation studies

The results of the studies to ascertain reasons for the differences in nutritive value of the Blairsville (B) and Experiment (E) turnip greens have been analyzed by the use of the "t" test to determine the effectiveness of each supplement.

The test for the presence of a toxic organic substance in the E greens was inconclusive. These data (diet and weight change) are as follows: 10% E, 114 gm; 10% B, 148 gm; 5% B, 134 gm; 5% E + 5% B, 127 gm. Thus, the dietary combination of 5% E + 5% B greens resulted in a mean weight

change which was 7 gm less than that obtained with a diet containing only 5% B greens, an unexpected finding since the addition of 5% E to 5% B greens would have nearly doubled the turnip-green protein and increased the other nutrients as well.

TABLE 3

Effect of supplement on growth and feed efficiency of rats fed Blairsville or Experiment turnip greens

SUPPLEMENT [1]	E DIETS [2]			B DIETS		
	Mean weight change	Increase due to supplement	Feed efficiency	Mean weight change	Increase due to supplement	Feed efficiency
	gm/8 wk	*%*	*gm feed/ gm gain*	*gm/8 wk*	*%*	*gm feed/ gm gain*
None – rep. 1 [3]	112 (8) [4]	....	5.50	139 (8)	....	4.92
None – rep. 2	114 (8)	....	5.55	148 (8)	....	4.81
Ash	114 (6)	2	5.55	153 (6)	3	4.94
Vitamin mix	137 (6)	22* [5]	4.70	163 (6)	10	4.78
Egg albumin (5%)	149 (6)	31**	4.47	156 (6)	12*	4.34
Methionine	152 (6)	33**	4.49	157 (6)	13*	4.42
Vitamin B_{12}	173 (6)	53**	4.82	184 (6)	32**	4.57
B_{12} + meth.	184 (6)	61**	4.11	181 (6)	30**	3.81

[1] See text, p. 348, for a description of the supplements.

[2] All diets contained 10% turnip greens grown at Experiment (E) or Blairsville (B) in 1953.

[3] Rep. 1 and 2 refer to field replications of turnip greens from each location. With the E diets, all supplements (except ash and vitamin mix) were added to rep. 2. With the B diets, all additions (except ash and vitamin mix) were made to rep. 1. The improvement was determined in each instance by comparing the results for the supplemented diet with those for its unsupplemented counterpart.

[4] Number of animals given within parentheses.

[5] *= significant at 5% level; **= significant at the 1% level.

The results of the remainder of the supplementation studies are presented in table 3 and may be summarized as follows:

(1) The difference between the greens was not in their mineral contents since the ash of one added to a diet containing the other did not alter the nutritive value of either the E or B diet. However, any mineral subliming at or below the ashing temperature (525° C.) would not have been tested.

(2) A vitamin mix (without vitamin B_{12}) significantly improved only diet E.

(3) Egg albumin, methionine or vitamin B_{12} improved both diets, the improvement in diet E being approximately twice that of diet B in all instances. Each of these supplements eliminated the previously observed difference between the greens. The improvement in both diets with additions of vitamin B_{12} was striking; its effect was about twice that of egg albumin or methionine. Vitamin B_{12} + methionine was no better than B_{12} alone.

The protein levels of the unsupplemented E and B diets were 13.5 and 14.1%, respectively, a difference attributable solely to the protein contents of the two turnip greens (E = 32.4%; B = 38.2%). The similarity in animal responses to dietary supplementation with egg albumin or methionine suggests that the principal contribution of the egg albumin was its methionine. The 5.0% level of egg albumin was no better than the 2.5% level; the latter would have contributed about 0.13% methionine (Block and Mitchell, '46). Before supplementation, diets E and B contained 0.17 and 0.18% methionine and 0.07 and 0.08% cystine, respectively. The difference is again to be found in the two turnip greens, as corn and cowpeas contributed a constant amount of both amino acids to all diets. Based on food consumption (table 3) and the analytical data given in table 4, the animals on diet B (replicate 1) received daily approximately 2.5 mg more methionine and 1.5 mg more cystine than those on diet E (replicate 2).

It is important to remember the low levels of sulfur amino acids in these diets when considering the effect of their supplementation with vitamin B_{12}. Although the sulfur amino acid content of each was slightly less than 0.3%, a marked improvement in growth was achieved solely by additions of vitamin B_{12}. In fact, the growth of the animals on diet B + B_{12} was not significantly different from that of the stock animals. Thus, while the improvement in growth and in food efficiency with methionine supplementation clearly indicated a methionine deficiency in both diets (table 3), the addition of vitamin B_{12} alone greatly improved their nutri-

tive quality. This sparing effect of B_{12} for methionine has been shown for the chick (Patrick, '50; Briggs et al., '50). The results of the present study emphasize the necessity of considering the vitamin B_{12} content of a diet before establishing the methionine requirement. The animal's need for vitamin B_{12} besides that relative to methionine metabolism was demonstrated in this study, since supplementation with vitamin B_{12} alone resulted in nearly twice the growth improvement obtained with methionine alone.

TABLE 4

Methionine, cystine and vitamin B_{12} contents of turnip greens grown at Blairsville or Experiment

TURNIP GREENS	METHIONINE [1]	CYSTINE [1]	VITAMIN B_{12} [2]		MOISTURE
			Ochromonas malhamensis	Lactobacillus leichmannii	
	mg/gm	*mg/gm*	*mμg/gm*	*mμg/gm*	%
B (1) [3]	4.30	2.52	6.2	7.4	4.2
B (2)	4.66	2.29	8.3	8.5	3.8
E (1)	3.34	1.98	0.23	0.5	4.3
E (2)	3.42	1.78	0.76	1.2	4.8

[1] Methionine and cystine were determined microbiologically using *Leuconostoc mesenteroides*. All values in this table are for the greens as fed with the moisture content indicated.

[2] Determinations with both organisms were made on the same sample extract. The *L. leichmannii* values have been corrected for desoxyriboside activity.

[3] B (1), B (2), E (1), and E (2) refer to field replications used at Blairsville (B) and Experiment (E).

Although there is no clear-cut evidence to date of the presence of true vitamin B_{12} (cyanocobalamin) in the leaves of higher plants, the possibility that at least a part of the difference between the greens might be due to their contents of a vitamin B_{12}-active substance was investigated. The results of microbiological assays of the turnip greens, presented in table 4, indicated the presence of significant amounts of vitamin B_{12} in the Blairsville greens. The findings with *Ochromonas* are of special interest since Coates and Ford ('55) have noted that this microorganism more closely resembles the animal in its response to the vitamin B_{12} family of compounds than any other.

While the Blairsville greens have been shown to contain a vitamin B_{12}-active substance, and only two naturally-occurring substances are now known which possess vitamin B_{12} activity for the animal and for *Ochromonas* — i. e., cyanocobalamin and vitamin $B_{12\ III}$ (Coates and Ford, '55) — further studies are needed before the identity of the vitamin B_{12}-active material in the B greens is established. Neither can it yet be concluded that this vitamin B_{12}-active substance was elaborated by the turnip plant itself. Two hypotheses might be suggested as alternative explanations for its presence in the turnip green sample. Both have experimental support and are in keeping with the generally accepted opinion that to date the only proved source of vitamin B_{12} is the microorganism. The first is that its presence could have resulted from soil contamination of the greens in the field. Vitamin B_{12} activity has been found in soils (Stephenson et al., '48; Robbins et al., '50), presumably produced by certain types of soil microorganisms (Burton and Lochhead, '52). It can only be reiterated that the greens were carefully washed and that the same personnel supervised the operation for both the E and B greens. A second hypothesis might be that the vitamin B_{12} was present in the greens as a result of bacteria living epiphytically on the turnip leaves. Ericson and Lewis ('54) have shown this situation to occur in several species of algae. Nevertheless, this vitamin B_{12} would be available to an animal or human eating the plant material as it is not removed by washing.

While both the supplementation studies and the analyses indicated that the principal difference between the greens was in their contents of vitamin B_{12} and methionine, the results of supplementation with a vitamin mix suggest a difference there also. The greater response of the animals on diet E to the mix (table 3) indicated that the E greens were more deficient than the B greens in at least one of the vitamins in the mix; or, that one of these vitamins spared another limiting nutrient in the greens. Dietary interrelationships among vitamin B_{12}, methionine, and several vitamins present in the

mix have been demonstrated with several animal species. Thus, vitamin B_{12} has been shown to spare, or be spared by, choline (Schaefer et al., '49a, b; Gillis and Norris, '49), pantothenic acid (Yacowitz et al., '51), folic acid (Schaefer et al., '50), and riboflavin (Hartman et al., '51; Cooperman et al., '52); similar relationships have been demonstrated for methionine and choline (Treadwell, '48), and methionine and pantothenic acid (Nelson and Evans, '49).

Thus, the small differences in the sulfur-amino acid and vitamin B_{12} contents, in favor of the B greens, could have resulted in a much greater nutritional effect for the animal if larger amounts of one or more of the vitamins known to spare methionine or vitamin B_{12} were also present in the Blairsville greens.

SUMMARY

A study of the relative nutritive value of several foods grown at widely differing locations demonstrated the superiority for the growing rat of turnip greens grown at Blairsville, Ga. in two different years. Turnip greens grown at two other locations — Experiment, Ga. and Raleigh, N. C.— were equivalent in nutritive value and definitely inferior to the Blairsville greens. No difference was observed in the nutritive value of corn and cowpeas which were tested similarly.

Supplementation studies, using these all-plant diets, to determine the reasons for the superiority of the Blairsville (B) over the Experiment (E) turnip greens showed that diets containing either greens were improved by the following supplements: egg albumin, methionine, vitamin B_{12}, and vitamin B_{12}+ methionine. Vitamin B_{12} was twice as effective a supplement as egg albumin or methionine, and vitamin B_{12} + methionine was no better than vitamin B_{12} alone. A complete vitamin mix (without vitamin B_{12}) significantly improved only diet E. The difference between the two greens was not in their mineral contents.

Vitamin B_{12}, or a substance with vitamin B_{12} activity for *Ochromonas malhamensis* and *Lactobacillus leichmannii,* was found in the B turnip greens; only negligible amounts were present in the E greens.

Microbiological analyses showed the B greens to contain slightly more methionine and cystine than the E greens.

This investigation showed that certain as-yet-undetermined factors associated with two different locations so influenced the composition of turnip greens grown there as to affect significantly their nutritive value for the animal. It also indicated that the principal difference between the two turnip greens was in that area of nutrition in which methionine, vitamin B_{12}, and several other vitamins have been shown to be interrelated.

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RELATIVE ROLES OF NIACIN AND TRYPTOPHAN IN MAINTAINING BLOOD PYRIDINE NUCLEOTIDES, NITROGEN BALANCE AND GROWTH IN ADULT RATS[1]

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In previous reports by Williams et al. ('50b, c) it was observed that physiological[2] levels of dietary tryptophan appeared to be more active than physiological[2] levels of niacin in stimulating synthesis of rat liver pyridine nucleotides (PN) when fed to animals depleted of liver PN by niacin-tryptophan-deficient or niacin-free, non-protein rations. On the other hand, when tryptophan or niacin was incorporated into the ration in *equimolar* amounts, over a wide concentration range, the two nutrients produced about the same concentration of liver PN, except at very high equimolar concentrations where tryptophan produced more liver PN than did niacin (Feigelson et al., '51). Burch et al. ('55) have reported that tryptophan contributed slightly less than niacin to DPN synthesis in red and white blood cells and in liver of rats previously fed a marginal amount of PN precursors. The main differences in the earlier studies in our

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[2] Levels customarily fed in rations or considered to be the requirement.

laboratory and the studies by Burch et al. are that in the latter a partially natural ration (Sherman Diet 13) containing 12.6% of protein (9% of which was casein) was employed throughout the experiment. Thus about 0.8 mg % of niacin and 88 mg % of tryptophan were present in the basal ration, to which further supplements of the two nutrients were added and their effects on blood and liver PN determined.

To extend the earlier studies in our laboratories using animals severely depleted of PN, the utilization of dietary tryptophan and niacin with respect to both blood PN synthesis and general protein metabolism has been investigated. The experiments were designed to determine which functions, *i. e.*, blood PN synthesis, growth, or nitrogen balance maintenance, take precedence when controlled amounts of tryptophan and niacin are supplied to niacin-tryptophan-deficient rats. They were also designed to reinvestigate the relative utilization of niacin and tryptophan in PN synthesis.

EXPERIMENTAL

The work reported in this paper is divided into two main sections: (I) a study of the interrelationships of blood PN levels, growth and nitrogen balance of niacin-tryptophan-deficient rats receiving graded levels of tryptophan without niacin; (II) a study of the response of blood PN levels and growth of niacin-tryptophan-deficient rats to constant, physiological levels of niacin, tryptophan, or tryptophan plus niacin.

Both blood PN [diphosphopyridine nucleotide (DPN) plus triphosphopyridine nucleotide (TPN)] and N′-methylnicotinamide (NMeN) levels were determined in most of these studies. NMeN showed no significant variations, but remained nearly a constant 10% of the total blood PN under all conditions studied, so the results for the sum of PN plus NMeN are reported here. Since both PN and NMeN are a reflection of the conversion of tryptophan to the niacin structure, this is an added reason for reporting the sum of these substances. The sum of PN plus NMeN has been calculated

using DPN as a standard and is expressed in this paper as micromoles of PN per milliliter of blood multiplied by the estimated body surface area of the rats. This method of expression is used since it should be an index of "total circulating" blood PN. A relatively constant relationship has been shown to exist between body surface area and total blood volume (Dreyer and Ray, '10), so the concentration of PN per milliliter of blood multiplied by the surface area should give a value which is proportional to the "total circulating" blood PN. Surface area was calculated from body weight data using the formula $S = Kw^{2/3}$, where $K = 11.36$ for the rat (Carmen and Mitchell, '26). In all experiments body weights of the rats were recorded at frequent intervals.

The composition of the basal ration used during the depletion periods for all experiments, and to which supplements were added during the experimental periods was as follows: zein, 2.0; diammonium citrate, 2.34; gelatin, 3.0; glycine, 3.0; DL-isoleucine, 0.616; DL-leucine, 1.425; L-lysine·HCl, 1.055; DL-methionine, 0.532; DL-phenylalanine, 0.708; DL-threonine, 0.968; DL-valine, 1.114; L-arginine·HCl, 0.041; L-histidine·HCl, 0.421; corn oil, 10.0; Salts 4 (Hegsted et al., '41), 4.0; choline chloride, 0.15; and sucrose, 68.38%. Vitamins were included to provide, in milligrams per 100 gm, thiamine hydrochloride, 0.5; riboflavin, 0.5; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002; and inositol, 10.0. Two drops of haliver oil, fortified to provide 1000 I. U. of vitamin A, 10 I. U. of vitamin D, 0.04 mg of menadione and 0.8 mg of α-tocopherol were administered weekly to each animal by mouth. This basal ration contained, by calculation, about 0.002% of tryptophan.

I. Effect of graded levels of tryptophan intake. Two experiments were conducted differing somewhat in experimental plan. In the first experiment three female and two male adult rats of the Sprague-Dawley strain, weighing about 220 gm, were used as their own controls. They were housed in individual, screen-bottom cages and fed the niacin-tryptophan-defi-

cient basal ration ad libitum for two months, for depletion. The rats were then transferred to individual metabolism cages, food consumption was measured and collection of urine and feces was started. Basal blood PN levels were determined at this time and throughout the following periods of supplementation when L-tryptophan was added to the ration at progressively increasing levels of 0.02, 0.08, 0.14, 0.20 and 0.30% of the ration, after the animals had been fed the levels for 8, 13, 15, 11 and 5 days, respectively.

In the second experiment 15 male and 15 female adult rats of the Sprague-Dawley strain, weighing about 235 gm, were housed as above and fed the niacin-tryptophan-deficient basal ration for 6 weeks, until their weight and blood PN values approximated those of the rats in the first experiment at the end of their depletion period. The animals were then separated into 5 groups of 6 animals each (three males and three females) and the basal ration was supplemented according to the following plan: group 1, no supplement; group 2, 0.02% of L-tryptophan; group 3, 0.08% of L-tryptophan; group 4, 0.14% of L-tryptophan; and group 5, 0.20% of L-tryptophan. The supplemented rations were fed for 30 days. Blood PN levels were determined on all animals the day before supplementation was started, on the 5th day and every 8th day thereafter. They were placed in individual metabolism cages for 72-hour intervals beginning every 8th day.

Blood for the PN analyses was obtained by excising a small portion of the tails of the rats and drawing the blood directly into a blood pipette. No anticoagulant was used. The PN content of the blood was measured by the method of Kring and Williams ('53).

Urine was collected under toluene with 1 ml of concentrated HCl added. The urine from each collection period was diluted to 200 ml. Fecal pellets were collected, covered with 95% ethyl alcohol plus one drop of concentrated HCl, and dried at 60°C. Nitrogen determinations of the ration, urine, and feces were carried out by the semi-micro Kjeldahl method of Hiller, Plazin and Van Slyke ('48).

II. Effect of niacin, tryptophan or niacin plus tryptophan supplementation. Two experiments of similar experimental plan but different duration were performed. In each experiment adult male and female rats of the Sprague-Dawley strain, weighing about 225 gm, were depleted for 6 weeks on the niacin-tryptophan-deficient basal ration, at the end of which time blood PN levels were determined. In the first experiment the depleted rats were then divided into three groups of 6, 4 and 6 rats each, equal numbers of males and females in each group, and the basal ration was supplemented with 0.2% of tryptophan, 2 mg % of niacin, or 0.2% of tryptophan plus 2 mg % of niacin, respectively. The supplemented rations were fed for 14 days, during which time blood PN analyses were made on the third, 5th, 8th and 14th days.

In the second experiment the depleted rats were divided into 4 groups of 6 rats each (three male, three female), three of the groups receiving the above supplements and one group receiving no supplement. The experimental period was 30 days and PN analyses were made on the 5th day and every 8th day thereafter.

RESULTS

I. Effect of graded levels of tryptophan intake. Despite the differences in experimental plan, the results of the two experiments were similar. Since no apparent differences existed between the results for males and females, the results for all animals were averaged together throughout this paper.

In the first experiment the rats were losing an average of 25 mg of nitrogen per rat per day when they received no tryptophan supplement, but they came into nitrogen equilibrium when 0.02% of tryptophan was added to the ration. As the tryptophan supplement was further increased to 0.08, 0.14, 0.20 and 0.30% of the ration, they showed a gradually increasing mean retention of nitrogen of 27, 45, 96 and 97 mg per rat per day, respectively.

During the two-month depletion period these rats decreased in average weight from 220 to 140 gm. There was a further

very slow decline in weight to 130 gm when the animals received a supplement of 0.02% of tryptophan in the ration, a maintenance of weight with 0.08% of tryptophan, and a steady increase in weight starting when the ration was supplemented with 0.14% of tryptophan and continuing throughout the rest of the experimental period.

The mean values for "total circulating" blood PN were somewhat erratic during the time when the animals received no supplement, 0.02, 0.08, or 0.14% of tryptophan supplement, varying between 0.25 and 0.35 μM per milliliter of blood × surface area. When 0.20% of tryptophan was added to the

TABLE 1

Mean nitrogen balance in milligrams of nitrogen per rat per day

GROUP [1] NO.	SUPPLEMENT TO DIET	DAYS [2]			
		1–3	9–11	17–19	25–27
	%				
1	none	−43.7	−22.5	−24.2	−25.7
2	L-tryptophan, 0.02	−23.2	−23.8	−24.6	−17.6
3	L-tryptophan, 0.08	2.7	5.4	12.7	17.8
4	L-tryptophan, 0.14	−9.0	50.2	87.0	95.0
5	L-tryptophan, 0.20	13.0	78.5	106.7	102.5

[1] Six rats per group.

[2] Inclusive.

diet, the "total circulating" PN values increased sharply and, with the addition of 0.30% of tryptophan to the diet, continued to increase to about 0.7 μM per ml blood times surface area. In another experiment, not reported here, the tryptophan supplement was increased to 0.7% of the ration but no further increase was observed in "total circulating" PN.

Thus, the niacin-tryptophan-depleted rats reached nitrogen equilibrium when 0.02% of tryptophan was added to the basal ration, an increase in body weight when the tryptophan supplement reached 0.14%, and an increase in "total circulating" PN only when 0.20% of tryptophan or more was added to the ration.

In the second experiment each of the 5 groups of rats received a different, but constant, level of tryptophan supplementation. The nitrogen balance data are presented in table 1. The animals receiving no supplement remained in negative nitrogen balance throught the 30-day period. Those rats receiving 0.02% of tryptophan supplement were also in negative nitrogen balance to almost the same extent as group 1. There was some suggestion, however, that the average daily

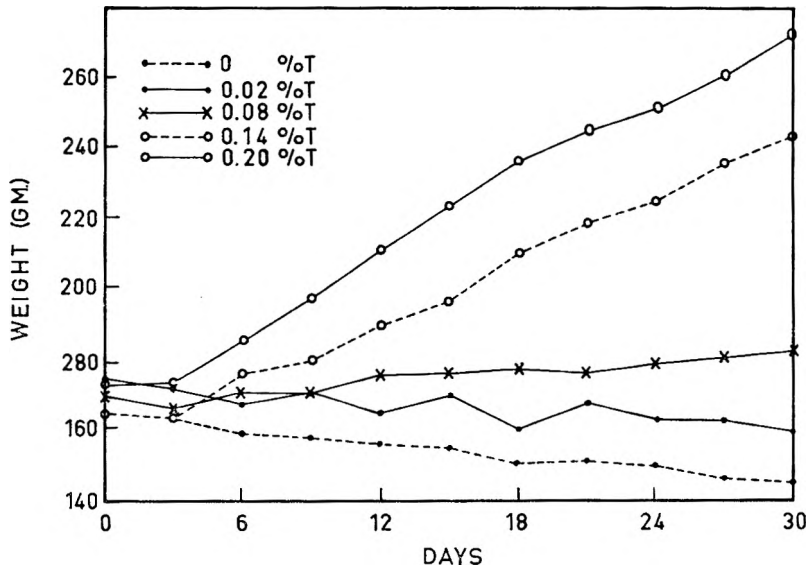

Fig. 1 Effect of different levels of dietary tryptophan on the weight of niacin-tryptophan-depleted rats.

nitrogen loss of group 2 was diminishing slightly by the last metabolism period. Nitrogen equilibrium or retention was evident in the group of rats receiving 0.08% of tryptophan, the amount of nitrogen retained increasing gradually over the 30-day period. The groups receiving 0.14 and 0.20% of tryptophan also stored increasing quantities of nitrogen throughout the period.

Figure 1 shows the mean weight changes of the animals receiving different levels of tryptophan. The rats receiving

no supplement continued to lose weight slowly. Supplementation of the ration with 0.02% of tryptophan was not sufficient to prevent a slight weight loss. After the first 10 days of the experimental period the group of rats receiving 0.08% of tryptophan gradually increased in weight. Group 4, receiving 0.14% of tryptophan, grew more slowly than did group

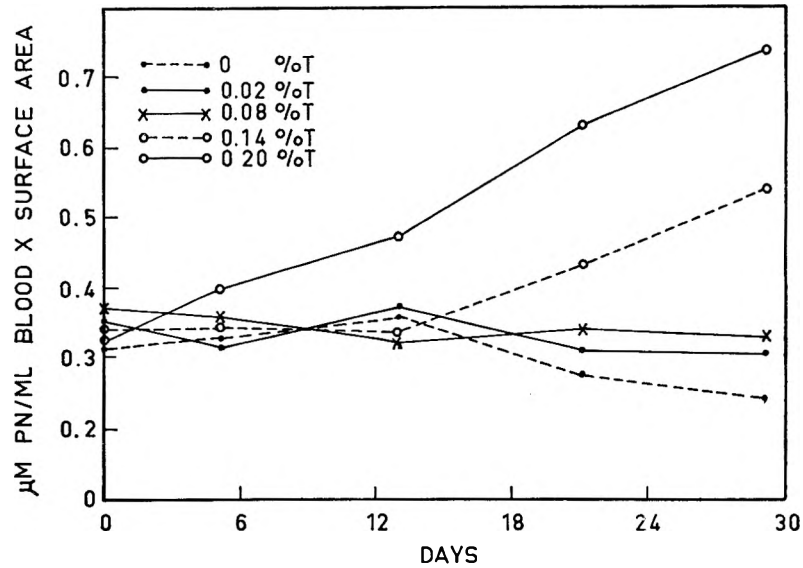

Fig. 2 Effect of different levels of dietary tryptophan on the "total circulating" PN (μM PN per ml blood × surface area) in niacin-tryptophan-depleted rats.

5, receiving 0.20% of tryptophan, during the first week on experiment. Thereafter, the rate of growth of the two groups was about the same.

The "total circulating" blood PN levels of the groups receiving no supplement, 0.02 and 0.08% of tryptophan are not significantly different and show a slight downward trend (fig. 2). During the first half of the experimental period the "total circulating" PN levels of group 4, 0.14% of tryptophan, followed the same pattern as groups 1, 2 and 3. However, some measurable synthesis of PN is apparent during the latter half of the experimental period, as shown by the increase

in PN levels as compared with group 1, the unsupplemented group. Only the rats supplemented with 0.20% of tryptophan demonstrated a marked increase in PN throughout the experimental period. The final mean PN value for this group was about 130% higher than the initial value.

II. Effect of niacin, tryptophan, or niacin plus tryptophan supplementation. The results of the 14-day and the 30-day studies were similar. In the shorter study the growth rates of the animals receiving tryptophan or tryptophan plus niacin were almost identical, the average weight gains over the 14 days being 38 and 40 gm, respectively. The group supplemented with niacin alone lost an average of 13 gm during the same period. In this same study the "total circulating" PN of the animals receiving tryptophan plus niacin increased slightly throughout the experimental period, that of the animals receiving tryptophan alone remained relatively constant, and that of the animals receiving niacin alone decreased slightly.

When this experiment was repeated with more rats and extended to a 30-day period, the mean weight changes of the rats which received only 2 mg% of niacin supplement were essentially the same as those which received no supplement at all. Both groups lost almost 20 gm during the 30-day experimental period. On the other hand, the groups which received tryptophan or tryptophan plus niacin also showed growth patterns similar to each other, both gaining slightly over 100 gm during the 30 days.

"Total circulating" PN levels (fig. 3) of the unsupplemented rats appeared to have increased slightly during the first half of the experimental period, but decreased slightly during the latter half. Although the animals which received niacin in the ration maintained quite a constant level of blood PN, some definite, however slight, synthesis of PN apparently occurred, as may be seen by comparison with the values of the unsupplemented group. Both tryptophan alone and niacin plus tryptophan in the ration provided for a substantial increase in "total circulating" PN, of the order

of 130%, in these niacin-tryptophan-depleted rats. It appears, then, that tryptophan alone or tryptophan plns niacin were equally effective in supporting PN synthesis over the 30-day period. However, when only the first two weeks of the period are considered there was a very slight indication that tryptophan plus niacin was superior to tryptophan alone.

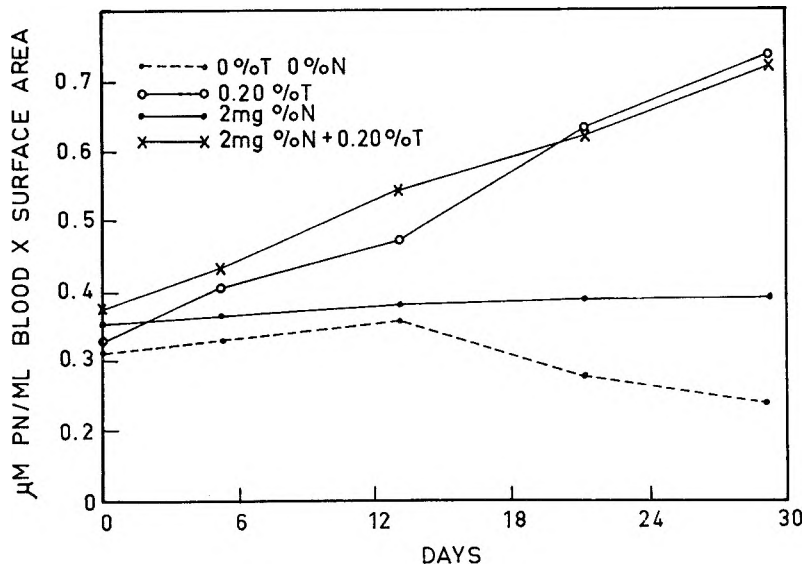

Fig. 3 Effect of daily supplements of 0.2 gm tryptophan, 2 mg niacin, 0.2 gm tryptophan plus 2 mg niacin per 100 gm of ration, and no supplement on the "total circulating" PN (μM PN per ml blood × surface area) in niacin-tryptophan-depleted rats.

DISCUSSION

The levels of tryptophan intake at which the greatest response in nitrogen balance, growth and PN synthesis were observed should give some insight concerning the preferential use of tryptophan in niacin-tryptophan-depleted rats. It is probable that supplemental tryptophan would go first to the metabolic function for which the demand was greatest after such a prolonged period of depletion. Thus, under the conditions of these studies, it appears that when niacin-trypto-

phan-depleted rats were fed limiting amounts of tryptophan in the ration, maintenance of nitrogen equilibrium was the first metabolic function of tryptophan to become evident as the level of tryptophan supplementation increased. In one experiment the depleted animals came into nitrogen equilibrium when 0.02% of tryptophan was added to the ration, and in the second experiment a level of 0.08% of tryptophan in the ration was required, although a trend toward nitrogen equilibrium was observed after about three weeks in the rats which had been receiving a 0.02% tryptophan supplement. In neither experiment could any increase in body weight or total blood PN be observed at the level of tryptophan at which nitrogen balance became evident. In fact, slight losses in body weight were still occurring.

The metabolic use of this nitrogen which was retained was not evident, as no corresponding increase in body weight was observed. However, whatever use was made of it, the need must have been filled by the time the tryptophan supplement was increased to 0.14% in the first experiment, at which time weight gains were observed. In the second experiment, also, definite weight gains were observed in the group receiving 0.14% of tryptophan in the ration. Some growth was also evident in the group receiving only 0.08% of tryptophan, after this supplement had been fed for two weeks.

Repletion of blood PN occurred with a further increase in the level of tryptophan supplementation to 0.20%, or, in the second experiment, after nitrogen retention and growth had been occurring for about two weeks in the group of rats receiving 0.14% of tryptophan.

Rose ('37) has suggested 0.20% of L-tryptophan as the requirement of the weanling rat. This same level is frequently used in rations for adult rats on the assumption that the tryptophan requirement of the adult rat should not be higher than that of the weanling rat. There is limited information available on the requirement of the adult rat for this amino acid. Cole and Robson ('51) have used maintenance of weight of adult female rats after recovery from tryptophan deficiency

as their criterion for adequacy of the amount of tryptophan in the ration. They found the tryptophan requirement to vary with the weight at which the animals were maintained and with the severity of the depletion. Consequently, they suggested a fairly wide range of 0.10 to 0.20% of tryptophan in the diet as an optimal level for adult rats. It is impossible to compare directly the work reported in this paper with that of Cole and Robson because of different experimental conditions. Also, the ration used here was niacin-deficient, as well as tryptophan-deficient. However, these studies indicated that 0.20% of L-tryptophan was adequate for nitrogen balance, growth and PN synthesis in adult rats of both sexes. By interpolation, however, it is probable that 0.10% of tryptophan, under the conditions of these experiments, would have supported only nitrogen balance and possibly maintenance of weight, but not optimal growth nor PN synthesis.

It became evident from the results of the second experiment that the level of tryptophan supplementation at which the various metabolic responses to tryptophan by niacin-tryptophan-depleted rats became apparent was a function of the length of time a level of tryptophan was fed, as well as the actual level of tryptophan itself. Therefore, that level could not be considered to be the tryptophan requirement for that response. However, the sequence of events in this response, rather than the actual level of tryptophan supplementation, remains significant and suggests a preferential use of limiting amounts of tryptophan by niacin-tryptophan-depleted rats as follows: *first,* maintenance of nitrogen balance; *second,* growth; and *third,* repletion of blood PN.

The second phase of these experiments, a comparison of the growth and "total circulating" PN levels of depleted rats receiving niacin, tryptophan, or niacin plus tryptophan in physiological amounts, indicated that when tryptophan was present at 0.20% of the ration, 2 mg% of niacin had very little further effect detectable in these experiments. The growth rates of the two groups of animals were almost identical in two separate experiments. Blood PN synthesis

appeared slightly greater in the animals receiving both niacin and tryptophan than in those receiving tryptophan alone during the first two weeks of supplementation, but when a full 30-day period was considered, this slight advantage was overcome. These results might be interpreted to support the results in the first phase of these experiments. If it it is true that nitrogen balance and growth take precedence over repletion of blood PN, then perhaps during the first two weeks of the experimental period 0.20% of tryptophan in the ration supplied only enough tryptophan to allow for these functions and for limited synthesis of blood PN. As body proteins then became repleted, more of this tryptophan may have become available for the repletion of "total circulating" PN. On the other hand, when both niacin and tryptophan were fed together, the tryptophan may have been used primarily for repletion of body proteins, whereas the niacin was available for some PN synthesis, thus showing a slight apparent advantage over the animals receiving tryptophan alone. This advantage was small and disappeared before the end of the 30-day experimental period.

It is interesting to note that niacin alone did not appear to support PN synthesis unless the niacin-supplemented group is compared with a group receiving no supplement at all. Then it can be seen that some, however slight, synthesis may have occurred, although it was not enough to increase "total circulating" PN, but merely enough to prevent further depletion such as occurred in the unsupplemented group. Another explanation of this might be that the presence of niacin in the ration in some way decreased the rate or necessity of breakdown of the already existing blood PN, rather than increasing the synthesis of PN. There was no evidence in these experiments to indicate which of these possibilities actually occurred.

From the results of these experiments it can be stated, as previously observed in these laboratories for PN synthesis in rat liver, that when tryptophan or niacin is added alone at physiological levels to the basal ration fed to PN-depleted

rats, tryptophan is utilized more readily than niacin for the synthesis of blood PN. An explanation for this greater conversion of tryptophan than niacin to PN cannot be offered at present. It is doubtful that the explanation lies in a decrease in activity of certain tissue enzymes necessary for the synthesis of PN, due to an amino acid (tryptophan) deficiency. Although non-protein rations have been shown to cause greater losses in tissue enzymes than individual amino acid deficiencies (Prigmore et al., '55), rats maintained on a non-protein ration were still able to synthesize liver PN quite readily (Williams et al., '50c).

SUMMARY

1. Young adult rats, depleted of blood pyridine nucleotides (PN) by a niacin-tryptophan-deficient ration, were fed supplements of L-tryptophan from 0 to 0.30% of the ration. Nitrogen equilibrium was evident on the lower levels of tryptophan supplementation, growth on the intermediate levels and synthesis of blood PN on the higher levels. This sequence suggests a preferential use of tryptophan in the order listed by niacin-tryptophan-deficient rats.

2. When similarly depleted rats were fed no supplement or constant physiological levels of tryptophan, niacin, or tryptophan plus niacin, those receiving niacin showed small weight losses comparable to the unsupplemented group, while those receiving tryptophan or tryptophan plus niacin showed comparable weight gains. "Total circulating" PN values of the niacin-supplemented group decreased slightly or were just maintained at a constant level only slightly above the unsupplemented group. "Total circulating" PN values of the tryptophan plus niacin-supplemented groups increased gradually, and in a two-week period were a little higher than the tryptophan-supplemented groups. At the end of a 30-day period this difference had disappeared. This supports the suggestion that at physiological levels tryptophan contributes to PN synthesis in niacin-tryptophan-depleted rats to a greater extent than does niacin.

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THE EFFECT OF VARIOUS FATS UPON EXPERIMENTAL HYPERCHOLESTEREMIA IN THE RAT[1]

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Numerous studies have demonstrated an effect of the amount and kind of dietary fat upon the level of serum cholesterol. The epidemiologic evidence presented by Keys et al. ('54, '56) has drawn attention to the possible role of total fat intake as a causative factor in hypercholesteremia and coronary heart disease. Groen et al. ('52), Kinsell et al. ('52), Ahrens et al. ('54), Beveridge et al. ('55), Bronte-Stewart et al. ('56) and various others (Van Itallie, '56) have shown that high levels of intake of certain fats cause a lowering of the serum cholesterol levels, presumably a favorable effect with regard to coronary disease and atherosclerosis. In general, unsaturation of the fat as indicated by high iodine numbers has been associated with lowering of the serum cholesterol values. Attempts to determine whether the causative factor is simply unsaturation *per se*, the presence of large amounts of the essential fatty acids, or factors associated with these characteristics are being actively pursued in many laboratories.

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In the past the isolation and identification of essential nutrients has almost invariably required the use of rapid biological assays. Appropriate bioassays may depend upon conditions which have no counterpart in human nutrition. For example, the rachitogenic diet for rats contains large amounts of calcium and a high ratio of calcium to phosphorus, the type of diet never encountered in human nutrition and not the type of diet associated with the development of rickets in children or osteomalacia in adults. The cholesterol-fed animal need not necessarily be inappropriate for the study of effects upon hypercholesteremia.

In a previous paper (Hegsted et al., '57) an assay procedure for the measurement of the effects of different fats upon serum cholesterol levels was developed using rats fed cholesterol and cholic acid. The effect of the different fats tested in the preliminary work was generally similar to the results obtained with human beings with one marked exception. Tung oil, a highly unsaturated oil, produced the highest cholesterol values of any fat tested. For the other fats tested there was a significant inverse correlation between the iodine number of the fat and the mean serum cholesterol. However, this correlation between cholesterol level and iodine number was not sufficiently close to permit the conclusion that this was the only factor of importance. Linseed oil, for example, with an iodine number of 180, produced higher serum cholesterol values than did corn and cottonseed oil with iodine numbers of 123 and 110, respectively. The standard errors of the mean cholesterol values were less than 5%, and it seemed certain that the departures from the line of regression were real and not due to error or chance variation within the groups.

This paper presents additional results using the assay described. The total data available at this time, including a few of the values presented in the previous paper, are those upon 50 different oils and combinations of oils fed to different groups of rats. The evaluation of this material leads us to the conclusion that the essential fatty acids (linoleic and

perhaps arachidonic acid) act together with the saturated fatty acids in causing low serum cholesterol values. The unsaturated non-essential fatty acids (oleic, linolenic[2], and eleostearic acid) apparently promote high serum cholesterol values and this action is counteracted by the essential and saturated acids.

EXPERIMENTAL

All of the assays reported have been done with adult male rats weighing approximately 250 to 300 gm when the experiments were initiated. The basic diet has been described (Hegsted et al., '57) and is a typical purified diet containing 10% casein. In the original studies, 20% of the diet was the fat or fat mixture under test. From the later work it appeared that similar results were obtainable with only 10% of fat in the diet, and in the interest of conserving material, the diet was changed to include only 10% of fat and the amount of glucose was increased by 10%. In the first experiments, 1% of cholic acid and 3% of cholesterol were added to the diets. However, after the quantitative studies upon the effects of various levels of cholesterol and cholic acid (Hegsted et al., '57), it seemed desirable to lower the amounts in order to avoid the tremendous hypercholesteremias encountered with some oils. Subsequently, 0.45% of cholic acid and 0.45% of cholesterol were added to the diets.

These changes in the experimental conditions prevent a consideration of all the data grouped together. Although in general similar degrees of hypercholesteremia are obtained in different groups of animals fed the same diet, it is impossible to duplicate exactly the experimental conditions since new groups of animals were used. Thus, each experiment, i. e., all of the groups of animals studied simultaneously, is considered separately. In all of the studies reported,

[2] The reasons for including linolenic acid as a non-essential fatty acid in this regard will be apparent from the results in the text. As is well known, there is discussion as to whether or not linolenic acid is an essential fatty acid when the activity is measured by other techniques, such as the rat growth method (Deuel and Reiser, '55).

unless otherwise specified, the groups contained 6 animals each.

During the experiment animals were bled from the tail into a small centrifuge tube, the blood allowed to clot, and the serum separated by centrifugation. Cholesterol determinations were done on duplicate aliquots of 0.02 ml of serum as described by Carpenter et al. ('57). At the termination of the study, the animals were either bled as described or blood was taken by syringe from the heart after anesthesia with the chest cavity opened. In most of the studies, the relative position of the various groups was established by the second week. Unless the group was large, as in the first experiment, averaging the values obtained from the second to 4th week gave a more secure mean with a smaller standard error than did the values of the 4th week alone.

The data utilized in this paper were obtained from the following experiments:

Experiment I. The diet contained 20% fat, 1% cholic acid and 3% cholesterol. The fats tested were tung, coconut, cottonseed, hydrogenated cottonseed, butter, linseed, corn and sardine. Twelve animals were used in each group and serum cholesterol values were determined at the 4th week. These results were reported in the previous paper (Hegsted et al., '57).

Experiment II. A diet of 20% fat, 0.45% cholic acid and 0.45% cholesterol was used. The oils tested were "Triolein"[3], triolein and safflower mixtures in the following proportions: 90 - 10, 75 - 25, 50 - 50, and safflower oil. Similar mixtures of triolein and coconut oil were also tested. Serum cholesterol values at 4th week were used.

Experiment III. The diet contained 10% fat and 0.45% of cholic acid and 0.45% cholesterol. The oils tested included

[3] A preparation high in oleic acid supplied by Emery Industries of Cincinnati. This material contained 3.6% linoleic acid, 8.2% saturated fatty acids, and 88.2% oleic acid, expressed as percentages of the total fatty acids present. The composition is thus similar to olive oil and "Triolein" is a misnomer.

triolein, 90 - 10 and 80 - 20 mixtures of triolein and safflower oil, the same mixtures of triolein and lard, as well as safflower oil and lard alone. Serum cholesterol values at second and 4th weeks were averaged.

Experiment IV. The diet was the same as in experiment III. The oils tested included triolein, olive, rapeseed, cottonseed, linseed, corn, tung, safflower, coconut, and various mixtures of these oils, in a total of 24 groups. The mean serum cholesterol values of the second, third and 4th weeks were averaged.

RESULTS AND DISCUSSION

As indicated in the previous paper and in the present introduction, the preliminary data showed that with the exception of tung oil, the oils which had been studied indicated a significant inverse relationship between the degree of hypercholesteremia produced and the iodine number. However, the relationship was not sufficiently close to be convincing, and the clear exception of tung oil indicated that perhaps this apparent relationship to unsaturation might be no more than a chance relationship depending upon the oils which we happened to study. It was believed that the assay might be improved and standardized if a diet could be devised with some basic or neutral oil as the fatty constituent, and various substitutions of this oil studied. We assumed that triolein, or a preparation containing only small amounts of essential fatty acids and saturated fats (which had been previously implicated as the most likely positive and negative agents) would be desirable. Experiment II was set up to test this hypothesis. We had assumed that saturated fatty acids as in coconut oil would elevate the serum cholesterol values whereas the essential fatty acid of safflower oil would decrease the serum cholesterol values. To our surprise we found the highest serum cholesterol values with the "Triolein" and additions of either safflower oil or coconut oil caused substantial reductions (table 1). Analytical data upon the oils used, and calculation of the linoleic acid content

or iodine numbers of the mixtures prepared (figs. 1 and 2) demonstrated with certainty that neither the iodine number nor the linoleic acid content of the fat could be implicated as the determinant factor.

Various manipulations of the analytical data were tried in an attempt to find some common denominator which might

TABLE 1

Relation of "Triolein" fat mixtures to serum cholesterol values

FATS TESTED	SATURATED FATTY ACIDS	ESSENTIAL FATTY ACIDS	UNESSENTIAL UNSATURATED ACIDS	SERUM CHOLESTEROL
	%	%	%	mg%
Experiment II				
20% Triolein	8.2	3.6	88.2	646
18% Triolein + 2% safflower	8.6	11.4	80.0	413
15% Triolein + 5% safflower	9.1	23.0	67.7	291
10% Triolein + 10% safflower	9.9	42.4	47.1	281
0 Triolein + 20% safflower	11.6	81.1	6.7	244
18% Triolein + 2% coconut	16.4	3.4	80.1	464
15% Triolein + 5% coconut	28.7	3.2	68.1	414
10% Triolein + 10% coconut	49.2	2.8	47.9	377
0 Triolein + 20% coconut	90.3	2.0	7.6	351
Experiment III				
10% Triolein	8.2	3.6	88.2	791
9% Triolein + 1% safflower	8.6	11.5	80.0	628
8% Triolein + 2% safflower	8.9	19.1	71.8	543
9% Triolein + 1% lard	11.1	4.6	84.4	671
8% Triolein + 2% lard	14.0	5.4	80.5	502
10% Lard	37.4	12.5	49.9	342
10% Safflower	11.6	81.1	6.7	300

Analytical data supplied by Dr. F. H. Mattson, The Proctor and Gamble Company.

explain the effect on serum cholesterol values. Of the various things tried, the number obtained by multiplying the content of essential fatty acid (linoleic acid) by the total content of saturated fatty acid appeared most satisfactory (figs. 3 and 4). When this same type of correlation was tried using the data from experiment I (fig. 5), it was apparent that in order to make linseed oil fit the curve, linolenic acid could not be

considered as an essential fatty acid. Similarly, in order to make sardine oil fit, arachidonic acid had to be included as an essential fatty acid.

With the above experience as background, experiment IV was devised to test the hypothesis that this product did indeed correlate inversely with the serum cholesterol values obtained. Twenty-four oils or mixtures of oils were used.

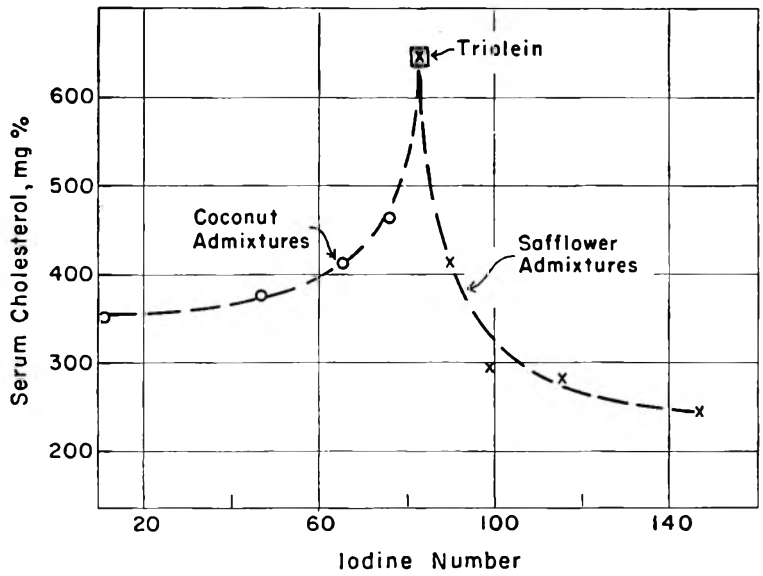

Fig. 1 Mean serum cholesterols of groups of animals which received "triolein" and various proportion of "triolein" mixed with either safflower oil or coconut oil plotted against the iodine numbers of the mixtures.

These were specifically chosen to give highly different contents of essential fatty acid (linoleic acid), saturated fatty acids of differing chain length including additions of palmitic and pelargonic acid, and of unsaturated non-essential fatty acid (oleic, linolenic, and eleostearic acid). These oils and combinations, their composition, the average cholesterol values obtained and the product of the essential fatty acid content times the saturated acid content are shown in table 2. The correlation between the product and the serum cholesterol values is shown in figure 6.

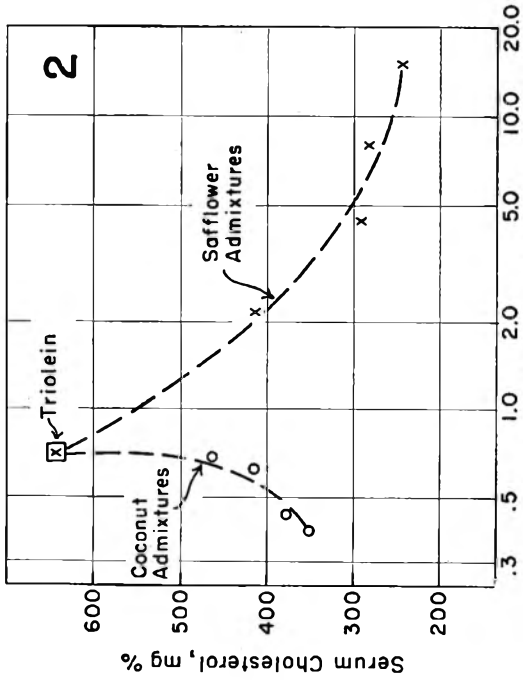

Fig. 2 The same data as in figure 1 (table 1). Linoleic acid contents of the diets plotted against the serum cholesterol values.

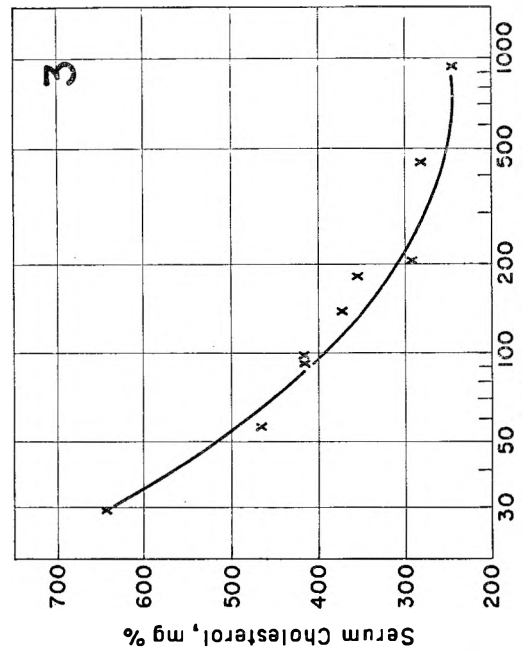

Fig. 3 A plot of the data obtained with mixtures of "triolein" with coconut and safflower oils showing the correlation of the product (% linoleic acid times % saturated acids) with the serum cholesterol values.

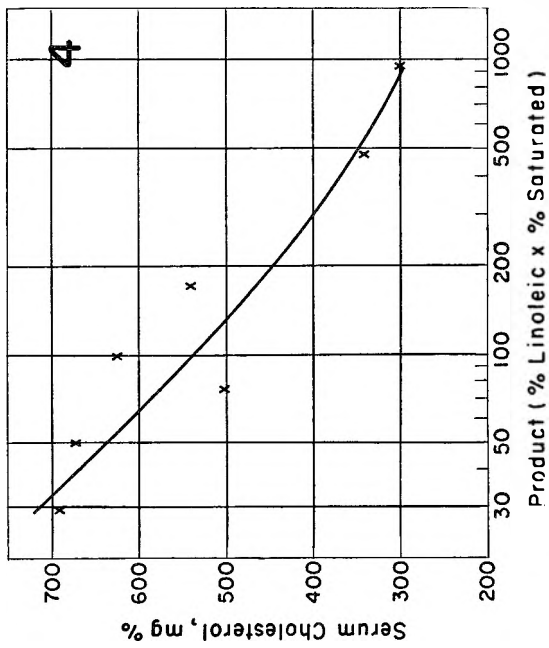

Fig. 4 A plot of the data obtained with mixtures of "triolein" with lard and safflower oils showing the correlation of the product (% linoleic acid times % saturated acids) with the serum cholesterol values.

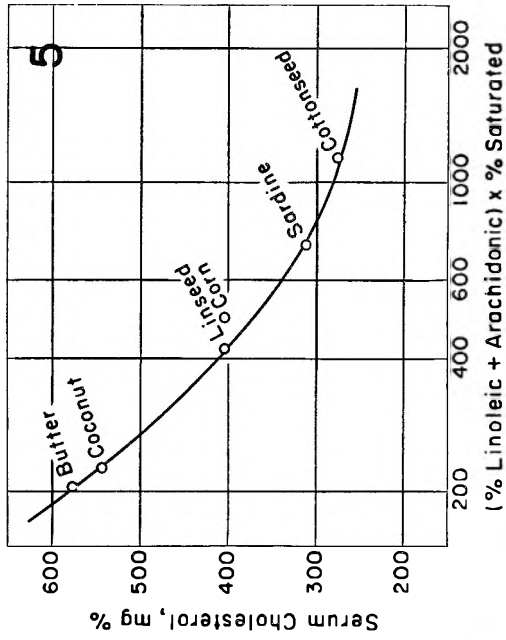

Fig. 5 Plot of data obtained previously (Hegsted et al. '57) showing the excellent correlation of the product with the serum cholesterol values. Linolenic acid not included as "essential" and arachidonic included to allow fit of data on linseed oil and sardine oil. (See text, p. 382.)

The insert in figure 6 shows the same data upon a log-log plot. It is readily apparent that the data conform to a straight line and that the fit is exceedingly good. The correlation coefficient is high, 0.94. The correlation coefficient squared, 0.885, gives the fraction of the variance that is accounted for by regression. Thus, only 11.5% of the variance

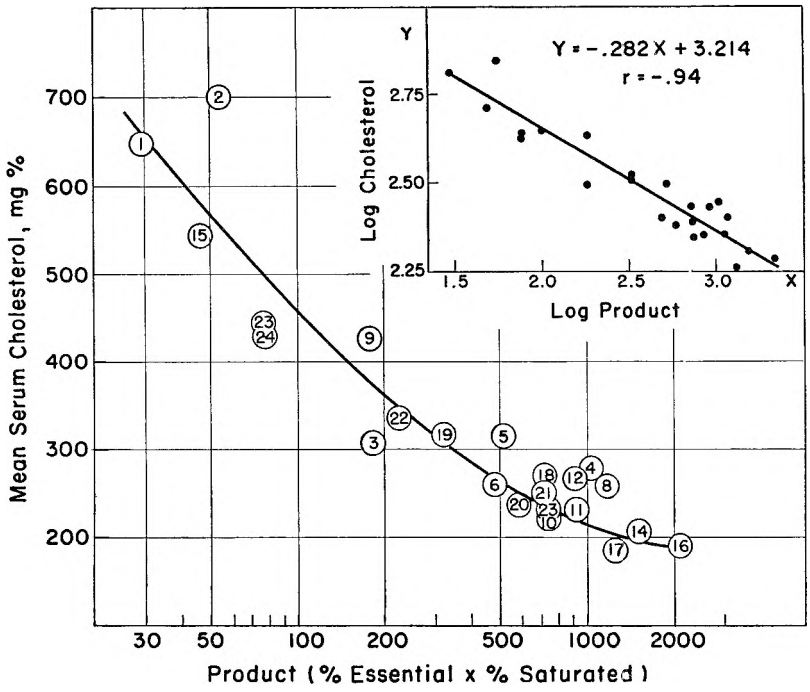

Fig. 6 Summary of data included in experiment V (table 2) showing the correlation between the calculated product and the mean serum cholesterol values. Insert shows same data upon a log log plot where it is linear.

is not explained. Since the standard errors of the mean cholesterol values were found to be equal to about $\pm 5\%$ of the mean, this is about as high a correlation as can be expected.

It should be noted that the analytical data employed for some of the oils were determined upon the samples used. For other oils average published figures had to be used. Whether the latter circumstance may account for any of the deviations

TABLE 2

Experiment IV. The effect of various oils and oil mixtures upon serum cholestrol

GROUP	FAT TESTED	I_2 NO.	(A) SATURATED ACIDS	(B) ESSENTIAL ACIDS	UNSATURATED NON-ESSENTIAL ACIDS	AVERAGE CHOLESTEROL	PRODUCT A × B
			%	%	%	mg %	
1	10% Triolein [1]	83	8.2	3.6	88.2	649	29.5
2	10% Olive [1]	82	11.2	4.9	83.9	701	54.9
3	10% Rapeseed [1]	110	10.3	18.3	71.0	309	188
4	10% Cottonseed	110	23.5	43.5	33.0	277	1022
5	10% Linseed	180	8.5	61.5	30.0	316	522
6	10% Corn	123	11.7	42.0	46.3	260	491
7	10% Tung	165	4.5	....	94.5	632	....
8	10% Safflower [1]	146	11.7	81.1	6.1	258	949
9	10% Coconut [1]	10	90.3	2.0	7.6	427	180
10	8% Coconut + 2% corn	32.6	74.5	10.0	15.3	221	745
11	5% Coconut + 5% corn	66	51.0	22.0	26.95	231	1122
12	2% Coconut + 8% corn	100	27.4	34	38.5	268	931
13	1% Coconut + 9% corn	112	19.5	38	42.4	226	743
14	5% Coconut + 5% Linseed	95	49.4	31.7	18.8	205	1565
15	5% Coconut + 5% tung	87.5	47.4	1.0	51.0	523	47.4
16	5% Coconut + 5% safflower	78	51.0	41.6	6.9	189	2120
17	5% Coconut + 5% cottonseed	60	56.9	22.7	20.3	184	1290
18	5% Safflower + 5% corn	134.5	11.7	61.6	26.2	269	721
19	5% Safflower + 5% tung [2]	155.5	8.1	40.6	50.3	318	329
20	2% Safflower + 8% corn	127.6	11.7	49.8	38.3	236	583
21	5% Safflower + 5% linseed	163	10.1	71.3	18.1	243	720
22	5% Safflower + 5% tung [2]	155.5	8.1	40.6	50.3	333	329
23	8% Triolein + 2% palmitic acid	66.4	26.6	2.9	71.4	444	77
24	8% Triolein + 2% pelargonic acid	66.4	26.6	2.9	71.4	436	77

[1] Composition of the oils used obtained by analysis. Data on other oils from table published by E. F. Drew and Co., Technical Products Division, New York.

[2] Inadvertent duplication of groups.

from the regression line is problematical. Also, it may be noted that the product of zero obtained for tung oil cannot be plotted on semi-logarithmic paper.

The results lead us to the conclusion that in some manner the essential fatty acids and the saturated acids act synergistically to prevent hypercholesteremia under these experimental conditions. Conversely, the unsaturated non-essential fatty acids like oleic acid promote high serum cholesterols; this action being counteracted by the essential and saturated acids. The mechanism of such action remains unknown. Speculation is possible, but probably not justified at the present time. The effects of different fats on cholesterol absorption (Lin et al., '55) may be partially responsible.

If the equation were strictly true, saturated and essential acids could be substituted for each other with equal effectiveness. Whether this is entirely true can only be proven by the use of oils higher in essential fatty acid and lower in saturated acids than any that we have available. Safflower oil with 81.1% linoleic acid and 11.6% saturated acid, giving a product of 941 is as far in this direction as we have been able to go. In any event, it may be noted that the product would be highest with an equal mixture of linoleic acid and saturated acids, devoid of oleic acid or other non-essential unsaturated acids. The nearest we have approached this is with an equal mixture of coconut and safflower oil (product 2120). This mixture and an equal mixture of coconut and cottonseed oil (product 1290) gave the lowest serum cholesterol values in the series, 189 and 184, respectively. It should be appreciated that the values for the products are plotted on semi-logarithmic paper in figure 6 and that actually a straight-line relationship is obtained upon log-log paper (see insert fig. 6). Thus, a change in the product from 1000 to 2000 would be expected to give a smaller change in the serum cholesterol values than a change in the product from 50 to 100 and the same difference as a change from 100 to 200. Considering the variability encountered in serum cholesterol values, even in rats under standardized con-

ditions, it is doubtful whether further definition of these small differences by the use of large groups of animals would be justified. We are more impressed by the general applicability of the theory over a wide range of oil compositions.

It may be noted that the addition of palmitic acid and pelargonic acid (a saturated 9-carbon acid) to "triolein" produced a significant lowering of the serum cholesterol and that these were equally effective. The applicability of the theory to these mixtures and the various fats, including coconut and lard, does not suggest any influence of chain length upon the action of the saturated fatty acids. Admittedly, this might be subjected to more rigorous proof.

It may also be noted that again in order to make linseed oil and various combinations of linseed with other oils "fit" the curve, the linolenic acid content cannot be considered as part of the essential fatty acids. Fish oils were not included in experiment IV because of the analytical difficulties in determining the composition. Thus, the sardine oil in experiment I is the only one tested in which arachidonic acid and higher polyunsaturated acids were major constituents. If the literature values for sardine oil composition are approximately correct, the data suggest that arachidonic acid is active, i. e., "essential", but that clupanodonic is not. The general "fit" of mixtures of products containing either oleic acid or eleostearic acid as the "non-essential unsaturated acids" as well as the results with linseed oil support the hypothesis that these may be generally grouped together.

Numerous other manipulations of the data were tried such as: the difference between the essential and non-essential fatty acid contents, the ratio of these, the sum of linoleic and saturated fatty acids, and weighting of various fractions in calculating the product. No other treatment of the data that we have tried gave as good a relation to the effect observed upon the serum cholesterol as the product described. A three dimensional plot of the data using saturated acids, essential fatty acids, and non-essential fatty acids as the three coordinates showed, as would be expected from the data presented,

that the lowest serum cholesterol values were in the region of equal mixtures of saturated and linoleic acid. This plot is not presented in the interests of conserving space.

SUMMARY

A large number of oils and combinations of oils were tested for their effects upon the serum cholesterol values in an assay based upon the cholesterol-cholic acid-fed rat. It appears that the oils may act upon this experimental system in a manner somewhat similar to those reported on the serum cholesterol values of human subjects.

The data show that the product obtained by multiplying the essential fatty acid content (linoleic and arachidonic acid) by the total saturated fatty acid content has a high degree of negative correlation with the serum cholesterol values produced. Thus, it is concluded that the "non-essential" unsaturated fatty acids (oleic, linolenic, eleostearic (and perhaps clupadonic) promote hypercholesteremia while this action is counteracted by the essential and saturated fatty acids. Insofar as we have been able to test the hypothesis, the essential fatty acids and the saturated fatty acids are equally active in this regard and substitute for each other.

ACKNOWLEDGMENT

We appreciate very much the assistance of the Research and Development Department of the Proctor & Gamble Company, Cincinnati, who supplied a number of the oils used and the analytical data upon various samples; and of Swift & Company, Chicago; Archer-Daniels-Midland Company, Minneapolis; and Emery Industries, Cincinnati, who also provided various samples.

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VITAMIN E DEFICIENCY IN THE MONKEY

II. TISSUE CONCENTRATIONS OF NUCLEIC ACIDS AND CREATINE [1]

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Certain of the signs of vitamin E deficiency in the monkey have been described (Dinning and Day, '57). These include muscular dystrophy, anemia, leucocytosis, an elevated excretion of creatine and allantoin and a reduced excretion of creatinine. Vitamin E-deficient rabbits exhibit derangements in the concentrations of tissue nucleic acids (Young and Dinning, '51) and muscle creatine (Goettsch and Brown, '32). It will be shown that in vitamin E-deficient monkeys similar aberrations in these tissue components were observed.

METHODS

The diet and general handling of the monkeys was the same as that previously described (Dinning and Day, '57). Eight monkeys were used in the present experiments. Four were vitamin E-deficient, having been fed the deficient diet for approximately 10 months. When they were selected for tissue analysis these animals exhibited the expected signs of vitamin E deficiency. The average peripheral erythrocyte count of the deficient monkeys was 1.8 million cells per microliter of blood.

Two other monkeys were fed the deficient diet until deficiency signs were apparent. At this time their average periph-

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[2] Studies carried out during the tenure of a Lederle Medical Faculty Award.

eral erythrocyte count was 2.0 million cells per microliter of blood. They were then each injected intraperitoneally with two 20 mg doses of α-tocopherol phosphate and the diet was thereafter supplemented with 20 mg of α-tocopherol acetate daily. The two animals were taken for tissue analysis two months and 3.5 months respectively after supplementation with vitamin E. At this time their average peripheral erythrocyte count was 5.9 million cells per microliter of blood and the animals appeared normal. These animals will be referred to as "recovered."

Two other monkeys were fed the basal diet supplemented with 20 mg of α-tocopherol acetate daily throughout the experiment. These animals will be referred to as "normal."

All 8 of the monkeys were approximately the same size, weighing between 2 and 3 kg each.

The animals were anesthetized [3] and the jugular vein was opened. Tissues were immediately frozen in a dry ice-acetone mixture and stored at −20° C. until taken for analysis.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined by the procedure of Schneider ('45). Muscle creatine from the muscles of the hind legs and heart creatine were determined by the procedure of Rose, Helmer, and Chanutin ('27).

RESULTS AND DISCUSSION

The tissue nucleic acid concentrations are given in table 1. The only tissues significantly affected by vitamin E deficiency were skeletal muscle and bone marrow. The DNA content of skeletal muscle from the deficient animals was elevated over that observed in normal or recovered monkeys. This is in agreement with observations made on rabbits (Young and Dinning, '51). Bone marrow RNA and DNA were greatly increased in vitamin E-deficient monkeys. Also, the ratio RNA/DNA was elevated in deficient animals. It is of interest that these same changes have been observed in bone marrow from pernicious anemia patients (Glazer et al., '54).

[3] Nembutal was used.

TABLE 1

Effect of vitamin E deficiency on the concentrations of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in monkey tissues. (The results are expressed as milligrams of nucleic acid per gram of wet tissue).

TISSUE	ANIMALS	RNA		DNA		RNA/DNA
		Mean	Range	Mean	Range	
Liver	Normal [1]	10.52	7.53–13.50	1.33	1.06–1.60	7.91
	Vit. E-deficient	7.72	5.70–11.50	1.85	0.91–2.40	4.17
	Recovered	8.43	6.45–10.40	1.46	1.34–1.58	5.71
Spleen	Normal	6.51	6.27– 6.75	6.37	5.70–7.04	1.02
	Vit. E-deficient	6.09	5.41– 6.92	5.10	4.50–5.80	1.19
	Recovered	6.27	4.83– 7.50	6.46	4.60–8.32	0.97
Kidney	Normal	4.88	4.20– 5.55	2.83	2.68–2.97	1.72
	Vit. E-deficient	4.25	3.10– 5.56	2.55	2.32–2.82	1.67
	Recovered	3.36	3.12– 3.60	2.69	2.32–3.06	1.25
Heart	Normal	4.59	4.55– 4.63	1.37	1.22–1.52	3.35
	Vit. E-deficient	4.47	4.24– 4.70	1.47	1.23–1.64	3.04
	Recovered	4.13	3.30– 4.96	1.25	1.23–1.26	3.30
Small intestine	Normal	4.81	4.32– 5.30	4.16	3.65–4.66	1.17
	Vit. E-deficient	4.34	3.86– 4.85	3.52	3.05–3.83	1.23
	Recovered	4.64	4.43– 4.85	3.20	3.13–3.26	1.45
Skeletal muscle	Normal	4.68	3.75– 5.60	0.64	0.62–0.66	7.31
	Vit. E-deficient	3.89	3.00– 4.65	1.46	0.84–2.2[illegible]	2.66
	Recovered	3.31	2.16– 4.45	0.78	0.59–0.97	4.24
Bone marrow	Normal	0.61	0.54– 0.67	1.54	1.09–1.98	0.40
	Vit. E-deficient	3.99	3.13– 5.17	5.53	4.40–7.75	0.72
	Recovered	0.62	0.59– 0.64	2.07	1.73–2.41	0.30

[1] "Normal" animals were fed the basal diet supplemented with 20 mg of α-tocopherol acetate daily.

"Vitamin E-deficient animals were fed the deficient diet for about 10 months.

"Recovered" animals were those fed the deficient diet until deficiency signs were evident. They were then given α-tocopherol acetate by intraperitoneal injection and also in the diet. The animals were taken for tissue analysis after two to 3.5 months of supplementation with vitamin E.

The data in table 2 indicate the effect of vitamin E deficiency on the total marrow DNA. For these experiments all the marrow from one femur from each monkey was weighed and its total DNA content determined. Since the DNA content should reflect the number of cells accurately it appears that the total number of marrow cells must be elevated approximately four-

TABLE 2

Effect of vitamin E deficiency on monkey bone marrow Desoxyribonucleic acid (DNA)

ANIMALS	MARROW WEIGHT	MARROW DNA
	gm/femur	*mg/femur*
Normal	2.04	2.9
Vitamin E-deficient	2.18	12.0
Recovered	1.21	2.5

TABLE 3

Effect of vitamin E deficiency on the concentration of muscle creatine in monkeys

ANIMALS	CARDIAC MUSCLE		SKELETAL MUSCLE	
	Mean	Range	Mean	Range
	mg creatine/100 gm		*mg creatine/100 gm*	
Normal	239	186–292	457	440–474
Vitamin E-deficient	222	190–262	243	209–273
Recovered	191	172–210	355	340–370

fold in the deficient monkeys. These data show that the anemia of vitamin E deficiency in the monkey is associated with a hyperplastic marrow.

Muscle creatine data are given in table 3. Vitamin E deficiency did not affect the concentration of heart creatine. The creatine concentration of skeletal muscle was considerably reduced in vitamin E deficiency. Of interest is the observation that the creatine content of skeletal muscle from the recovered animals was not completely restored to normal.

These results show that, based on nucleic acid concentrations, the tissues most drastically affected by vitamin E deficiency in the monkey are skeletal muscle and bone marrow.

This is in agreement with the gross signs of deficiency, the most obvious of which are muscular dystrophy and anemia.

SUMMARY

The concentrations of tissue nucleic acids and muscle creatine were determined on vitamin E-deficient, normal, and recovered monkeys. Vitamin E deficiency resulted in an elevated concentration of skeletal muscle desoxyribonucleic acid (DNA) and an elevated concentration of bone marrow DNA and ribonucleic acid (RNA). The bone marrow RNA/DNA ratio was elevated in vitamin E-deficient monkeys. All these changes were reversed with tocopherol therapy. Skeletal muscle creatine was reduced in vitamin E-deficient monkeys and only partially restored toward normal in recovered animals.

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NECROGENIC POTENCY OF YEASTS GROWN ON VARIOUS MEDIA [1]

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The use of yeasts in diets that induce massive hepatic necrosis in rats has been reviewed by Schwarz ('55) and Goyco ('56). Vitamin E and the S-containing amino acids protect against this type of disorder, and the degree of necrosis also seems to depend upon the kind of yeast used: British bakers' yeast vs. American brewers' yeast, (György et al., '50); torula vs. brewers' yeast (Schwarz, '51a, b). These latter differences did not appear to be associated with differences in the vitamin E content of the various yeasts (low in all cases). Lindan and Work ('51) found that a necrogenic yeast did not differ substantially in methionine and cystine content from a non-necrogenic one. In 1951, Schwarz proposed the presence of an unidentified protective "factor 3" in the non-necrogenic yeasts. Goyco ('56) has attempted to correlate necrogenic potency of various yeasts with their biological value, as measured by the rate of regeneration of liver. The present paper describes a rapid method for inducing massive hepatic necrosis in rats and demonstrates that the necrogenic potency of both *T. utilis* and *S. cerevisiae* can vary with the medium on which the yeast is grown.

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METHODS

The procedure used in all experiments was as follows: weanling male rats of the Holtzman strain were housed in individual wire cages and fed and watered ad libitum. During the initial week the animals received a depletion diet consisting of the following in grams per kilogram: casein 200; dextrin 630; salts [2] 40; lard 90; cod-liver oil 10; and cellulose 30. The vitamins in milligrams per kilogram were: choline 2000; inositol 1000; calcium pantothenate 20; niacin 10; menadione 4; riboflavin 3; thiamine 2; pyridoxine 2.5; biotin 0.1; folic acid 0.2; and vitamin B_{12} 0.001.

The animals were then distributed according to weight into groups and fed the experimental diets ad libitum. In these diets the yeast being tested replaced casein and an appropriate amount of dextrin of the depletion diet (see footnotes in tables of results for actual composition). All diets contained 18% of crude protein (N $\times$ 6.25) except in those experiments in which the effect of protein level was under study. The experiments were usually ended 35 days after the animals were placed on the yeast diets.

The livers of animals that had recently died were examined for gross pathological changes, and histological studies were carried out to establish whether necrosis had occurred. Weights were recorded only for the initial two weeks on the experimental diets since death usually occurred during the third week.

Erythrocyte hemolysis was determined by the method of György and Rose ('52), alloxan-cysteine in a 1:1 molar ratio being used instead of dialuric acid.

EXPERIMENTAL

Hepatic necrosis

At the beginning of the depletion period the rats weighed 45 to 55 gm and their erythrocytes showed virtually no hemolysis (0 to 2.5%). After one week the weights had increased

[2] Wesson.

to 75 to 85 gm and hemolysis ranged from 75 to 100%. According to this latter criterion, therefore, the rats were substantially depleted of vitamin E before being placed on the experimental diets.

TABLE 1

Survival of rats fed torula-SSL and brewers' by-product yeast as the sole source of protein in diets deficient in vitamin E

EXP. NO.[1]	PROTEIN SOURCE [2]	SURVIVAL	MEDIAN SURVIVAL TIME
			days
I	Torula-SSL	0/5	22
II	Torula-SSL	0/8	21
III	Torula-SSL	0/8	22
IV	Torula-SSL	0/8	16
V	Torula-SSL	0/8	18
VI	Torula-SSL	0/8	18
VII	Torula-SSL	0/8	39
VIII	Torula-SSL	0/8	18
IX	Torula-SSL	0/8	18
X	Torula-SSL	0/8	43
XI	Torula-SSL	0/8	21
XII	Torula-SSL	0/8	23
	All torula-SSL	0/93	24 ± 9
	All brewers' yeast	54/54	–

[1] These groups constitute the controls for all other data presented.

[2] All diets contained 18% of crude protein (N x 6.25). This required 400 gm of torula yeast and 351 gm of brewers' yeast per kilogram. In addition, the diets contained in grams per kilogram: Wesson salts 40, lard 90, cod liver oil 10, cellulose 30, complete vitamins as in the depletion diet (see text) and dextrin to 1000 gm.

Vitamin E-free diets containing torula-SSL yeast [3] as the sole source of 18% of dietary protein caused the death of all rats in 12 separate series; the average of the median survival times of the depleted rats was 24 ± 9 days (table 1). The survival times were longer in two series (VII and X) in which a different lard was used which presumably contained residual amounts of vitamin E.

[3] Torula-SSL yeast (*Torulopsis utilis*) was feed grade yeast grown aerobically on spent sulfite liquor. It contained 45% of crude protein (N × 6.25). Obtained through the courtesy of Dr. P. L. Pavcek, Lake States Yeast Corp., Inc., Rhinelander, Wis.

The average weekly gains on the torula-SSL diet were 10 to 18 gm for the first week and 28 to 32 gm for the second. The rats appeared thrifty and gained weight until just prior to the onset of a comatose condition which in every case observed led to death within 4 to 6 hours. The symptoms seen during this final period were similar to those described by Himsworth ('47) and György et al. ('50). Gross examination of the livers showed marked areas of hemorrhage with no apparent pattern of distribution. Histological examination of the livers of a few of the animals taken shortly after death revealed necrotic changes similar to those described by Fite ('54), mainly a diffuse karyolysis and karyorrhexis of liver cell nuclei with relatively small changes in the cytoplasm.

When brewers' by-product yeast [4] was tested under the same conditions as the torula-SSL yeast, no deaths were observed in 7 trials even though the experimental period was in one case prolonged to 70 days (table 1). The rats gained weight and appeared normal; examination of the livers of the rats at the end of the experimental period showed no signs of any pathological changes.

Level of torula

Schwarz ('51b) has reported that the incidence of hepatic necrosis does not depend on the level of torula yeast fed. Abell et al. ('49) on the other hand, observed a decreased incidence of necrosis as the level of a necrogenic Saccharomyces yeast was increased. In the present study the level of torula-SSL yeast in the diet ranged from 31 to 67%, corresponding to 14 and 30% of dietary protein, respectively. At levels of yeast corresponding to 18% of total protein or less, none of the rats survived (table 2). Intermediate levels of yeast produced intermediate and variable degrees of protection. Survival was 75% at the highest level of torula-SSL fed. Improved survival in the presence of increasing amounts of

[4] Brewers' by-product yeast (*Saccharomyces cerevisiae*) was obtained from the Jos. Schlitz Brewing Co., and from the Pabst Laboratories, Milwaukee, Wisconsin. It contained an average of 50% of crude protein (N × 6.25).

torula-SSL in the diet would argue against the presence of a toxic principle in this yeast. This degree of protection was also observed (table 2, exp. XI) when the 18% torula protein diet (40% torula-SSL yeast) was supplemented with 0.4% of DL-methionine, an amount calculated to bring the methionine level of the diet to that in the high protein diet.

Protective factors

In agreement with others (Hock and Fink, '43), Goyco and Asenjo '54, Schwarz '51a, Dam and Granados '51) death due to hepatic necrosis was prevented by the feeding of cystine, methionine, α-tocopherol or methylene blue (table 3). Similar protective action resulted when 0.01 M diphenyl-*p*-phenylenediamine (DPPD) was added to the diet. However, no protection resulted from butylated hydroxytoluene either added to the medium in which torula yeast was grown [5], or included in the diet at a level of 0.01 M. Lipoic acid (0.015%) was also without effect against the onset of hepatic necrosis.

Effect of medium

The observed difference in necrogenic potency between torula and brewers' by-product yeast might have been due either to innate differences between the two species of yeast or to differences in the way these yeasts are ordinarily grown. The latter possibility was tested by growing pure strains of *Torulopsis utilis* (Wisconsin strain 3) and *Saccharomyces cerevesia* (S-101-Brewers') under identical conditions of aeration and in media of controlled composition [6]. The characteristics of the yeasts as well as the results of the necrosis-indu-

[5] Butylated hydroxytoluene, added to the spent sulfite liquor in which torula yeast was grown, at the rate of 22.5 gm of Shell "Ionol" per 500 pounds of torula yeast in the cream stage. Obtained from Dr. P. L. Pavcek, Lake States Yeast Corp., Rhinelander, Wis.

[6] We are particularly indebted to Drs. A. J. Wiley and L. M. Whitmore, Jr., of the Sulphite Researchers Manufacturing League, Appleton, Wis., and to Dr. H. J. Peppler of the Red Star Yeast Corp., Milwaukee, Wis. for these yeasts.

TABLE 2

Effect of the level of torula-SSL yeast on survival of rats

EXP. NO.	LEVEL OF TORULA YEAST IN THE DIET [1]	SURVIVAL	MEDIAN SURVIVAL TIME	PROTECTION
	gm/kg		*days*	*%*
III	310	0/7	17	0
IV	310	0/8	19	0
A11	400	0/93	24 ± 9	0
III	490	4/8	18	50
IV	490	0/8	19	0
V	490	3/8	26	37.5
II	580	3/8	24	37.5
IV	580	0/8	23	0
V	580	3/8	26	37.5
V	670	6/8	23	75
XI	670	6/8	22	75
XI	400 + 0.4% DL-Methionine	6/8	22	75

[1] The increasing levels of yeast were added at the expense of the dextrin. In addition the diets contained in grams per kilogram: Wesson salts 40, lard 90, cod liver oil 10, cellulose 30 and vitamins as in the depletion diet (see text).

TABLE 3

Factors protective against liver necrosis

EXP. NO.	ADDITIONS TO TORULA-SSL BASAL [1]	SURVIVAL	PROTECTION
			%
A11	None	0/93	0
IX	+ 0.4% DL-methionine	6/8	75
I	+ 0.5% DL-methionine	10/13	77
VI	+ 1.0% DL-methionine	8/8	100
VI	+ 0.5% cystine	8/8	100
I	+ 3 mg % α-tocopherol	8/8	100
VI	+ 2 mg % α-tocopherol	8/8	100
VI	+ 0.015% lipoic acid	0/8	0
X	+ 0.01 M DPPD [2]	7/8	87.5
XII-XIV	+ 0.01 M DPPD	16/16	100
X	+ 0.01 M methylene blue	3/8	37.5
XII	+ 0.01 M methylene blue	6/16	100
X	+ BHT [3] in the medium	0/8	0
XII	+ BHT in the medium	0/16	0
XII	+ 0.01 M BHT [3]	0/6	0

[1] As in footnote 2, table 1.

[2] Diphenyl-*p*-phenylenediamine.

[3] Butylated hydroxytoluene. (See footnote 3 in text for details of addition to the medium.)

TABLE 4

Effect of growth medium on anti-necrogenic properties of T. utilis and S. cerevisiae

EXP. NO.	BATCH [1]	GROWTH MEDIUM	AEREATION	CRUDE PROTEIN (N x 6.25)	LEVEL IN DIET [2]	SURVIVAL	MEDIAN SURVIVAL TIME	PROTECTION
				%	*gm*		*days*	%
A. Torulopsis utilis								
VIII	—	Spent sulfite	+	45.0	400	0/8	19	0
IX	—	Spent sulfite	+	45.0	400	0/8	18	0
XII	—	Spent sulfite	+	45.0	400	0/8	23	0
VIII	A	Synthetic [3]	+	44.7	405	0/8	19	0
IX	A	Synthetic [3]	+	44.7	405	0/8	20	0
VIII	A	Synthetic [3]	—	35.7	504	0/8	18	0
IX	A	Synthetic [3]	—	35.7	504	0/8	20	0
VIII	A	Beer wort [4]	—	39.5	456	9/9	—	100
IX	A	Beer wort [4]	—	39.5	456	8/8	—	100
XII	B	Beer wort [4]	+	45.2	400	8/8	—	100
B. Saccharomyces cerevisiae								
VIII	—	Beer wort	—	49.6	363	8/8	—	100
IX	—	Beer wort	—	49.6	363	8/8	—	100
XII	—	Beer wort	—	48.0	375	8/8	—	100
VIII	A	Synthetic [3]	+	52.0	346	3/8	27	37.5
IX	A	Synthetic [3]	+	52.0	346	0/8	23	0
XII	B	Synthetic [3]	+	31.8	562	5/8	28	62.5
VIII	A	Synthetic [3]	—	52.2	345	3/8	26	37.5
IX	A	Synthetic [3]	—	52.2	345	0/8	25	0

[1] Samples marked A were grown by Drs. L. M. Whitmore and A. J. Wiley of the Sulphite Researchers' Manufacturing League, Appleton, Wisconsin, under the following conditions:

Strain of Yeast Medium	*S. cerevisiae* Synthetic	*S. cerevisiae* Synthetic	*T. utilis* Synthetic	*T. utilis* Synthetic	*T. utilis* Beer wort
Air rate, CFM	30 → 18	0	30 → 23	0	0
Temperature–°C	30	6 – 16	30	5 – 9	7 – 9
pH	4.4 – 4.8	4.0 – 4.2	3.9 – 5.5	3.4 – 3.8	4.0 – 4.2
Length of fermentation hrs.	44 – 48	70 – 72	14 – 20	91 – 93	42 – 44
Vol. % moist yeast	0 – 3.5	3.4 – 3.8	0 – 11	6.2 – 8.0	5.0
Operating volume, gal.	210 – 273	115 – 118	135 – 220	170 – 192	89 – 96
Wt. dry yeast	9 lb. 15 oz.	9 lb. 10 oz.	10 lb. 5 oz.	10 lb. 10 oz.	8 lb. 0 oz.

The synthetic medium used was that described by Maxon and Johnson ('53). Part of the *T. utilis* grown aerobically in the synthetic medium was stored overnight in a refrigerator, then after addition of beer wort (obtained from a commercial brewery) was held anaerobically for two days before harvesting.

Samples marked B were grown in the Red Star Yeast Laboratories, Milwaukee, Wisconsin, under the direction of Dr. H. J. Peppler. The *S. cerevisiae* yeast was grown on a synthetic medium (Maxon and Johnson, '53), in six 10" diameter, agitated type fermenters. They were inoculated with 122 gm dry weight of yeast, the average yield being 1000 gm dry weight per fermenter.

For the *T. utilis* 210 lbs. of beer wort of 17.5° Brix and pH 5.3 (obtained from a local brewery before the hops had been added) was fed to a 30" fermenter along with the following: 2.1 lbs. NH_4OH (29% NH_4OH), 3.2 lbs. NH_4HSO_4, and 0.59 lbs. $NH_4H_2PO_4$. The yield was 5.77 lbs. of dry yeast, from 2.68 lbs. of pitching yeast. The beer wort contained 9.2% invert sugar.

[2] In addition: Wesson salts 40, lard 90, cod liver oil 10, cellulose 30, vitamins as in the "vitamin E depletion diet" (see text) and dextrin to make 1000 gm. All diets were made to contain 18% of crude protein (N x 6.25).

cing experiments are shown in table 4. When incorporated into vitamin E-low diets torula yeast grown on a synthetic medium (Maxon and Johnson, '53) under either anaerobic or aerobic conditions proved to be as necrogenic as torula grown on spent sulfite liquor. The median survival times of the rats in a series of trials ranged from 18 to 23 days (table 4, lines 1-7). On the other hand, survival was 100% when the rats were fed either of two batches of torula grown on beer wort (lines 8-10).

The importance of medium on the nutritional qualities of yeast was also apparent in the response of low-vitamin E rats to Saccharomyces grown in various ways. Survival was 100% when the yeast was grown on beer wort (commercial brewers' yeast) whereas many of the rats failed to survive on Saccharomyces grown on a synthetic medium (table 4, lines 14-18), the percentage of survival in these latter groups ranging from 0 to 62.5.

DISCUSSION

These data support the suggestion of Schwarz that a protective factor is present in some yeasts and not in others. The protective principle or an essential precursor apparently comes from the medium, beer wort being a good source. According to this view the protective principle must be deficient in a simple synthetic medium, in spent sulfite liquor, and apparently also in the molasses used for the production of necrogenic samples of Puerto Rican yeasts. This effect of medium rather than of strain or species of yeast might also have contributed to the differences in necrogenic potency between British and American yeasts recorded by György.

Innate differences between strains and species of yeast cannot be ruled out completely, however, since in the present experiment survival was better on *S. cerevisiae* grown on synthetic medium (11/40) than on *T. utilis* grown on this medium (0/32). On the other hand, survival was 100% when either of the two yeasts was grown on beer wort (table 4).

The multiple nature of factors previously reported to minimize necrosis is illustrated by the following list: vitamin E,

cystine, methionine, methylene blue, aureomycin, "factor 3" in brewers' yeast and elsewhere. The present experiments show that the necrogenic or protective potency of yeast depends on the medium, that a species (*S. cerevisiae*) previously regarded as protective, becomes necrogenic when grown on synthetic medium, and that a species (*T. utilis*) previously regarded as necrogenic becomes protective when grown on beer wort.

That autooxidation may be involved in the necrogenic syndrome is suggested by the number of antioxidants on the list of protective factors, to which DPPD may now be added (table 3). It is conceivable that the natural protective principle also belongs to this class of substances and that the synthetic antioxidants merely protect it or vitamin E or both. A measure of specificity among antioxidants, however, is suggested by the failure of butylated hydroxytoluene (BHT) to protect against necrosis (table 3) even though this antioxidant has been reported to diminish the incidence of encephalomalacia in low-vitamin E chicks (Bunnell et al., '55).

Since these experiments were completed Schwarz and Foltz ('57) have reported that selenium prevents necrosis in rats fed torula yeast.

SUMMARY

1. Death due to hepatic necrosis occurred rapidly and in 100% incidence in vitamin E-depleted rats fed *Torulopsis utilis* grown on spent sulfite liquor as the sole source of protein. Comparable diets containing brewers' by-products yeast did not produce necrosis. Survival was increased by increasing the level of the torula-SSL yeast in the diet.

2. *Torulopsis utilis* and *Saccharomyces cerevisiae* grown on a synthetic medium were both found to be necrogenic, while these same yeasts grown on commercial beer wort were both found to be non-necrogenic.

3. Liver necrosis was prevented by the addition of methylene blue or diphenyl-*p*-phenylenediamine (DPPD) to the diet; butylated hydroxytoluene was ineffective.

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THE ADDITION OF SMALL AMOUNTS OF DEFATTED FISH FLOUR TO WHOLE YELLOW CORN, WHOLE WHEAT, WHOLE AND MILLED RYE, GRAIN SORGHUM AND MILLET

I. INFLUENCE ON GROWTH AND PROTEIN EFFICIENCY
II. NUTRITIVE VALUE OF THE MINERALS IN FISH FLOUR[1]

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In a recent communication (Sure, '57) it was reported that the addition of small amounts of defatted fish flour [2] to the proteins in milled wheat, corn and rice results in enormous gains in body weight and protein efficiency. In this study results are submitted on the effect of additions of small amounts of defatted fish flour to whole yellow corn, whole wheat, whole and milled rye, grain sorghum and millet on growth and protein efficiency. In addition, results are given on the nutritive value of the minerals in fish flour. The fish flour used was from a mixture of carp, smelts and whitings processed by the method of Levin ('52), later modified by Levin and Finn ('55), the composition of which is given in the recent paper by the author (Sure, '57).

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[2] Supplied by The VioBin Corporation, Monticello, Illinois.

TABLE 1

The addition of small amounts of defatted fish flour to whole yellow corn, whole wheat, whole rye, milled rye flour, grain sorghum and millet. Influence on growth and protein efficiency.

(Experimental period was 10 weeks, with 12 rats in each group. Results are means.)

TYPE OF RATION	AMOUNT IN RATION	PROTEIN	GAINS IN BODY WEIGHT		TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO [1]	
			Actual	Increase			Actual	Increase
	%	%	*gm*	%	*gm*	*gm*		%
Adding defatted fish flour to whole yellow corn								
Whole yellow corn	76.2	7.0	21.5 ± 4.2 [2]	–	500.0	35.0	0.61 ± 0.03	–
Whole yellow corn Fish flour	75.2 1.0	7.69	71.1 ± 6.4	230.7	691.6	53.2	1.34 ± 0.05	119.7
Whole yellow corn Fish flour	73.2 3.0	9.07	87.0 ± 6.9	304.7	680.0	61.7	1.41 ± 0.06	131.1
Whole yellow corn Fish flour	71.2 5.0	10.04	135.9 ± 8.4	532.1	800.8	80.4	1.69 ± 0.07	177.0
Adding defatted fish flour to whole wheat flour								
Whole wheat flour	64.0	8.0	65.2 ± 6.7	–	666.2	53.3	1.22 ± 0.03	–
Whole wheat flour Fish flour	63.0 1.0	8.66	104.6 ± 7.9	60.4	741.9	64.2	1.63 ± 0.07	33.6
Whole wheat flour Fish flour	61.0 3.0	9.96	142.7 ± 9.2	118.8	900.6	89.7	1.59 ± 0.06	30.0
Whole wheat flour Fish flour	59.0 5.0	11.28	182.5 ± 11.5	180.0	969.8	109.4	1.67 ± 0.07	36.9
Adding defatted fish flour to rye flour								
Whole rye flour	72.8	8.0	93.8 ± 7.7	–	714.2	57.1	1.64 ± 0.06	–
Whole rye flour Fish flour	71.8 1.0	8.6	133.2 ± 8.8	42.0	807.8	69.5	1.92 ± 0.09	17.1
Whole rye flour Fish flour	69.8 3.0	10.02	158.2 ± 9.4	68.6	847.9	85.0	1.86 ± 0.09	13.4

Whole rye flour Fish flour	67.8 5.0	11.36	156.1 ± 9.6	66.4	845.3	96.0	1.64 ± 0.07	0.0
			Adding defatted fish flour to milled rye flour					
Milled rye flour	78.0	6.0	43.0 ± 4.8	–	551.3	33.4	1.29 ± 0.04	–
Milled rye flour Fish flour	77.0 1.0	6.35	96.2 ± 7.7	123.7	697.3	44.3	2.17 ± 0.11	68.2
Milled rye flour Fish flour	75.0 3.0	8.12	128.4 ± 8.1	198.6	785.4	61.0	2.10 ± 0.13	62.9
Milled rye flour Fish flour	73.0 5.0	9.55	147.6 ± 9.2	243.3	856.2	81.8	1.80 ± 0.09	39.5
			Adding defatted fish flour to grain sorghum					
Grain sorghum	77.0	8.0	19.0 ± 3.4	–	484.7	38.8	0.49 ± 0.02	–
Grain sorghum Fish flour	76.0 1.0	8.68	56.3 ± 5.1	196.3	600.8	52.1	1.08 ± 0.04	120.4
Grain sorghum Fish flour	74.0 3.0	10.04	113.4 ± 7.3	496.8	784.5	78.8	1.43 ± 0.06	191.8
Grain sorghum Fish flour	72.0 5.0	11.39	142.3 ± 8.6	648.9	855.1	97.4	1.47 ± 0.05	200.0
			Adding defatted fish flour to millet					
Millet	78.3	7.0	32.2 ± 4.7	–	557.4	39.0	0.82 ± 0.05	–
Millet Fish flour	77.3 1.0	7.69	55.2 ± 5.6	71.4	649.6	50.0	1.10 ± 0.07	34.1
Millet Fish flour	75.3 3.0	9.11	82.4 ± 7.1	155.9	782.6	71.3	1.15 ± 0.06	40.2
Millet Fish flour	73.3 5.0	10.41	174.3 ± 11.8	441.3	945.1	98.4	1.77 ± 0.08	115.8

[1] Gains in body weight per gram of protein intake.

[2] Standard deviation.

EXPERIMENTAL

Influence on growth and protein efficiency. This study was carried out on the Wistar strain albino rats. There were 12 animals in each group, the sexes being equally divided. The animals were about 26 days old when started on experiments and weighed 50 to 54 gm each. The experimental period was 10 weeks. The animals were weighed once weekly and accurate records were kept of food consumption. The protein content of the whole yellow corn was 9.1%; of the whole wheat, 11.9%; of the whole rye, 10.8%; of the milled rye, 7.8%; of the grain sorghum, 10.4%; and of the millet, 9.0%. Fish flour was fed at three levels, 1, 3 and 5%, at the expense of the grain. The balance of the rations consisted of cellu flour, 2%, for roughage; Sure's salts no. 1 ('41) 4%; vegetable shortening 7%; cod liver oil 2%; wheat germ oil 1%; and cerelose (glucose) to make 100%. All the rations contained an abundance of the B vitamins (Sure, '53, '54). The fat-soluble vitamins A, D and E were furnished by the cod liver oil and wheat germ oil in the rations. The results of this investigation are summarized in table 1 and are expressed as gains in body weight per gram of protein intake, which indicates the protein efficiency ratio (PER). The data on the nutritive value of the minerals in fish flour are given in table 2.

The results given in table 1 are self-explanatory. It is evident from table 1 that the addition of fish flour in small amounts to whole yellow corn is accompanied by large increase in body weight and protein efficiency. The addition of 1% fish flour was followed by 3.5-fold increased growth and 119.7% increase in PER; the addition of 3% fish flour resulted in over 4.5-fold increase in body weight and 131.1% increase in PER; and the addition of 5% produced almost 7-fold increase in body weight and 177% increase in PER. At 1% level of fish flour as a supplement to whole wheat there was almost as high a PER as there was at 5%, although on the latter there was optimum growth, i.e., a three-fold increase. Again, 1% fish flour proved to be the optimum for PER when used as a

supplement to whole rye but the optimum growth was obtained on the 3% level. In the case of milled rye, 1% fish flour also produced the optimum PER but maximum growth was secured on the 5% level. In the case of grain sorghum, 3% fish flour addition resulted in the optimum PER with some increased growth on the 5% level. The greatest responses to growth and protein efficiency ratios were secured when 1, 3, and 5% fish flour were added to the millet. The optimum level for both growth and PER was 5%.

According to Martin and Leonard ('49), sorghum is a chief food grain in a large portion of Africa and parts of India, Manchuria and China. It also is grown in nearly all countries in the southern half of Europe and Asia, and in Central America, South America and Australia. As a world food grain, sorghum ranks third, being exceeded only by wheat and rice. Consequently, fish flour should have great potentialities as an efficient protein supplement to plant foods in the battle against kwashiorkor in large sections of the world where a deficiency of good quality proteins exist.

Millet is one of the oldest of cultivated crops. It was grown in China as early as 2700 B.C. While the millets are minor crops in this country, they are used extensively in the Orient, particularly in China and India; hence, it would be of interest to determine clinically the value of fish flour as a protein supplement to millet when used as a cereal for infants and growing children in Asia.

Nutritive value of the minerals in fish flour. For the study of the nutritive value of the minerals in fish flour two products were used: one, a mixture of smelts, carp and whitings; and another, which was a mixture of hake and merluza from Valparaiso, Chile.[3] This product contained 61.2% protein; 9.7% fat, and 15.2% ash. After repeated extraction with petroleum ether at room temperature the Chilean fish flour contained 67.6% protein; 0.7% fat and 16.0% ash. By using 25.0% of

[3] Supplied by Mr. Donald Sabin of The United Nations Children's Emergency Fund of the United Nations, New York.

TABLE 2

Nutritive value of minerals in fish flours

(Experimental period was 8 weeks with 20 rats in each group. Results are means) 17.2% protein in each ration.

TYPE OF RATION	AMOUNT IN RATION	GAINS IN BODY WEIGHT	TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO [1]
	%	gm	gm	gm	
Purified casein	20.0	144.6 ± 8.7 [2]	629.7	108.3	1.34 ± 0.05
Sure's salts No. 1	4.0				
Chilean fish flour [3]	25.0	168.6 ± 9.5	733.5	126.2	1.34 ± 0.06
(minerals in fish flour)					
Purified casein	20.0	150.2 ± 8.9	627.3	107.9	1.40 ± 0.5
Sure's salts No. 1	4.0				
Domestic fish flour [4]	22.1	167.6 ± 9.8	720.8	124.0	1.35 ± 0.05
(minerals in fish flour)					

[1] Gains in body weight per gram of protein intake.

[2] Standard deviation.

[3] Hake and merluza.

[4] Whitings, smelts and carp, supplied by The VioBin Corporation, Monticello, Illinois.

the Chilean fish flour and 22.1% of the domestic product [4] there was introduced 4.0% minerals and 17.2% protein into the rations. By feeding 20.0% purified casein [5] (containing 86% protein) the ration carried 17.2% protein which was supplemented with 4.0% of Sure's salt mixture no. 1 (Sure, '41). With such an experimental set up it is possible to evaluate the relative biological value of the minerals as well as the proteins in fish flours and that of Sure's salts no. 1 and the chief protein in milk. The rations also contained cellu flour, vegetable shortening, cod liver oil, wheat germ oil and glucose, in the amounts given in the first section of this paper. The animals were 27 days old when started on the experiments and weighed 50 to 54 gm each. There were 20 animals in each group and the sexes were equally divided. The rations contained adequate amounts of vitamins A, D and E and were supplemented separately from the rations with an abundance of the B vitamins. The duration of the experimental period was 8 weeks.

It will be noted from table 2 that the growth of the animals on the fish flours at the 17.2% level of protein intake was superior to that obtained with purified casein and the protein efficiency ratios equalled that of the casein. Since the fish flours carried all the minerals in the rations, it is evident that they provided the needed salts at least equal in nutritive value to that furnished by Sure's salt mixture no. 1 which for many years has proved adequate for very good growth of the albino rat.

SUMMARY

Data have been presented on the influence of addition of 1, 3 and 5% defatted fish flour to whole yellow corn, whole wheat, whole and milled rye, grain sorghum and millet on growth and protein efficiency. The greatest responses were secured with grain sorghum, millet and whole yellow corn. The minerals in two dehydrated and defatted fish products were found to be of excellent nutritive value for growth of the albino rat.

[4] See footnote 2, page 409.

[5] Supplied by The Nutritional Biochemicals Corporation, Cleveland, Ohio.

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THE EFFECT OF A LOW ENVIRONMENTAL TEMPERATURE ON THE WEIGHT AND FOOD CONSUMPTION OF THIAMINE-DEFICIENT RATS [1]

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The effect of low temperatures on the requirement of the rat for thiamine has been studied from the standpoint of survival time and total thiamine needs. Hegsted and McPhee ('50), using growth of adults as their criterion, found that rats required more thiamine per kilogram of body weight at 55°F than at 78°F. Relating this to observed food intakes, they calculated that the requirement for thiamine per 1000 Cal. was greater in the cold. Ershoff ('50) compared the survival time of rats receiving a thiamine-deficient diet at 2°C with that of rats at 23°C. He found that there was a significant decrease in survival time at 2°C. Grab and Lang ('44) have also shown that a dietary deficiency of thiamine impaired the resistance of the rat to cold.

Since most published thiamine requirements are now based on food intake, or more specifically on non-fat caloric intake, and since a major effect of cold on rats seems to be an elevation of caloric intake, we became interested in comparing rats in the cold with warm rats when thiamine was based on food intake. If the thiamine needs were greater per unit of food intake in the cold, it seemed that cold rats receiving suboptimal levels of thiamine in their diet would fare poorly com-

[1] This paper was presented before the American Physiological Society, Federation Meetings, Chicago, Illinois, April, 1957.

pared with warm rats receiving identical levels of thiamine. At the same time, we planned to study carefully the food intake of all rats during the experiment, in view of the well known anorectic effect of a thiamine deficiency.

EXPERIMENTAL

Fifty male Sprague-Dawley albino rats, ranging in weight from 220 to 300 gm, were divided into two groups of 25 each. One group was placed in a cold room held at 5 $\pm$ 2°C; the other group was maintained at 25°C. Wire-bottom cages were used and the rats were randomized as to position in the cage rack. All rats were fed a commercial food [2] for a period of one week in order to allow them to adjust to the lowered temperature. Following this, all rats were maintained on a thiamine-free diet until growth ceased or fluctuated daily around a central point. The thiamine-free diet had the following composition: [3] vitamin test casein, 18%; sucrose, 68%; vegetable oil, 10%; U. S. P. Salt Mixture No. 2, 4%. The vitamin mixture supplied 2000 units of vitamin A, 222 units of vitamin D, 11.1 mg of α-tocopherol, 100 mg of ascorbic acid, 11.1 mg of inositol, 166.5 mg of choline chloride, 5 mg of menadione, 11.1 mg of *p*-aminobenzoic acid, 10 mg of niacin, 2.22 mg of riboflavin, 2.22 mg of pyridoxine hydrochloride, 6.66 mg of calcium pantothenate, 44 µg of biotin, 200 µg of folic acid, and 3 µg of vitamin B_{12} per 100 gm diet.

Cessation of growth occurred at 7 to 10 days after the thiamine-free diet was offered. On the 10th day, each group of 25 rats was divided into 5 sub-groups, consisting of 5 rats each, which received a supplement of thiamine orally to give the following levels: 0.3, 0.4, 0.5, 0.6, and 2.0 µg/gm food. The group receiving 2.0 µg/gm food was assumed to be in adequate thiamine nutriture and was used as a positive control in each temperature group. The animals were weighed periodically throughout the experiment. Thiamine was mixed into the food

[2] Friskies, Albers Milling Company, Division of Carnation Company, Los Angeles, California.

[3] Purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

slurry daily and the diet was offered ad libitum, refusals being measured daily.

During cold exposure, the symptoms described by Ershoff ('50) occurred. Erythema appeared in the ears within two weeks, followed by necrosis and subsequent sloughing of the necrotic tissue. Hardening of the tail occurred in most of the rats and several deaths may have been due to excessive blood loss brought about by tail chewing. There seemed to be no correlation between thiamine levels in the diet and these symptoms.

The experiment was allowed to proceed for 18 days, at the end of which some of the more thiamine-deficient rats had lost 40% of their original weight. All rats were then placed on a complete diet and recovered satisfactorily, both at 5°C and at 25°C.

RESULTS AND DISCUSSION

Table 1 shows the weight data of the rats at the two environmental temperatures. The initial weights of the rats in the cold room were significantly lower than those in the warm room due to weight loss in the rats during acclimatization to 5°C. A calculation of the correlation coefficients between initial weight and weight gain or loss revealed that the weight changes were influenced by the initial weight. In order to partial out this effect, the results were subjected to an analysis of covariance (Snedecor, '46). The probability values for each level of thiamine intake were obtained in this way.

At none of the five levels of dietary thiamine intake were the weight changes of the cold rats significantly different from those of the warm rats. At two levels, the differences approached significance at the 5% level: at 0.3 μg/gm food, the weight losses of the rats at 25°C were somewhat greater and at 2.0 μg/gm, the weight gains of the rats at 25°C were greater than those of the rats at 5°C.

These data are presented graphically in figure 1, using percentage weight gain or loss on the ordinate and micrograms of thiamine/gram food on the abscissa. A calculation of the regression equations showed that the slopes of the lines were

practically identical, indicating that the weight loss increments for the various levels of thiamine were not affected by exposure of the rats to cold. The regression lines are not significantly different, as determined by an analysis of variance.

If the requirement is taken to be the intersection of the regression line with the line representing maximum growth, there would be an apparent reduction of the thiamine require-

TABLE 1

Weight changes of semi-adult, thiamine-deficient rats as affected by environmental temperature [1]

THIAMINE	5°C			SIGNIFICANCE OF DIFFERENCE BETWEEN WEIGHT CHANGES FOR 5° AND 25° AT PROBABILITY LEVEL	25°C		
	Rat No.	Initial weight	Weight change		Rat No.	Intial weight	Weight change
ug/gm food		*gm*	*gm*			*gm*	*gm*
0.3	1	236	−84		1	251	−99
	2	241	−56		2	271	−81
	4	228	−69	$0.05 < P < 0.10$	3	266	−87
	5	210	−67		4	266	−89
					5	279	−91
0.4	6	238	−75		6	281	−100
	7	221	−27		7	256	−82
	8	233	−71	$0.05 < P > 0.10$	8	267	−50
	10	236	−47		9	279	−63
					10	253	−60
0.5	11	263	−75		11	287	−49
	12	228	−33		12	258	−48
	13	231	−46	$0.05 < P > 0.10$	13	214	−37
					14	269	−51
					15	267	−59
0.6	16	243	−15		16	262	− 7
	17	222	−13		17	283	−51
	18	227	−17	$0.05 < P > 0.10$	18	246	−33
	19	228	−17		19	270	−50
	20	231	−26		20	236	−37
2.0	21	204	+17		21	284	+51
	22	211	+30		22	265	+56
	23	245	+42	$0.05 < P < 0.10$	23	284	+61
	24	238	+21		24	269	+54
	25	238	+21		25	292	+55

[1] Eighteen day experiment.

ment per gram of food at 5°C, the requirement being 0.9 μg of thiamine per gram of food. On the other hand, if the theoretical maximum growth of the rats at 5°C is considered to be equal to that of the rats at 25°C, the requirement of both groups of rats becomes 1.05 μg of thiamine per gram of food. This compares well with the rat requirement of 1 μg/gm of food cited by Brody ('45). However, it is higher than the 0.7

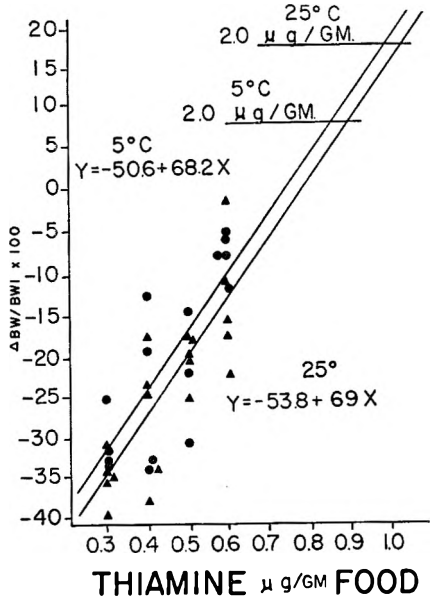

Fig. 1. Relation between thiamine levels and percentage weight changes. ● 5°C; ▲ 25°C.

μg/gm at 15°C reported by Robinson ('43). It is readily apparent, from an examination of these regression lines and the data on table 1, that exposure to cold at 5°C did not increase the needs of the rat for thiamine per gram of food, when the weight change of semi-adult rats was used as the criterion of the deficiency.

Another possible index of thiamine deficiency is anorexia. By the end of the preliminary period, i. e., after having ingested a thiamine-free diet for 10 days, all rats exhibited a

TABLE 2

Food consumption of semi-adult, thiamine-deficient rats as affected by environmental temperature

	THIAMINE	WEEK 1 AVERAGE INTAKE	Week 2 AVERAGE INTAKE	DIFFERENCE	MEAN FOOD CONSUMPTION 18 DAYS	
	μg/gm food	*gm*	*gm*	*%*	*gm*	
	0.3	115	89	−23	253	(4) [1]
	0.4	138	106	−24	299	(4)
5°	0.5	144	106	−26	304	(3)
C.	0.6	157	132	−16	351	
	2.0	177	189	+ 7	436	
	0.3	39	33	−15	97	
	0.4	46	26	−43	100	
25°	0.5	58	40	−31	123	
C.	0.6	68	51	−26	143	
	2.0	119	124	+ 4	284	

[1] Numbers within parentheses indicates survivors. All other values are for 5 animals.

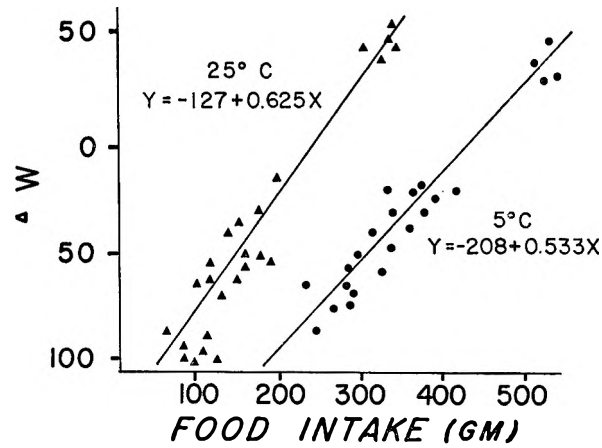

Fig. 2. Relation between total food intakes and weight changes. ● 5°C; ▲ 25°C.

marked decrease in appetite. Food intakes, however, were not measured until the beginning of supplementation. Table 2 shows the percentage decreases in food consumption of the second week of the experiment compared with the first week. Anorexia did not seem to be accelerated in the cold.

Since cold seemed to have no effect on the weight changes in these rats, it is evident that the rats at 5°C must have kept pace with the rats at 25°C by eating more food. In order to obtain information about the relation of food intake to weight changes and to account for the variation within groups, the graphs shown in figure 2 have been constructed. Although the thiamine levels are not plotted on this graph, they are implicit in the progressive decreases in food intake and loss or gain in weight. From this graph, it can be seen that loss or gain of weight in both groups of rats is a straight line function of food intake. Weight loss in thiamine-deficient rats thus appears to be directly related to decreases in food intake. At these levels, thiamine deficiency did not have any gross effect on the utilization of food for changes in weight. This is particularly true of the cold rats. In the warm rats, there seemed to be a sudden decrease in food utilization at very low levels of thiamine intake.

The deficiency of thiamine, in this experiment, apparently restricted the food intake of each group of rats in the same way as it might have been restricted by an investigator. The horizontal distance between the two lines represents food eaten by the rats to supply their cold-induced extra energy requirements. This increment amounted to about 10 gm of food, or 43 Cal. per day and seemed not to be grossly affected by the level of calories ingested, the latter being influenced by the level of thiamine in the diet. While the thiamine deficiency restricted the food intake of each group, it did not affect the distance between the lines. That this food increment is constant can also be deduced from the regression lines in figure 1, for if the appetite of the cold rats were failing to the same proportional extent as the appetite of the warm rats, the energy deficit of the cold rats would be much greater than it is in this experiment, and the cold rats would lose much more weight at a given level of thiamine. If this were true, the regression lines would diverge as the level of thiamine decreased.

In order to explain these results, it might be useful to divide the appetite of these rats into two portions: one which is based

on the normal energy and adult growth requirements and another portion stimulated by increased caloric needs in the cold. It appears that the portion of the appetite stimulated by the increased energy expenditure in the cold is not subject to the anorexia induced by a thiamine deficiency.

SUMMARY

Weight changes and food intakes were measured in rats kept at two environmental temperatures and receiving adequate and suboptimal levels of thiamine in the diet.

There were no significant differences in weight loss or gain between the rats held at 25°C and at 5°C when the level of thiamine, expressed as micrograms per gram of food, was the same.

The animals kept at 5°C ate an approximately constant increment of food above the intake of those kept at 25°C. This increment amounted to about 10 gm, or 43 Cal. daily.

An attempt is made to explain this constant increment by suggesting a fraction of the appetite which is not affected by a thiamine deficiency at 5°C.

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EFFECT OF ASCORBIC ACID AND OF ORANGE JUICE ON CALCIUM AND PHOSPHORUS METABOLISM OF WOMEN [1]

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In spite of an appreciable amount of work in this area, the relation of ascorbic acid to calcium and phosphorus utilization remains unclear. In a report of what would appear to be the earliest investigation of this problem Chaney and Blunt ('25) noted improved utilization of calcium in two girls 10 and 11 years of age when orange juice was added to the diet. On the other hand, in studies on preschool children, Daniels and Everson ('37) and Watson and others ('45) found no evidence of improved calcium utilization when orange juice or pure ascorbic acid were added to several levels of milk intake. As the result of a study of adult men, Steggerda and Mitchell ('46) reported that the utilization of the calcium of milk is not appreciably or consistently modified by the ingestion of sodium citrate, citric acid, or orange juice in considerable amounts. They also stated that in some of their subjects sodium citrate and ascorbic acid induced profound disturbances in calcium metabolism subsequent to a 20-day period of dosage. Generally, this was characterized by a phase of increased calcium retention followed, or preceded, or both, by a period of increased excretion.

Studies in which young rats were used as the experimental animals are equally contradictory. Lanford ('39) reported improved utilization of calcium when moderate amounts of

[1] Paper no. 3572, Scientific Journal Series, Minnesota Agricultural Experiment Station.

orange juice were added to a wheat and milk diet. Mallon and co-workers ('42, '46, '52) noted increased calcium retention with a fresh grapefruit juice supplement to a basal diet of whole wheat flour and dried whole milk, but not with lemon juice, lime juice, orange juice, or commercially canned tomato juice.

The present study was undertaken for the purpose of investigating the effect of the ingestion of moderate amounts of orange juice and of ascorbic acid on calcium and phosphorus metabolism in adult women. These minerals were supplied in amounts which were calculated to be slightly below the minimal needs for maintenance, since it was thought that at these levels any effects of the supplements would be more apparent. The vitamin C supplements were added in amounts which would provide the National Research Council's Recommended Dietary Allowance for this vitamin.

PROCEDURE

Twelve college women, ranging in age from 19 to 30 years, served as subjects. To insure that only healthy individuals participated, each student was given a physical examination by the University Student Health Service. No student receiving any kind of medication was included in the study.

The basal diet was planned to be adequate in all nutrients with the exception of calcium, phosphorus, and ascorbic acid. The diet was calculated to provide daily an average of 2075 calories, 55 gm protein, 350 mg calcium, 750 mg phosphorus and 22 mg ascorbic acid. Five menus were planned and these were repeated for each of 9 5-day balance periods. A typical day's menu is given in table 1. Sugar was allowed ad libitum; however, a record was kept of the amount consumed by each subject. In addition, for those subjects whose energy requirement exceeded the caloric content of the basal diet, ice box cookies low in calcium were allowed ad libitum. The amounts of calcium and phosphorus contributed by the cookies were included in the balances, thus resulting in small variations in the calcium and phosphorus intakes among the subjects. Dis-

tilled water was used for food preparation and drinking purposes and chemically pure sodium chloride, as seasoning. This salt also was used as a dentifrice by the subjects, since many tooth pastes and powders contain significant amounts of calcium.

During the first three periods of the study all subjects received the same basal diet (basal I). During the next three periods, (supplemented periods), 6 of the subjects selected at

TABLE 1

Typical menus for a day [1]

BREAKFAST		LUNCH		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Apricots, canned	100	Baked egg, with	54	Grape juice	100
Bacon, (raw wt.)	30	ham (cooked) and	60	Beef, round, cooked	50
Toast, white bread	50	cream, light	15	Carrots, cooked	75
Butter	12	Lettuce, head	40	Rice, cooked	100
Cream, light	15	French dressing	9	Gravy	35
Jam	20	Bread, Vienna	40	Salad, molded gelatin	30
Coffee		Plums, canned	100	with cottage cheese	20
		Tea		lettuce	15
				mayonnaise	9
				Bread, Vienna	20
				Butter	6
				Gingerbread with	70
				whipped cream	30
				Tea	

[1] Sugar was consumed ad libitum, but the amount was measured.

random (group B) were given 25 mg of pure crystalline ascorbic acid at each meal, and the remaining 6 subjects (group A) were given 65 gm of reconstituted frozen orange juice at each meal. This amount of orange juice was calculated to provide 25 mg of ascorbic acid. Unopened cans of orange juice which had been held in the frozen state were analyzed 6 months later. The total ascorbic acid intake, based on these analyses, averaged 65.8 mg or 88% of the amount planned. However, since these samples had been held for some time before analysis, it is probable that some loss had occurred in the interim and the

actual difference in ascorbic acid was not as great as it would appear. The last three periods (basal II) were a duplication of the first three. The subjects were given daily 1 mg of riboflavin and 400 I. U. of vitamin D [2] throughout the study to insure adequate amounts of these nutrients.

Servings of each of the foods were weighed on a torsion balance to the nearest tenth of a gram. Food composites for analysis were prepared at the time the foods were served to the subjects.

Total fecal collections were made using carmine to mark the periods. Acid digests of the food and fecal composites were prepared following the procedure of Stearns ('29), and an aliquot of each was retained for future analysis. Urine collections for each subject were measured daily and one-fifth of the day's total volume was acidified with 10 ml of concentrated HCl. At the end of each period an aliquot of the well-mixed composite was stored for later analysis.

Stearn's ('29) modification of the McCrudden method was used for calcium determination and the Fiske and Subbarow ('25) method, for phosphorus.

The statistical procedures employed for testing the significance of the observed differences were analysis of variance and Student's "t" test. The association between calcium and phosphorus was tested by the determination of the coefficients of correlation.

RESULTS AND DISCUSSION

Calcium. Table 2 contains a summary of the calcium data for the two groups of subjects. Period 1 for group B is based on 5 subjects, since participation in the study was not begun by one subject until the 4th day. The first three periods for each group served for the adjustment of the calcium metabolism of the subjects to the intake level of the basal diet. It may be noted that both groups were in negative balance during periods 1 and 2 and in positive balance in period 3 of basal

[2] The authors acknowledge their indebtedness to Mead Johnson and Company for the vitamin D used in this study.

I. Since this shift from a negative to a positive balance was in large measure the result of a marked reduction in the fecal excretion of calcium, the data for the individual subjects were carefully scrutinized to ascertain whether or not there was evidence that this was attributable to imperfect separation of the carmine-marked feces into their respective periods. Since the 9 periods of this study were continuous, it is obvious that imperfect separation of the specimens could result in high or low values in the period either preceding or following any given period. Inspection of the data for the individual subjects in group A showed that all 6 excreted more calcium in the feces in period 2 than in period 3. For one subject, in particular, the shift was very marked, the fecal calcium excretion in period 3 amounting to only 39% of that in period 2. A similar comparison of the data for periods 3 and 4 shows that all but one had lower fecal losses of calcium in period 3 than in period 4.

A similar scrutiny of the fecal excretion data for the subjects in group B showed a higher value for 5 of the 6 subjects in period 2 than in period 3 and for 4 of the subjects in period 4 than in period 3. It would seem unlikely that this consistency in low fecal excretion of calcium in period 3 is attributable to errors in the separation of the specimens, hence it may be assumed that it represents a physiological adjustment to the low calcium intake.

Considerable variation among the subjects was noted in calcium and phosphorus absorption and retention. To test whether or not the two groups of subjects differed significantly in their response to the basal diet, the analysis of variance was applied to the absorption and retention data in periods 1, 2, and 3 (basal I). The results of this test indicated that the two groups did not differ significantly. However, because of the observed individual variations it was thought that each group should serve as its own control. Consequently, in the interpretation of the findings, the basal II periods for each group were used for this purpose.

During periods 4, 5, and 6 when 195 gm of orange juice were added to the basal diet of group A and 75 mg of crystalline ascorbic acid to the diet of group B, there was a shift to a slightly negative calcium balance in period 4, followed by positive balances in periods 5 and 6. These positive balances were appreciably greater for the group receiving orange juice than for that given ascorbic acid.

In the basal II periods (periods 7, 8, and 9), both groups again were in negative calcium balance, the ascorbic acid group showing the greater negative balances.

To test whether or not the retentions during the periods when orange juice and ascorbic acid supplements were given were significantly greater than during the following (basal II) periods, an analysis of variance was run using the data for periods 5 and 6 and 8 and 9, periods 4 and 7 being considered adjustment periods to the change in regimen. The results of this test indicated that for both groups the difference in calcium retention between these periods was significant at the 5% level.

The greater calcium retention in periods 5 and 6 as compared with periods 8 and 9 was due to greater absorption which, for the orange juice group, was significant at the 1% and for ascorbic acid group at the 5% level. For the orange juice group lower urinary excretion of this mineral was also noted. However, a comparison of the urinary calcium excretion during the first basal periods with that during the orange juice periods shows that, associated with the improved absorption, was an increase in the urinary excretion. The high urinary excretion during the second basal periods as compared with the orange juice periods would seem to confirm Steggerda and Mitchell's observation that "sodium citrate and orange juice may induce profound disturbances in the calcium metabolism of adult men subsequent to a 20-day period of dosage." It should be mentioned, however, that these authors used two and a half times as much orange juice daily as was given in the present study. Shifts in calcium absorption and urinary excretion also were observed for the ascorbic

TABLE 2

Effect of orange juice and of ascorbic acid on mean daily calcium and phosphorus balances

DIET	PERIOD	CALCIUM					PHOSPHORUS				
		Intake	EXCRETION		Balance	Absorption	Intake	EXCRETION		Balance	Absorption[1]
			Fecal	Urinary				Fecal	Urinary		
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
					Group A (6 subjects)						
	1	338	365	96	-123	-27	843	319	626	-102	524
Basal I	2	312	280	96	-64	32	824	300	609	-85	524
	3	337	198	100	39	139	783	220	631	-68	563
Basal + 195 gm	4	342	236	108	-2	106	832	269	615	-52	563
orange	5	352	192	103	57	160	822	219	628	-25	603
juice	6	372	227	108	37	145	812	241	622	-51	571
	7	333	220	120	-7	113	786	237	590	-41	549
Basal II	8	344	215	120	9	129	820	231	603	-14	589
	9	316	232	113	-29	84	768	248	582	-62	520
					Group B (6 subjects)						
	1	337[2]	296[2]	103[2]	-62[2]	41[2]	841	291[2]	571[2]	-21[2]	550[2]
Basal I	2	310	243	85	-18	67	818	278	580	-40	540
	3	338	184	92	62	154	785	212	616	-43	573
Basal + 75 mg	4	325	238	103	-16	87	803	261	601	-59	542
ascorbic	5	336	219	114	3	117	795	234	611	-50	561
acid	6	354	219	110	25	135	781	243	557	-19	538
	7	333	232	112	-11	101	786	260	562	-36	526
Basal II	8	347	240	112	-5	107	827	249	568	10	578
	9	318	256	117	-55	62	773	258	587	-72	515

[1] Obtained by deducting fecal excretion from intake.
[2] Mean for 5 subjects.

acid group, but the effects were less pronounced. This would seem to indicate that the citric acid in orange juice was a factor in the effect of this food on calcium absorption.

To test whether or not the orange juice was more effective in increasing calcium absorption than the pure ascorbic acid, a Student's "t" test was applied to the absorption data for periods 5 and 6. For the orange juice group the mean daily calcium absorption was 153 mg and for the ascorbic acid group, 126 mg. The difference, however, was not statistically significant. Similarly, although the mean daily calcium retention during periods 5 and 6 for the orange juice group was 47.0 mg and for the ascorbic acid group, 14.0 mg., due to wide individual variations, these means also were not significantly different.

Phosphorus. Table 2 gives a summary of the phosphorus data. It is evident that the intake level used in this study was slightly below the requirement for these subjects, since for both groups the mean balance in every period was negative, with the exception of period 8 for the ascorbic acid group when a low positive balance was recorded. The fact that the phosphorus intake was a little higher in period 8 than in either period 7 or 9 may account for this positive balance.

A comparison of the phosphorus data for the two groups of subjects for periods 5 and 6 with those for periods 8 and 9 indicates that neither orange juice nor ascorbic acid influenced phosphorus absorption or retention. Furthermore, no statistically significant differences in phosphorus absorption or retention were noted between the basal I periods (2 and 3) and either the corresponding Supplemented or the basal II periods.

To ascertain the effect of orange juice and of ascorbic acid on the relationship between calcium and phosphorus utilization, coefficients of correlation were computed using the corresponding absorption and retention data for periods 2 and 3, 5 and 6, and 8 and 9. The results of these computations (table 3) show that during the basal I periods for both groups there was a high correlation between calcium and phosphorus ab-

sorption, with a lower correlation during the Supplemented (ascorbic acid and orange juice) and basal II periods. Using $r^2 \times 100$ as the criterion, it would appear that during basal I periods 76% for group A and 60% for group B of the variation in the absorption in calcium was associated with the variation in phosphorus. During the supplemented periods these values are 64 and 36% for group A and group B, respectively, and in the basal II periods, 25 and 37%.

TABLE 3

Coefficients of correlation and the association in percent between calcium and phosphorus absorption and retention

	ABSORPTION			
	r	$r^2 \times 100$	r	$r^2 \times 100$
		%		%
Periods	Group A (6 subjects)[1]		Group B (6 subjects)[2]	
Basal I	0.8734	76	0.7769	60
Supplemented	0.7998	64	0.5955	36
Basal II	0.4987	25	0.6052	37
		Retention		
Basal I	0.6937	48	0.4552	21
Supplemented	0.8080	65	0.6632	44
Basal II	0.8905	79	0.7966	64

[1] Orange juice (65 gm) with each meal during Supplemented Periods.

[2] Crystalline ascorbic acid (25 mg) with each meal during Supplemented Periods.

Conversely, the pattern for retention appears to be that initially there was a low correlation between calcium and phosphorus which increased when ascorbic acid was added to the diet either in the form of orange juice or as pure crystalline ascorbic acid, with further increases during the basal II periods. The values for $r^2 \times 100$, given in table 3, again reflect this shift in association. These alterations in the relationship between calcium and phosphorus absorption and retention are in keeping with the statistically significant changes noted in calcium utilization, despite the fact that phosphorus utilization remained unchanged. These findings, however,

readily raise the question as to the form in which the calcium was retained since, on the average, the subjects were in negative phosphorus balance during most of the study.

SUMMARY

The effects of orange juice and of ascorbic acid on calcium and phosphorus metabolism were investigated using 12 college women as experimental subjects. The study was divided into 9 5-day periods. During the first three periods (basal I), all subjects were on the basal diet which provided a mean of 336 mg calcium, 806 mg phosphorus, and a calculated 22 mg ascorbic acid daily. During the second three periods, for half of the subjects a supplement of 65 gm of orange juice was provided at each meal and for the other subjects, 25 mg crystalline ascorbic acid. During the last three periods, the subjects were again on the unsupplemented basal diet (basal II).

For both groups of subjects, calcium absorption was significantly greater during the periods of supplementation than during the basal II periods. Urinary excretion of this element was increased both during the periods of supplementation and the basal II periods which followed when compared with that of the basal I periods.

Supplementation of the basal diet with orange juice or crystalline ascorbic acid resulted in calcium retention which was significantly greater than during the succeeding periods when only the basal diet was given.

Although the orange juice group absorbed and retained a somewhat greater amount of calcium than did the ascorbic acid group the differences were not statistically significant.

Neither orange juice nor ascorbic acid in the amounts given in this study influenced significantly phosphorus utilization by these subjects.

Calcium and phosphorus absorption were highly correlated during the basal I periods, but lower coefficients of correlation were obtained during the supplemented and basal II periods. Conversely, during the basal I periods there was a low cor-

relation between calcium and phosphorus retention, which increased when either orange juice or crystalline ascorbic acid was added to the diet, with further increases during the basal II periods.

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ASSAY OF BIOLOGIC VALUE OF MILK PROTEINS BY LIVER XANTHINE OXIDASE DETERMINATION

I. POWDERED PRODUCTS

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The possibility of changes in nutritional value because of modification of milk proteins in modern methods of dairy technology has been widely investigated. Pasteurized milk, homogenized milk, evaporated milk, spray- and roller-dried whole milk and non-fat dry milk solids have been studied. A highly heated milk product has received special study by Tomarelli, Linden and Bernhart ('52). Questions involved concern the type of milk processing, the choice of animal and method of assay, storage effects, and the extrapolation of results to human nutrition. In a review of the subject by Heineman ('53), discrepancies among the findings of various laboratories were described, and the need for further study emphasized.

As shown by Scott and Norris ('49), human milk will not support rat growth, although its failure to do so is incompletely understood. Of the reasons advanced to explain this failure to thrive, those most plausible relate to the protein and lactose components. The protein level, 10% of total calories, falls below that required by the growing rat. A review by Fisher and Sutton ('49) points out that high lactose intake induces chronic diarrhea in the animal. Vitamins, minerals and possible unknown nutritional factors also deserve consideration.

In their first report (Elvehjem, '54), the Committee on Amino Acids of the National Reserch Council recommended that special emphasis be placed on the study of amino acids in human nutrition, pointing out that there is little information on either the requirements for infants or the composition of infant foods with regard to amino acids. They suggested that pertinent data might be derived from animal experimentation, including the effects of amino acids on tissues, enzyme activity and liver function. A later report (Allison, '56) raises again the question of an appropriate reference pattern of amino acids — current data will not permit the unqualified use of either a natural protein or a pattern based on determined requirements. The point was also made that limited knowledge of the intake of breast milk made calculation of requirement for the infant uncertain.

Evaluation of the proteins of milk products designed for infant feeding through modification of cow's milk to resemble human milk in composition has not been attempted by nitrogen balance studies in small animals because separate collection of fecal pellets and urine is impossible, due to diarrhea secondary to the relatively high lactose intake. However, these products have been shown to support growth of weanling rats (Scott and Norris, '49; Baur, '56[1]). Cox, Mueller and Ellingson ('55) have studied the protein efficiency of infant formula products as a function of protein level, and suggested the importance of careful studies on the effect of manufacturing techniques on the nutritive value of these foods.

An indirect approach to studying the biologic value of proteins in milk products designed for infant feeding was suggested by the work of Litwack, Williams, Chen and Elvehjem ('52) and Litwack, Williams, Fatterpaker, Chen and Elvehjem ('53). These investigators related liver xanthine oxidase activity in both young adult and growing rats to the quantity and quality of protein in the diet. Allison ('55) has recently reviewed the methods of evaluation of the

[1] Unpublished data.

biologic value of proteins, and pointed out the good correlation found with activity of liver enzymes, particularly xanthine oxidase.

The determination of liver xanthine oxidase has also been used to assay dietary riboflavin by Decker and Byerrum ('54). Vitamin B_{12} and molybdenum are also known to increase liver xanthine oxidase activity. These influences, however, can be minimized in young adult rats by feeding a stock diet that will build good body stores of these substances prior to feeding the experimental diet.

It was felt that, if stimulation by other factors were minimized, determination of liver xanthine oxidase would provide a reliable index of the nutritional value of variously heat-treated milk proteins, egg protein and vegetable protein. Total liver nitrogen, total liver solids and protein efficiency were determined in an attempt to further validate this method.

MATERIALS AND METHODS

Adult male, Sprague-Dawley strain rats, 80 to 85 days of age, on a stock diet from weaning, and weighing 263 $\pm$ 14 gm were fed various diets ad libitum, sacrificed, and their livers analyzed.

Several groups of rats were fed the various test feedings for 9 days. Other groups were first fed nitrogen-free diets for a 9-day depletion period; some were sacrificed at once, others placed on the test diets for 9 days.

Two powdered cow's milk products — infant foods A[2] and B[3], a powdered soybean base product[4] designed for infant

[2] Infant food A – Similac: Cows milk protein 13.75%, lactose 53.4%, fat 26.85%, (mixture of corn, coconut, olive, milk fat and coca butter), minerals 4.00% including milk ash plus added Na, K, and Ca salts, fish liver oil conc., aseorbic acid, niacin and thiamine.

[3] Infant food B – Olac: Cows milk protein 23.0%, carbohydrate 51.4% (lactose 30.4%, maltose 12.0%, dextrins 9.0%), fat 18.5% (corn oil 17.3%, milk fat 1.2%) minerals 4.9%, vitamin A palmitate and calciferol.

[4] Soybean base food – Mull-Soy: Soy protein 24.0%, carbohydrate 35.9% (sucrose and dextrose), soy fat 30.0%, minerals 5.4% including soy ash plus added Na and Ca salts, soy lecithin.

use, defatted whole egg protein, acid-precipitated casein, vitamin-free test casein, gluten and zein were the proteins or protein sources tested. The composition of infant food A formed the model for the construction of the other diets. Infant food B and the soybean-based infant food were brought to an approximation of this composition by the addition of lactose and a dextrin-maltose mixture[5], vegetable oils[6] and salts[7]; lactose, vegetable oils and salts were added to the pure proteins. All diets were supplemented with 0.5% of a vitamin-trace element mixture[8], and sufficient A and D concentrate to bring the levels respectively to 300 and 2,100 U. S. P. units/100 gm diet. Diets containing some of these proteins at various levels below 13.8%, accomplished by dilution with cornstarch, were also tested. The nitrogen-free diet offered the same nutritional elements as infant food A, with the substitution of cornstarch for the milk protein.

Liver xanthine oxidase activity was determined by the method of Litwack, Bothwell, Williams and Elvehjem ('53). Liver nitrogen was determined by micro-Kjeldahl technique, and total liver solids were determined by drying an aliquot of homogenate at 105°C.

Significance of differences was determined by application of the Student "t" test.

RESULTS AND DISCUSSION

Determinations of liver xanthine oxidase response in rats transferred from the stock ration directly to the experimental diets are summarized in table 1. The activity of liver xanthine oxidase is expressed in micromoles of xanthine disappearance (oxidized) per hour per gram of liver and per 100 gm of rat.

[5] Dextri-Maltose.

[6] Corn oil only used with infant food B; a mixture of corn, cocoanut, and olive oils with the soybean base food.

[7] Salt mixture, U. S. P. XIII, No. 2.

[8] Mixture in grams: $FeSO_4 . 7H_2O$, 12.0; $MnSO_4 . 4H_20$, 14.4; $CuSO_4 . 5H_2O$, 0.78; choline chloride, 10.0; Ca pantothenate, 0.05; thiamine. HCL, 0.10; riboflavin, 1.0; niacin, 2.0; folic acid, 0.005; vitamin B_6, 0.05; inositol, 1.0; biotin, 0.005; cerelose, 39.0.

Infant food products A and B, containing whey proteins in addition to casein, produced a significantly greater liver xanthine oxidase response than casein alone, or than the vegetable proteins. The soybean-base infant food yielded values greater than those of gluten or zein. Casein-fed,

TABLE 1

Liver xanthine oxidase activity of rats fed diets containing various proteins at 13.8% by weight [1]

PROTEIN	XANTHINE OXIDASE RESPONSE [2]		LIVER NITROGEN CONTENT	PROTEIN INTAKE
	Per gm of liver	Per 100 gm of rat		
			mg N/gm fresh tissue	*gm/100 gm body weight/day*
Whole egg [3]	18.6 ± 3	61.9 ± 12	30.1	0.630
Infant food A	13.1 ± 6	42.8 ± 18	27.8	0.655
Infant food B	13.0 ± 3	51.1 ± 11	27.4	0.822
Soybean-base infant food	9.7 ± 4	36.1 ± 12	26.6	0.751
Acid-precipitated casein [3]	8.4 ± 4	26.8 ± 13	28.5	0.751
Vitamin-free test casein [3]	7.7 ± 0.4	28.3 ± 4	26.2	0.544
Gluten [3]	7.7 ± 3	27.4 ± 12	27.1	0.544
Zein [3]	3.4 ± 3	11.1 ± 8	25.3	0.630

[1] Rats per group ranged from 4 to 10 in number.

[2] Xanthine disappearance in micromoles per hour.

[3] Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

gluten-fed, and soybean-fed rats showed no differences in liver xanthine oxidase activity. The feeding of zein gave the lowest liver xanthine oxidase activity and total liver nitrogen content.

In liver xanthine oxidase activity per 100 gm of rat, there was no significant difference between the rats fed whole egg and those fed infant food B. Infant food A-fed rats differed significantly from the rats fed whole egg protein, but not from those fed infant food B. Rats fed infant food B and the soybean base product showed a significant difference, although this difference does not exist on the basis of liver

weight. The lactose content of both infant food B and the soybean-base infant food was less than that of infant food A, and the animals on the former diets manifested a less severe degree of diarrhea than those fed infant food A. More infant food B and soybean-base food were consumed during the test feeding period, and these animals had larger livers in proportion to body weight. This combination of factors may account for the higher xanthine oxidase response per 100 gm of rat observed in the group fed infant food B.

The influence of differing dietary levels of certain of the proteins on liver xanthine oxidase activity is shown in table 2. Decrease in liver xanthine oxidase activity and in total liver nitrogen accompanied reduction of dietary proteins. At the lower levels of protein intake, all the proteins failed to support adequate liver xanthine oxidase activity.

Comparison of the influence or effectiveness of proteins was arbitrarily considered from 4 aspects (table 3). One approach considered a linear relationship between dietary protein and liver xanthine oxidase activity at all levels of observation. The slope of the line expressing this relationship was derived by the method of least squares. Distortion was produced by the weighting of a disproportionate number of readings in the lower range of protein percentage. Consequently, these observations did not show the significant relationship found by Litwack, Williams, Chen and Elvehjem ('52) in the data from a similar study using completely synthetic diets with greater differences in protein content.

A second method derived a xanthine oxidase response index from a two point analysis of the data. The difference in enzyme activity between two levels of protein intake was divided by the difference in percentage of the diet as protein. The levels of protein intake selected for whole egg and infant food A were 13.8 and 6.9%; for the vitamin-free test casein, 13.8 and 8.5%.

The third and 4th methods of comparison considered relative enzyme activities per gram of liver and per 100 gm of rat at a dietary protein level of 13.8%.

Effect of dietary level and type of protein on liver xanthine oxide activity [1]

PERCENTAGE OF THE DIET AS PROTEIN	*Xanthine oxidase response* [2]		LIVER NITROGEN CONTENT	LIVER SOLIDS	PROTEIN INTAKE
	PER GM OF LIVER	PER 100 GM OF RAT			
			mg N/gm fresh tissue	%	*gm/100 gm body weight/day*
Whole egg					
13.8	18.6 ± 3	61.9 ± 12	30.1	32.25	0.630
10.3	12.5 ± 4	45.4 ± 11	24.9	30.88	0.554
6.9	2.9 ± 2	10.8 ± 8	23.9	30.89	0.526
3.4	1.5 ± 1	6.0 ± 3	20.9	29.30	0.369
Infant food A					
13.8	13.1 ± 6	42.8 ± 18	27.8	31.24	0.655
10.3	3.8 ± 3	12.3 ± 10	25.1	31.14	0.648
8.5	5.1 ± 4	16.5 ± 12	23.4	30.75	0.443
6.9	1.2 ± 1	4.2 ± 4	22.0	30.25	0.506
4.3	2.8 ± 1	9.6 ± 4	23.2	29.37	0.223
3.4	1.3 ± 1	5.0 ± 4	21.1	30.03	0.281
2.1	0.9 ± 0.3	3.1 ± 2	20.8	29.66	0.136
Vitamin-free test casein					
13.8	7.7 ± 0.4	28.3 ± 4	26.2	30.68	0.544
8.5	3.7 ± 1	21.4 ± 19	23.2	31.16	0.398
4.3	2.1 ± 0.5	7.6 ± 2	22.7	29.67	0.239
2.1	1.1 ± 0.8	3.9 ± 1	20.8	30.14	0.133

[1] Number of rats per group 4-6, except for infant food A at 13.8% protein level where 10 animals were used.
[2] Xanthine disappearance in micromoles per hour.

TABLE 3

Comparisons of liver xanthine oxidase response to various proteins

PROTEIN	XANTHINE OXIDASE RESPONSE VALUES BY THE METHOD OF LEAST SQUARES		XANTHINE OXIDASE RESPONSE INDEX [1]		XANTHINE OXIDASE RESPONSE PER GM LIVER AT 13.8% PROTEIN		XANTHINE OXIDASE RESPONSE PER 100 GM RAT AT 13.8% PROTEIN	
	Observed	% of response to casein	Observed	% of response to casein	Observed response	% of response to casein	Observed response	% of response to casein
					μM/hr		*μM/hr*	
Whole egg	1.77	316	2.28	296	18.6	242	61.9	219
Infant food A	0.88	157	1.73	225	13.1	170	42.8	151
Vitamin-free test casein	0.56	100	0.77	100	7.7	100	28.3	100

[1] Derived from two point analysis: $\frac{\text{difference in xanthine oxidase response}}{\text{difference in \% of diet as protein}}$

All 4 methods of comparison ranked the proteins tested in the same relative order of response.

According to Prigmore, Bothwell and Williams ('55), the feeding of a nitrogen-free diet for a 9-day period results in minimum liver xanthine oxidase activity. On diets containing various amino acid mixtures, complete restoration of activity occurs without histidine, and partial restoration of activity occurs on methionine- or lysine-free diets. It was postulated that the missing amino acids required in the synthesis of liver enzymes are donated from body stores. Nasset and Davenport ('55) and Nasset, Schwartz and Weiss ('55) have demonstrated that the amino acids found in the gastrointestinal tract of normal animals during digestion are qualitatively similar, whether the test meal is egg albumin or zein. Deficits in ingested proteins may be temporarily corrected from body stores of the missing amino acids. The amino acid mixture available for absorption is determined only in part by the nature of the ingested protein. Because this and associated phenomena might reduce the value of the xanthine oxidase response as a measure of the biologic value of a protein in the normal rat, it was considered that the protein-depleted animal might better serve as a test subject.

Rats placed on nitrogen-free intake for 9 days showed markedly lower liver xanthine oxidase and lower total liver nitrogen than the rats on the other test diets. Feeding of the test proteins to depleted rats for 9 days increased liver xanthine oxidase responses and total liver nitrogen, but to levels lower than those of the nondepleted rats on the test diets. Relative order of response was the same (table 4). Liver nitrogen content, although somewhat lower, showed the same pattern as in the nondepleted rats.

Rats fed egg protein manifested greater liver xanthine oxidase activity than rats fed infant food A, casein, gluten or zein. Whole egg protein, as 10.3% of the diet, gave liver xanthine oxidase values and total liver nitrogen levels comparable to those of powdered infant food A at 13.8% protein.

TABLE 4

Liver xanthine oxidase activity and efficiency of various proteins following protein depletion

PERCENT OF DIET AS PROTEIN	XANTHINE OXIDASE RESPONSE [1]		LIVER NITROGEN CONTENT	LIVER SOLIDS	PROTEIN INTAKE	PROTEIN EFFICIENCY [2]
	per gm of liver	per 100 gm of rat	*mg N/gm fresh tissue*	%	*gm/100 gm body weight/day*	
Nitrogen-free 0.25	0.9 ± 1	6.2 ± 3	24.5	30.67	0.013	——
Nitrogen-free 0.21	1.2 ± 1	4.5 ± 4	23.0	29.26	0.010	——
Whole egg 13.8	14.4 ± 5	50.2 ± 16	27.0	30.56	0.569	5.27
10.3	8.7 ± 4	31.0 ± 11	24.2	31.58	0.625	6.08
Infant food A 13.8	9.1 ± 5	31.8 ± 16	25.5	32.21	0.919	5.87
Vitamin-free test casein 13.8	6.1 ± 4	21.6 ± 13	23.0	31.90	0.998	3.61
Acid-precipitated casein 13.8	4.8 ± 4	17.6 ± 13	26.4	31.76	0.859	2.54
Gluten 13.8	3.8 ± 3	13.9 ± 9	22.5	31.02	0.812	1.48
Zein 13.8	1.0 ± 1	3.1 ± 2	23.7	30.36	1.007	-2.19

[1] Xanthine disappearance in micromoles per hour.

[2] Grams gain/day/grams of protein/100 gm body weight.

If it is assumed that the egg protein has a biologic value of 100, a value of 75 can be calculated for infant food A. Similarly, vitamin-free casein can be given a value of 62, and acid-precipitated casein and gluten a value of 50.

TABLE 5

Order of biologic value of various proteins, from data of repletion study

PROTEIN	LIVER XANTHINE OXIDASE RESPONSE	LIVER NITROGEN	PROTEIN [1] EFFICIENCY	COMPOSITE RANKING
		mg N/gm liver		
Whole egg	1	1	2	4
Infant food A	2	3	1	6
Vitamin-free test casein	3	5	3	11
Acid precipitated casein	4	2	4	10
Gluten	5	6	5	16
Zein	6	4	6	16

[1] Grams gain/day/grams of protein/100 gm body weight.

TABLE 6

Comparison of methods of evaluating the biologic value of proteins

PROTEIN	LIVER XANTHINE OXIDASE RESPONSE		PROTEIN [1] EFFICIENCY		LIVER NITROGEN CONTENT	
	Determined	Relative	Determined	Relative	Determined	Relative
					mg N/gm liver	
Whole egg	14.4	236	5.27	146	27.0	117
Infant food A	9.1	149	5.87	163	25.5	111
Vitamin-free test casein	6.1	100	3.61	100	23.0	100
Acid precipitated casein	4.8	79	2.54	70	26.4	115
Gluten	3.8	62	1.48	41	22.5	98
Zein	1.0	16	-2.19		23.7	103

[1] Grams gain/day/grams of protein/100 gm body weight.

Mean weight loss during the 9 day depletion period approximated 20% of body weight. The efficiency of a dietary protein in repleting body stores of protein has been calculated as grams of weight gain/day/gm protein intake/100 gm body weight, and the results of the repletion diets are shown in table 4. There is no significant difference between the

efficiency of egg protein and that of infant food A. The other 4 proteins tested showed much lower efficiency.

An attempt to rank these proteins in order of biologic value is shown in table 5. That most effective by each criterion is given number 1, that least effective, the highest number in the order. Adding the indication of order in each column gives a total rating in the last column. Whole egg protein and the protein of infant food A show similarly high ranking; indicating their high relative biologic value, compared to that of the other proteins tested.

Using vitamin-free test casein, which represents among these proteins the standard, pure test material, as a reference of effectiveness, the values shown in table 6 were calculated. The range achieved by this comparative treatment would seem to make liver xanthine oxidase activity the more sensitive indicator of the biologic value of a protein.

SUMMARY AND CONCLUSIONS

1. Because the biologic value of milk proteins in diets prepared for infant feeding that resemble human milk in composition is difficult to evaluate by nitrogen balance methods in the rat, an indirect approach to the resolution of this problem was sought in the measurement of liver xanthine oxidase activity.

2. Whole egg protein and milk proteins, as processed for powdered infant foods, fed to rats either protein-depleted or transferred from a stock diet, give liver xanthine oxidase values in keeping with known biologic values reported for these two types of protein.

3. Liver xanthine oxidase activity reflects the biologic value of dietary proteins.

4. A decrease in liver xanthine oxidase is paralleled by a decrease in the content of total liver nitrogen.

5. Liver xanthine oxidase activity permits study of protein biologic value of diets that normally produce diarrhea in the rat.

6. Prior protein depletion of the adult rat does not increase the sensitivity of liver xanthine oxidase activity as an index of protein quality.

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EXCRETION OF CERTAIN NUTRIENTS BY YOUNG COLLEGE WOMEN CONSUMING SELF-SELECTED DIETS [1]

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The previous reports from this laboratory of the self-selected diets of young Texas college women indicated that these "taller" than average women consumed fewer calories (Davis and Scoular, '57) less protein (Scoular et al., '57a) but more calcium phosphorus and magnesium (Scoular et al., '57b) than the National Research Council's recommended daily allowances ('53). All of the subjects seemed to maintain their weights on this lower calorie level and 66% were in positive protein balance on the lower protein intakes, although many subjects had negative calcium, phosphorus and magnesium balances on the higher mineral intakes. Since these balances were reported only as positive or negative in relation to intakes, the amounts excreted seem worthy of evaluation. In an earlier study of college women on self-chosen diets, McKay et al., ('42) determined the nitrogen, calcium and phosphorus in the feces and urine of their subjects in order to obtain the reported balances, but did not publish the actual amounts excreted. Leverton and Marsh

[1] Originated with Faculty Research Funds and financed with Williams-Waterman Grants during 1954–1956.

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('42), however, published the actual values from which the following percentages were calculated: 79% of the calcium, 41% of the phosphorus and 12% of the protein intake were in the feces and 19, 56, and 81%, respectively, in the urine. Additional data are needed on the excretion of nutrients by subjects consuming self-selected diets. Consequently, it is the purpose of the present study to report the fecal and urinary values obtained for calories, protein, calcium, phosphorus and magnesium during the first 10 years of the present long-time balance study of young Texas college women consuming self-selected diets.

PROCEDURE

The technique used in collecting the food and excreta for analysis was first reported by Holt and Scoular ('48) and more recently by Scoular et al., ('57b). Since the present study involves only the excreta, a brief review of its collection is given here. The 24-hour urine collections were made directly into amber gallon bottles for each of the 5 days of the balance period. After the daily urinary volume was measured and recorded, aliquots were removed and pooled for analysis. Carmine was used as the fecal marker and the usual care in separation of the marker and the feces for each 5-day period was observed. The 5-day fecal collections were weighed, then macerated in a Waring Blendor before aliquots were removed for analysis. These aliquots were dried in a hot-air oven at a temperature below 100°C and the water content determined before grinding in a mortar. One-gram duplicate samples were then burned in a Parr oxygen bomb calorimeter and their calorie values calculated.

Similarly, aliquots of the wet feces were dried before determining the nitrogen (macro Kjeldahl method, Association of Official Agricultural Chemists, '50), calcium (Scott's oxalate method, Furman, '39), phosphorus (molybdate method, Furman, '39) and magnesium (ammonium phosphate method, Association of Official Agricultural Chemists, '50). These same determinations were made directly upon the

urine. The results of both the fecal and the urinary determinations were calculated to obtain the daily average output which was used in obtaining the balances reported previously (Scoular et al., '57a, b) and again in this paper.

RESULTS AND DISCUSSION

With the initiation of the present long-time study of young Texas college women, 17 to 27 years of age, a considerable variation was observed in both the volume of urine and in the wet weight of the feces of the subjects during each 5-day period. These variations seemed unrelated to the diet or to the external temperature. The daily urinary volumes were usually quite similar for a given subject during the 5-day balance period, but varied markedly between subjects. As previously stated, the balances reported by Scoular et al., ('57a, b) were designated merely as positive or negative in comparing the nutrient intakes provided by the self-selected diets. The present report deals with the actual quantity of these nutrients (calories, protein, calcium, phosphorus and magnesium) in the feces and urine of the subjects consuming these diets.

Feces. In the process of preparing fecal samples for burning in the Parr bomb calorimeter, the percentage of moisture in the feces of 117 subjects was determined for 5-day balance periods. Neither the percentage of fecal water nor the total wet weight of the feces was related to the total calories excreted. The percentage of the average total caloric intake excreted in the feces varied little, namely 4 to 7% with an average of 5%. On the dried weight basis, the calorie value was similar for the 50 to 60% and the 80 to 90% water content (4.7 and 4.8 Cal./gm dried weight). Both height and weight of the subjects were used in comparing the fecal excretion of calories and the former was found to give a more uniform basis of comparison just as in the previous comparison of the caloric intake of 93 of these subjects (Davis and Scoular, '57). The average daily wet weight of feces was different for the subjects of the same height consuming the

same foods with the exception of variable amounts of milk during the 5-day period. Error due to separation of the feces from the carmine marker was at a minimum since only three individuals made the separation in this 10-year period of time.

Using height in centimeters as the common reference point, table 1 was constructed to give the calories, protein, calcium,

TABLE 1

Average daily fecal calories, protein, calcium, phosphorus and magnesium compared with wet fecal weight per centimeter of height

WET WEIGHT feces/cm	CALORIES (N=117) [1]	PROTEIN (N = 147) [1]	CALCIUM (N = 129) [1]	PHOSPHORUS (N = 125) [1]	MAGNESIUM (N = 86) [1]
gm	*Cal./gm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*
0.10–0.20			3.7		0.30
0.20–0.30	0.35	15	3.7	0.40	0.15
0.30–0.40	0.45	24	5.1	0.63	0.61
0.40–0.50	0.56	38	5.3	0.91	0.62
0.50–0.60	0.66	38	6.2	0.86	1.15
0.60–0.70	0.73	51	6.4	1.33	0.73
0.70–0.80	0.73	50	7.3	0.89	0.76
0.80–0.90	0.70	39	7.6	1.70	1.20
0.90–1.00	0.74	55	6.2	1.20	0.55
1.00–1.10	0.55	40			
average	0.61	40	5.7	0.99	0.67
γ [2]	0.69	0.75	0.91	0.86	0.61
γ [3]	0.254	0.208	0.228	0.228	0.283

[1] Number of subjects used in obtaining coefficient of correlation, γ.

[2] Coefficient of correlation.

[3] γ, significant at 1% level.

phosphorus and magnesium per centimeter of height with reference to the wet fecal weight per centimeter. From inspection of the table, it is evident that the calorie value of the feces per centimeter increased with an increase in the wet weight of the feces per centimeter. Since the fecal nitrogen was not determined for all of the 171 subjects reported by Scoular et al. ('57a) only those subjects (147) for whom the determinations were made, were included in table 1. Again, as the wet weight of the feces increased, the amount of fecal protein increased. Similar increases in fecal calcium,

phosphorus and magnesium are evident with increased wet weight of feces for the subjects (N) reported by Scoular et al. ('57b) as selecting foods containing unusually high amounts of these elements. The coefficients of correlation (γ) for each nutrient with the wet fecal weight were calculated and are given at the bottom of each column together with those from Snedecor ('53) for the 1% level of significance. All of the coefficients are highly significant being two to 4 times the 1% level.

The average percentages of the nutrients in the feces are 5 of the calories, 16 of the protein, 61 of the calcium, 29 of the phosphorus and 26 of the magnesium intake as compared to Leverton and Marsh's ('42) 12% of the protein, 79% of the calcium and 41% of the phosphorus intakes of their subjects on self-chosen diets. The protein percentage of the feces in the present study is slightly higher than that obtained by Leverton and Marsh ('42) for Nebraska women and is probably due to the greater consumption of vegetable protein (Scoular et al., '57a). The percentage of calcium in the feces is less for the Texas subjects although Bronner and Harris ('56) and Brine and Johnston ('55) found that with larger intakes of calcium more was in the feces. The Texas women averaged more calcium per day than the Nebraska women (1.41 compared to 0.857 gm), yet excreted less in the feces. Walker et al. ('48) reported data on adult humans from which the percentages of calcium, phosphorus and magnesium of the feces were calculated to be 79, 39 and 77%, respectively. The phosphorus content of the feces in the present study is lower than that reported by either Leverton and Marsh ('42) or Walker et al. ('48). The latter authors are the only ones providing a basis for the comparison of fecal magnesium and again the fecal magnesium of the present study is much lower, 26 as compared to 77%. Consequently, the negative balances reported by Scoular et al. ('57a, b) cannot be said to be due to excessive fecal losses.

Urine. In table 2, the urinary protein, calcium, phosphorus and magnesium per centimeter are tabulated for increas-

ing volumes of urine per centimeter of height for the subjects of table 1. The coefficients of correlation are again compared with the 1% level of significance (Snedecor, '53) at the bottom of each column. All of the correlations are significant at the 1% level although those for calcium and magnesium are not as high as those for protein and phosphorus. The greater solubility of the latter may account for this difference.

TABLE 2

Average daily urinary protein, calcium, phosphorus and magnesium compared with urine volume per centimeter of height

URINE VOLUME	PROTEIN (N = 147) [1]	CALCIUM (N = 129) [1]	PHOSPHORUS (N = 125) [1]	MAGNESIUM (N = 86) [1]
ml/cm	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*
3–4	163	1.6	2.6	1.1
4–5	226	3.3	2.8	3.3
5–6	207	3.1	3.1	1.7
6–7	234	3.2	2.9	3.8
7–8	287	3.8	3.1	3.9
8–9	268	3.2	4.0	2.8
9–10	322	5.1	3.4	2.6
10–11	253	4.7	2.4	3.5
11–12	420	5.2	4.2	2.9
12–13	491	2.0	2.5	3.8
13–14	446	3.5	5.1	1.1
14–15				
15–16	276	3.3	5.7	6.0
average	299	3.5	3.5	3.0
γ [2]	0.73	0.33	0.71	0.40
γ [3]	0.208	0.228	0.228	0.283

[1] Number of subjects used in obtaining coefficient of correlation, γ.
[2] Coefficient of correlation.
[3] γ, significance at 1% level.

Leverton and Marsh's ('42) subjects are calculated to have excreted 81% of the protein, 19% of the calcium and 56% of the phosphorus of their diets in the urine as compared to 83, 52 and 50%, respectively, in the present study. The higher urinary calcium excretion of the Texas women which is almost three times that of the Nebraska women (52 compared to 19%) is probably due to the higher intake. According to Knapp ('47) the mean urinary calcium always tends to increase with age and with intake. The two groups of subjects

were similar in age range but the Texas women selected foods higher in calcium than those selected by the Nebraska women. Statistically there is evidence that young Texas college women consuming self-selected diets excrete more of the nutrients when they produce large volumes of urine.

Balances. In table 3 the balances are given for protein, calcium, phosphorus and magnesium distributed into the two age groups used in evaluating food intake (Davis and Scoular, '57, Scoular et al., '57a, b). Group I includes the women under 20 years of age and group II those 20 years of age and over. The reason for tabulating the balances according to age of the subjects is based on the fact that certain differences had been noted between the intake and the number of negative balances occuring in the two age groups. Forty-one per cent of group I had negative protein balances in comparison with 30% for group II on slightly higher average intakes (Scoular et al., '57a), while the negative balances of group I were 60% of the calcium, 14% of the phosphorus and 50% of the magnesium balances compared to 37, 42 and 27%, respectively, for group II (Scoular et al., '57b). Inasmuch as the actual degrees of the positive and negative balances were being compared with intakes per unit of height it seemed advisable to determine whether the amounts ingested influenced these group differences. The coefficients of correlation are compared with the 1% level of significance of Snedecor ('53) as in tables 1 and 2. The coefficients of correlation exceed the 1% level of significance for all nutrients under group I but only for protein and calcium in group II. The coefficients of correlation for phosphorus and magnesium under group II are not significant at either the 1% or the 5% levels. The positive protein balances for both groups I and II may be assumed to increase with increased protein intake. The coefficient of correlation for group I is very significant but not as high as that for group II. On the basis of the actual daily weight of protein ingested, the older women (group II) consumed more for each height represented (Scoular et al., '57a) than the younger women (group I).

TABLE 3

Average daily balances for protein, calcium, phosphorus and magnesium compared with intake based on height

PROTEIN BALANCE			MINERALS BALANCE						
				Group I			Group II		
Intake	Group I [1]	Group II [1]	Intake	Ca [2]	P [3]	Mg [4]	Ca [2]	P [3]	Mg [4]
mg/cm	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*	*mg/cm*
100–150	45		0.0– 1.0			–1.7			–0.5
150–200	68	–106	1.0– 2.0			–2.8			–1.0
200–250	–66	–139	2.0– 3.0						
250–300	31	13	3.0– 4.0	–2.4	2.0	0.8		0.4	2.0
300–350	107	10	4.0– 5.0	1.3	0.9	2.5	–1.3	3.0	2.0
350–400	92	31	5.0– 6.0	–1.8	1.7	2.0	–6.1	0.9	1.2
400–450	148	90	6.0– 7.0	–0.3	1.4	2.0	5.0	1.3	5.0
450–500	81	92	7.0– 8.0	–0.6	1.3	1.8	1.9	1.5	3.0
			8.0– 9.0	2.6	2.0	3.6	1.4	1.7	4.0
			9.0–10.0	1.8	8.0		–2.3	4.0	
			10.0–11.0	1.5	7.0		1.0		
			11.0–12.0	1.9	6.0		–2.8	6.0	
			12.0–13.0	5.3	6.6		3.3		
			13.0–14.0	4.5			5.0		
			14.0–15.0	3.0	9.3	5.0			1.0
			15.0–16.0		8.0	3.0	2.5		1.0
average	63	–1.3	8.0	1.4	4.7	1.6	0.7	2.4	1.8
γ [5]	0.500	0.885		0.724	0.790	0.691	0.370	0.122 [6]	0.237 [6]
γ [7]	0.267	0.354		0.325	0.325	0.393	0.325	0.325	0.393

[1] Group I, 90 subjects; group II, 57.
[2] Group I; 66 subjects; group II, 63.
[3] Group I, 63 subjects; group II, 62:
[4] Group I, 42 subjects; group II, 44.
[5] Coefficient of correlation.
[6] Not significant at either 1% or 5% levels.
[7] γ, significance at 1% level.

This difference is further indicated by the lower γ (only twice the level of significance) for group I, 0.50 as compared to 0.885 for group II (two and one-half times 1% level). Since the percentage of the protein intake in the feces (16% as compared to 12% in the Nebraska study) was similar for all subjects it is assumed that if the younger subjects (group I) selected more protein rich foods that they too would have more positive protein balances.

That the increased intakes of calcium, phosphorus and magnesium per unit of height for group I is statistically significant with coefficients double the 1% level suggests that the selection of foods high in these minerals is desirable. McKay et al. ('42) calculated the coefficients of correlations for intake and retentions as 0.73 for nitrogen, 0.50 for calcium and 0.58 for phosphorus. They concluded that the results showed that place, age and weight differences were not significant for their 124 subjects and that intake was significantly related to retentions. In the present study age differences may be involved but the geographic ones do not exist. Weight has been shown to give low correlations with the nutrient intake of the subjects of the present study. However, the older subjects (group II) have lower coefficients of correlation for all three minerals with those for phosphorus and magnesium lacking significance at both the 1% and 5% levels. The calcium balances appear to be dependent upon the intake in each group, the higher the intake per centimeter of height the more positive the balance. The phosphorus and magnesium ingestion levels of group II were not comparable to those of group I (notably lacking at the 10, 12, 14 and 15 mg/cm levels of phosphorus intakes whereas subjects of group I consumed such levels of intake). The lack of correlation between magnesium intake and balance for the older subjects may be of little importance. Too few such balance studies have been reported to draw conclusions. The larger number of negative phosphorus balances of group II reported by Scoular et al. ('57b) are clearly due to the lower levels of intake per centimeter although the average daily total

was higher than that indicated by the National Research Council ('53) as being desirable. From these data the tendency of the older subjects (group II) to select foods providing less phosphorus should be discouraged.

SUMMARY

The fecal and urinary excretions of nutrients (calories, protein, calcium, phosphorus and magnesium) by young college women consuming self-selected diets have been compared on the basis of height in centimeters.

The greater the wet fecal weight per unit of height, the greater the fecal nutrient loss. Similarly, the higher the volume of urine per unit of height, the greater the urinary nutrient loss.

The 16- to 20-year olds (group I) who consumed a high level of intake per unit of height had more positive balances. This was also true of group II (over 20 years) with respect to protein and calcium intake but not of phosphorus and magnesium.

The average percentage of calories, protein, calcium, phosphorus and magnesium intake in the feces was 5, 16, 61, 29 and 26%, while the average percentage of protein, calcium, phosphorus and magnesium in the urine was 83, 52, 50 and 26, respectively.

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EFFECT OF YEAST AND OF YEAST EXTRACTS ON LIVER NECROSIS AND HEMOLYSIS BY DIALURIC ACID OF RED BLOOD CELLS OF RATS ON A NECROGENIC DIET [1,2]

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The intravascular hemolysis caused by injection of alloxan in rats on a vitamin E-deficient diet can be prevented by feeding vitamin E or it can be reduced in incidence and severity by adding yeast supplements to the diet (Rose and György, '49). This report deals with further indications of a protective effect of dietary yeast in reducing damage associated with vitamin E deficiency. Red blood cells of rats on vitamin E-deficient diets are readily hemolyzed *in vitro* by dialuric acid (Rose and György, '50). This defect of the cells can be corrected by feeding vitamin E to the rats (Rose and György, '52) or, as this report shows, it can be corrected by supplementing the vitamin E-deficient diet with yeast or yeast extracts. These yeast supplements or yeast extracts also prevented hemorrhagic liver necrosis in rats fed a vitamin E-deficient necrogenic diet.

METHODS

Male weanling rats of the Sprague-Dawley strain weighing between 40 and 50 gm were given the basal necrogenic Y5H diet used in previous studies (György, Stokes, Goldblatt and

[1] A portion of the information in this publication has been presented in abstract form in Federation Proc., *13:* 210, 1954.

[2] Supported in part by the Subcommittee on Liver of the Advisory Committee on Metabolism, through Army Contract no. DA-49-007-MD-78, Office of the Surgeon General of the United States Army.

Popper, '51) or the Y5T diet in which torula yeast[3] was substituted for the British bakers' yeast[4]. The diet consisted of yeast (British bakers' or torula), 18%; cornstarch, 79%; salt mixture U. S. P. II, 3%; cod liver oil, 1.4 ml; peanut oil, 6 ml. A vitamin supplement consisting of thiamine, 20 μg; riboflavin, 25 μg; pyridoxine, 20 μg; calcium pantothenate, 100 μg; and menadione, 20 μg dissolved in 1 ml of water was offered daily to each rat. On the basal diets the rats as a rule succumbed with liver necrosis within 100 days. Rats were offered 8 gm of diet daily and food intake was recorded. Supplementation with yeast was made by substitution for equal weights of starch. Vitamin E supplements were fed weekly or every two days in aliquots of mixed tocopherols[5] diluted in peanut oil to furnish either 10 mg of α-tocopherol/rat/week or 0.35 mg/rat/week.

Yeast extracts of British bakers' yeast or of Fleischmann's bakers' yeast (type 5009) were prepared by refluxing one kilogram of yeast in 10 liters of 95% ethanol for 8 hours. The alcoholic extract was allowed to stand at 5° C. for 24 hours and was then filtered. The combined filtrates of 5 such batches were concentrated under reduced pressure and the alcohol was distilled off. Aliquots of the residue were lyophilized and used in the feeding experiments. Approximately 200 gm of dry material was obtained from the alcoholic extract of 5 kg of yeast. Ether extracts were prepared by suspending the alcohol-soluble residue in a 10% (W/V) solution of water and ethyl ether, adjusting to pH 8.0 with 4N NaOH, and continuously extracting the suspension with ether for 48 to 72 hours. Approximately 30 gm of dry material was obtained from the ether extract of 200 gm of the alcohol-extracted residue of yeast. The ether solution was dried over sodium sulfate and filtered, the filtrate was concentrated *in vacuo* in an atmosphere of nitrogen and the residue was suspended in a 50% ethanol solution. Aliquots of this suspension were mixed with portions of the necro-

[3] Lake States Yeast Corporation, Rhinelander, Wisconsin.
[4] Distillers Ltd. Co., Glasgow, Scotland.
[5] Distillation Products Industries, Rochester, New York.

genic diet so that 6 gm of diet (which was the average daily intake) contained 50 mg of dry weight of ether extract.

The percentage of hemolysis was determined according to the method described by Rose and György ('52). The action of dietary yeast supplements on the resistance to hemolysis of the erythrocytes of the rats was consistent but only partial and it was found necessary to decrease the amount of hemolytic agent to 25 μg for all groups in these experiments to demonstrate the effect. In the experiments in which the alcoholic extracts of yeast and ether extracts of alcohol-soluble yeast fraction were fed, the erythrocytes of all rats were, however, tested with the 50 μg level of dialuric acid. Red blood cells of rats in all groups in one experiment were tested simultaneously in duplicate using the same solution of dialuric acid. Readings of duplicates usually checked within 10% hemolysis. Repeat tests on blood samples of rats taken at a few days' interval gave essentially the same results.

The *in vitro* protective effect of yeast extracts was determined according to the method of Rose and György ('52) with slight modifications. A weighed sample of yeast extract was dissolved in alcohol to make a solution containing 1 mg/ml. Aliquots of this solution containing various quantities of yeast extract were distributed in a series of Wasserman tubes and the alcohol was evaporated by immersing the tubes in boiling water for a few minutes. To each tube was then added 0.1 ml of 0.05 M phosphate buffer of pH 7.4, 0.12 ml of a 5% suspension in saline of erythrocytes from a vitamin E-deficient rat and 40 μg of dialuric acid in 0.04 ml of phosphate buffer. The tubes were incubated at 37° C. for 15 minutes and were then left to stand at room temperature for two and a half hours after which the minimum quantity of extract preventing hemolysis was determined. Activity was expressed as the number of "hemolysis prevention" units per milligram, one unit being the minimum weight of extract preventing hemolysis by dialuric acid of erythrocytes from vitamin E-deficient rats.

Reverse-phase paper chromatography of the ether extract of the residues of the alcoholic extracts of yeast were carried

out according to Eggitt and Ward ('53). Chromatograms were first examined for fluorescent substances by exposing them in the dark to the light of an ultraviolet lamp equipped with a long-wave filter, and then developed by spraying with the Emmerie-Engel reagents (0.5% α, α', dipyridyl and 0.2% ferric chloride, both dissolved in alcohol). A spot active in the hemolysis-prevention test was located by cutting the chromatogram into 10 equal parts from the origin to the front and by eluting each section with 4 ml of ethanol. After removal of the alcohol by heating the tubes in a boiling-water bath, the residues of aliquots of these eluates were tested for their ability to protect vitamin E-deficient erythrocytes from hemolysis by dialuric acid.

RESULTS

"In vivo" effects of yeast and yeast extracts on rats on necrogenic diets. Red blood cells from rats on a diet containing 40% British bakers' yeast are less susceptible to the hemolytic action of dialuric acid than the red blood cells of rats on the basal necrogenic diet which contains only 18% bakers' yeast (table 1, exps. 1 and 2). The protection afforded to the erythrocytes of rats receiving dietary supplements of yeast is only partial, but it is consistent and the difference between the average hemolysis and that of the control group is statistically significant ($P < 0.01$) in all experiments. The increased resistance to hemolysis of the red blood cells of rats receiving the additional yeast is not related to the corresponding increase in sulfur amino acids in the diet, since the addition of cystine in an amount equivalent (0.3%) to that of the methionine plus the cystine (György, Rose, Tomarelli and Goldblatt, '50) present in the additional yeast does not result in an increased protection of the erythrocytes. A higher level of supplemental cystine (1%), in contrast to the lower level, protects rats from liver necrosis [6] but does not increase the resistance of the erythrocytes (table 1, exps. 1 and 2). Adequate dietary supplements of vitamin E (10 mg/week) completely prevent the sus-

[6] Diagnosis of liver necrosis was based on histological examination of all specimens by Dr. Harry Goldblatt (Mt. Sinai Hospital, Cleveland, Ohio).

TABLE 1

Effect of various supplements on (a) the susceptibility to hemolysis by dialuric acid of erythrocytes of rats fed necrogenic diets and (b) the incidence of liver necrosis

EXP.	BASAL DIET	SUPPLEMENTS TO BASAL DIET	AV. HEMOLYSIS [1]	MORTALITY [2]	AV. TIME ON DIET AT DEATH	LENGTH OF EXP.
			%		*Days*	*Days*
1	Y5H	None	76 ± 2 [3]	8/8	32 ± 5 [3]	120
		0.3% cystine (20 mg/day)	61 ± 2	8/8	82 ± 4	
		Vitamin E (10 mg/week)	0	0/6	—	
		22% British bakers' yeast (1.32 gm/day)	43 ± 1	0/8	—	
2	Y5H	None	78 ± 2	6/10	108 ± 4	162
		1% cystine (60 mg/day)	81 ± 2	0/8	—	
		Vitamin E (50 μg/day)	57 ± 2	3/10	153 ± 3	
		22% British bakers' yeast	37 ± 3	0/10	—	
3	Y5T	None	Not deter.	10/10	38 ± 3	75
		1% cystine	79 ± 2	0/8	—	
		Alcoholic yeast ext. (160 mg/day) [4]	24 ± 6	6/10	43 ± 4	
		None	Not deter.	10/10	45 ± 3	
		Alcoholic yeast ext. (320 mg/day) [4]	Not deter.	0/10	—	
4	Y5T	None	Not deter.	9/10	63 ± 3	160
		1% cystine	82 ± 7	0/10	—	
		Alcoholic yeast ext. (250 mg/day)	10 ± 1	0/10	—	
		Ether ext. of alcoholic ext. (50 mg/day) [5]	6 ± 1	0/10	— [6]	

[1] Average hemolysis determined on 25th day in exp. 1, 160th day in exp. 2, 38th day in exp. 3 and 74th day in exp. 4.

[2] Fractions indicate number of rats dead with liver necrosis/rats started.

[3] Mean ± standard error of the mean.

[4] In those experiments the same alcoholic extract of British bakers' yeast was used. Three hundred twenty milligrams of alcoholic extract is equivalent to about 6 to 8 gm of dry yeast.

[5] In this experiment extracts of Fleischmann's bakers' yeast were used. Two hundred fifty milligrams of alcoholic extract is equivalent to about 6 gm of yeast, 50 mg of ether extract of the alcohol soluble fraction represents about 8 gm of yeast.

[6] After 112 days the rats were taken off the ether extract and continued to receive the basal diet. Between 15 and 42 days after discontinuation of ether extract supplement 8 animals died with liver necrosis. Fifteen days after the ether extract supplement was discontinued average hemolysis was 95%.

ceptibility to hemolysis by dialuric acid of the red cells of rats on the basal diet, but a suboptimal level of vitamin E (50 μg/day) provides only partial resistance, a degree of protection similar to that afforded by a diet containing 40% instead of 18% of yeast (table 1, exps. 1 and 2). Alcoholic extracts of yeast and ether extracts of the residues of these alcoholic extracts have an even more marked effect than the yeast supplements in increasing the resistance to hemolysis of the red blood cells of the rats. Thus a daily supplement of 50 mg of the ether extract almost completely reverses the susceptibility to hemolysis by dialuric acid of the erythrocytes of the rats on the vitamin E-deficient necrogenic diet (table 1, exps. 3 and 4).

The data of table 1 further show that increasing the yeast content of the necrogenic diet from 18 to 40% also prevents liver necrosis in rats. These findings are in agreement with observations by McLean and Beveridge ('52). A protective principle against liver necrosis in yeast can apparently be extracted with alcohol and ether. Indeed, as shown in table 1 (exps. 3 and 4), a supplement of 320 mg of alcoholic extract of British bakers' yeast or 250 mg of extract of Fleischmann's bakers' yeast protected the rats from liver necrosis. Ether extraction of the alcohol-soluble material of yeast resulted in a further enrichment in protective constituents. Thus a daily supplement of 50 mg of the ether extract of the residue of an alcoholic extract of Fleischmann's bakers' yeast afforded complete protection against liver necrosis (table 1, exp. 4).

"In vitro" effects of yeast extracts on vitamin E-deficient erythrocytes. Small quantities of α-tocopherol added to suspensions of erythrocytes from rats on a vitamin E-deficient diet protect the cells from hemolysis by dialuric acid. Yeast extracts were found to have a similar *in vitro* effect. Thus a minimum of 10 mg of aqueous lyophilized extract of British bakers' yeast or 0.2 mg of alcoholic extract or 0.005 to 0.01 mg of an ether extract of the residue of an alcoholic extract afforded a protection from hemolysis to a standard suspension of vitamin E-deficient cells similar to that afforded by 0.0005 mg of tocopherol. Yeast extracts, in contrast to α-

tocopherol, were active in the *in vitro* test without the addition of Tween 80, required to solubilize the tocopherol in the aqueous reaction system. Yeasts of various types were found to differ in their ability to protect vitamin E-deficient red blood cells. Thus, as can be seen from table 2, the alcoholic extracts of Fleischmann's brewers' and bakers' yeasts contain higher amounts of hemolysis-preventing activity than torula and British bakers' yeast.

The effect of dietary yeast supplements and yeast extracts in increasing the resistance of erythrocytes and protecting

TABLE 2

Hemolysis-prevention activity in the ethanol extracts of various yeasts[1]

TYPE OF YEAST	DRY WEIGHT OF ALCOHOLIC EXTRACT	UNIT/MG	TOTAL UNITS
	gm		
Torula	6.7	1	6,700
British bakers'	3.1	5	15,500
Fleischmann's bakers'	6.7	5	33,500
Fleischmann's brewers'	5.7	16	91,000
d α-Tocopherol		2000	

[1] One hundred-gram samples of each of the 4 types of yeasts were simultaneously refluxed in 95% ethanol for 8 hours. Aliquots of the filtrates of these extracts were tested for their hemolysis-preventing activity.

rats from liver necrosis would suggest the possible presence in yeast of small amounts of vitamin E. However, vitamin E has not been reported to be present in yeast. Ether extracts of the residue of alcoholic extracts of yeast and of alcoholic extracts saponified with KOH under nitrogen were run on reverse-phase paper chromatograms. Small amounts of the tocopherols (5 μg) mixed with 0.5 mg of the yeast extracts and run on paper chromatograms could readily be demonstrated after developing the paper with the Emmerie-Engel reagents. No spots of an Rf corresponding to those of alpha- (Rf 0.17), beta-(Rf 0.38), gamma-(Rf 0.36) or delta-tocopherol[7] (Rf 0.53) run in parallel on the same chromatograms were

[7] The samples of beta, gamma and delta tocopherol were kindly provided by Dr. P. L. Harris (Distillation Products Industries, Rochester, New York).

however detected in as much as 1 mg of the alcohol or ether extracts of yeast. A blue fluorescent spot of Rf 0.84 which gave a positive Emmerie-Engel reaction was, however, consistently found in chromatograms of these extracts of British bakers' yeast or of Fleischmann's bakers' yeast. This substance was shown to be the factor responsible for the activity of the ether extracts in protecting suspensions of vitamin E-deficient erythrocytes from hemolysis by dialuric acid. The residue of the alcohol eluate of the section of the chromatogram incorporating the blue fluorescent spot in contrast to the residues of the eluates of all other sections of the paper was found to be the only one active in protecting vitamin E-deficient erythrocytes from the hemolytic action of dialuric acid. Thus, using the protective activity of fractions of yeast extracts as a guide, it became possible to isolate the substance responsible for the *in vitro* protection of vitamin E-deficient erythrocytes. The isolation of this substance from yeast is the subject of a separate report.

DISCUSSION

Dietary supplements of yeast or extracts of yeasts, when given to rats on a necrogenic diet, have an effect similar to that of vitamin E, namely, increasing the resistance of erythrocytes to hemolysis by dialuric acid and decreasing or preventing the incidence of liver necrosis. Available information is insufficient to determine whether these two effects are related to the same factor or factors in the yeast supplements. Similarly, the relationship of the alcohol- and ether-soluble factors in bakers' yeast to Factor 3 of Schwarz ('51a) found in brewers' yeast is unknown. Yeast extracts added to suspensions of vitamin E-deficient red blood cells protect them from the hemolytic action of dialuric acid. Fleischmann's brewers' and bakers' yeasts were most active in this test. It is of interest that these two types of yeast are not suitable to induce dietary liver necrosis in rats, while torula and British bakers' yeast are used routinely for this purpose (György *et al.*, '50) and (Schwarz, '51b). The activity in the *in vitro* test

of the yeast extracts is due to a blue fluorescent substance which gives a positive Emmerie-Engel test and which is different from the tocopherols. The role of this substance in preventing liver necrosis in rats on necrogenic diets and in reversing the susceptibility to hemolysis by dialuric acid of the red blood cells of these rats remains to be determined.

SUMMARY

Dietary supplements of British bakers' yeast, Fleischmann's bakers' yeast, alcoholic extracts of yeast, or the ether extracts of the residue of the alcoholic extracts prevented or decreased the incidence of hemorrhagic liver necrosis in rats on diets which regularly induce this fatal liver injury. Like vitamin E, these extracts of yeast, when fed as dietary supplements, also increase the resistance to *in vitro* hemolysis by dialuric acid of the erythrocytes of the rats on the vitamin E-deficient diets. In contrast, dietary supplements of cystine which protected rats from liver necrosis did not reverse the characteristic susceptibility to hemolysis by dialuric acid of the erythrocytes of the rats.

The same alcoholic extracts of yeast or ether extracts of the alcohol-soluble fractions of yeast when added to red blood cell suspensions of vitamin E-deficient rats, like vitamin E, protected them from the hemolytic action of dialuric acid. The active principle in the extracts protecting the vitamin E-deficient erythrocytes was found to be a blue fluorescent substance which gives a positive Emmerie-Engel reaction and is different from alpha-, beta-, gamma-, or delta-tocopherol.

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THE ROLE OF LYSINE IN THE GROWTH AND FEATHER PIGMENTATION OF TURKEY POULTS

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Lysine is an amino acid which is essential for the growth of turkey poults and appears to be specifically needed for the prevention of depigmentation of their flight feathers (Vohra and Kratzer, '57). The effect of a deficiency of lysine on growth is probably due to an impairment in the synthesis of protein. It is not known whether the depigmentation of poult feathers in a deficiency of lysine is related to impaired protein synthesis or is a result of a deficiency of some vitamin or non-peptide hormone which may be derived from lysine.

The experiments with a single delayed supplementation of the required amino acid to an amino acid-deficient diet have proved useful in elucidating the "protein" and "extra-protein" function of amino acids (Geiger, '50). If the growth of poults and the pigmentation of their feathers are both related to the "protein" function of lysine, then a single daily dose of lysine to the lysine-deficient poults should neither support growth nor prevent depigmentation. This is because there is no appreciable storage of amino acids in the body and they will not be available for protein synthesis when lysine is made available at a later time. However, if an "extraprotein" function is involved, the presence or absence of other amino acids should not influence the re-

sponse to lysine and normal pigmentation should result from a single daily dose of lysine.

Work with other amino acids has confirmed the value of this experimental method. A single supplement of tryptophan was less effective for the growth of rats than feeding this amount in smaller doses more frequently (Berg and Rose, '29). Rats also failed to maintain a positive nitrogen balance when given delayed injections of tryptophan (Elman, '39). A delayed supplement of tryptophan to niacin-deficient, instead of tryptophan-deficient, rats gave increased growth (Geiger, '47; Geiger et al., '49). This was explained by the fact that tryptophan was utilized in the synthesis of niacin by the animal and this pathway might be independent of the need of tryptophan for protein synthesis.

The delayed supplementation of lysine in rats on a lysine-deficient diet did not support growth (Geiger, '47; Henderson and Harris,'49). Only a limited utilization of lysine was observed when injected subcutaneously or intraperitoneally in contrast to the more effective utilization when lysine was mixed in the diet of the rats (Conrad and Berg, '37). However, good recovery in rats had been observed when lysine was injected subcutaneously while the other essential amino acids were fed orally. The injection of the missing component increased the consumption of the deficient diets (Frazier et al., '47).

EXPERIMENTAL

The lysine-deficient diet had the following composition in grams per 100 gm: sesame seed oil meal, 57.0; ground corn, 29.0; tribasic calcium phosphate, 4.5; soybean oil, 2.0; vitamin mix, 2.0; fish solubles, 2.0; choline chloride (25%), 0.8; cornstarch, 3.4; iodized salt, 0.8; vitamin A in dry carrier (10,000 units/gm), 0.1; vitamin D in dry carrier (1500 units/gm), 0.1; vitamin E in dry carrier (44 units/gm), 0.1; $MnSO_4$, 0.025; biotin, 0.00002; and vitamin B_{12}, 0.000001. The vitamin mixture contained the following in grams: riboflavin, 2.0; thiamine-HCl, 2.0; pyridoxine-HCl, 2.0; calcium pantothenate, 6.0;

niacin, 20.0; folic acid, 1.0; vitamin K, 2.0; and cornstarch, 4000.0. In the lysine-supplemented ration, DL-lysine monohydrochloride was added at a level of 1.35% to replace an equivalent amount of starch.

Broad Breasted Bronze turkey poults which had been fed a commercial ration for 2 to 3 days after hatching and sexing were used in the present study. Each treatment was carried out in duplicate with 5 to 8 birds per group. A total of three trials were carried out.

In two trials an attempt was made to determine the feed consumption per bird per day. To do this, the feeders were weighed each day without applying any correction for any spillage or other waste by the birds. For the first 3 to 4 days the birds consumed about 4 gm of feed per day and for the rest of the experimental period the maximum feed consumption was about 8 gm daily. Based on this feed consumption, the amount of DL-lysine monohydrochloride that had to be administered daily to provide the equivalent to 1.35% of the diet was found to be 54 and 108 mg respectively. Slightly more lysine than this was administered by the daily doses since numbers 5 and 4 gelatin capsules were used which contained an average of 61 and 119 mg of DL-lysine monohydrochloride respectively. Each bird received a daily supplement of 61 mg lysine for the first 4 days and 119 mg for the rest of the experimental period.

In each trial the birds were divided into 5 groups which were studied in duplicate. Groups A and B had the lysine-deficient and lysine-supplemented diets respectively available to them at all times. Group C was given a single daily supplement of lysine but the lysine-deficient diet was available all the time. Groups D and E did not have access to the lysine-deficient diet for 4 hours; however, the group E was given a single daily supplement of lysine just after the removal of the feed. The trials were carried out for periods varying from 16 to 19 days and the birds were weighed twice every week. At the end of the trials, the birds were scored for any depigmentation in their flight feathers. The score varied from 0 to 4 for black to completely white emerging feather follicles.

TABLE 1

The effect of a single supplement of lysine compared to lysine mixed in the feed on the growth and feather pigmentation of turkey poults

			EXPERIMENT NO.					
			1		2		3	
DURATION OF THE TRIAL IN DAYS			16		17		19	
Group	Supplement	Feeding time	Average daily gain per bird	Color score	Average daily gain per bird	Color score	Average daily gain per bird	Color score
		hrs.	%		%		%	
A	None	24	3.83 (11/11) [1]	4.0	3.46 (15/15)	3.9	3.60 (14/16)	4.0
B	Lysine in diet	24	6.52 (12/12)	0.3	6.83 (16/16)	0.0	6.26 (14/16)	0.0
C	Single lysine capsule daily	24	4.10 (12/12)	2.6	4.52 (9/9)	2.1	3.91 (16/16)	4.0
D	None	20	3.46 (10/10)	4.0	3.68 (13/16)	4.0	3.60 (13/16)	4.0
E	Single lysine capsule daily	20	4.42 (10/10)	2.7	4.09 (16/16)	2.9	4.09 (15/15)	4.0

[1] The figures within parentheses indicate the number of birds surviving/total birds started on the experiment.

RESULTS AND DISCUSSION

From the results in table 1 it appears that the removal of the feed for 4 hours (from 10 a.m. to 2 p.m.), as in groups D and E, did not affect the growth of the turkey poults adversely when compared to groups A and C in which the feed had been available all the time. The pigmentation of the flight feathers was also comparable in the corresponding groups. The administration of the amino acid in the diet was far superior to a single supplement of an equivalent amount of lysine to the birds for growth as well as feather pigmentation. In all experiments, the administration of a single daily dose of lysine gave slightly better growth than no supplementation, and pigmentation of the flight feathers was slightly improved, except in experiment 3. The turkey poults can store some feed in their crops, and when a single daily supplement of lysine was administered, the other essential amino acids might have been available in the crop to allow a partial protein synthesis. This may explain the partial improvement noticed in the trials 1 and 2.

The fact that a single daily supplement of lysine is inferior to the same amount of lysine present in the feed where other amino acids are also present for growth and feather pigmentation in turkey poults strongly suggests a "protein-function" of lysine. This is further confirmed by the fact that these experiments did not separate the function of lysine for the formation of pigment from its requirement for growth. If lysine were to act like a precursor of some vitamin which were to be involved in the development of pigment, a single supplement of it should have improved the color of the feathers more than the growth of the birds. If lysine is a precursor of some hormone, then one would expect that hormone to be a polypeptide. As a part of the polypeptide chain, lysine could play an important role both for growth and for feather pigmentation.

SUMMARY

The administration of lysine in a single daily dose to turkey poults was inferior both for growth and for feather pigmentation when compared to the same amount of lysine mixed in

the diet. Growth and pigmentation were closely associated. It is postulated that the role of lysine in pigment formation is through its necessity for protein synthesis rather than an "extraprotein" function.

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THE IMPROVEMENT OF THE PROTEIN QUALITY OF WHITE RICE BY LYSINE SUPPLEMENTATION

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The improvement in nutritive quality of the protein of a food or feed attainable by amino acid supplementation is dependent upon many factors. Certain principles governing successful supplementation have evolved as a result of many studies carried out in recent years. Such principles include the recognition of the essential amino acid requirement of the species or individual which is to benefit from the improved protein and the amino acid composition of the food to be improved. The key step, then, involves supplementation of the food with the first limiting essential amino acid to the extent of bringing the total amount of the first limiting amino acid in balance with the second limiting amino acid according to the species' requirements. While this procedure assures a protein of superior nutritive quality, the organism consuming it will obtain benefit from the improvement only when the diet includes energy from non-protein sources in an amount sufficient to assure utilization of the balanced portion of the portion of the protein for tissue synthesis and repair.

Application of these sound principles has been rewarding in several instances, e. g. in studies on the lysine supplementation of bread (Rosenberg and Rohdenburg, '52) and on the methionine supplementation of corn-soybean oil meal diets for the growing chick (Baldini and Rosenberg, '55). In the present work it has been demonstrated that the protein quality of white rice can be significantly improved by the addition of

relatively small amounts of lysine as the sole supplementary amino acid. This is in agreement with the concept that lysine is the first limiting amino acid in white rice (Mitchell and Block, '46) and that only small amounts of supplementary lysine are necessary to balance this amino acid against the second limiting amino acid according to the rat's requirements as stated by Rose ('37) and modified by Rao, Metta and Johnson ('57).

TABLE 1

Composition of basal diet

INGREDIENT	AMOUNT
	%
Ground rice	90.0
Vitamin mix [1]	1.5
Salt mix [2]	3.0
Crude soybean oil	3.0
Cod liver oil	1.5
Diammonium citrate (or rice)	1.0
Total	100.0

[1] At the 1.5% level, Vitamin Mix supplies per 100 gm diet:

Choline chloride	150.0 mg	Vitamin B_{12} (0.1% triturate)	1.500 mg
Inositol	75.0 mg		
PABA	75.0 mg	Thiamine	0.750 mg
α-Tocopherol acetate	7.5 mg	Pyridoxine. HCl	0.750 mg
Niacin	3.0 mg	Menadione	0.375 mg
Riboflavin	1.5 mg	Folic acid	0.075 mg
Ca Pantothenate	3.0 mg	Biotin	18.750 μg

[2] Hubbell, Mendel and Wakeman, '37.

EXPERIMENTAL PROCEDURE

Five growth experiments involving the supplementary value of lysine in rice diets have been carried out with weanling animals from our colony of hooded rats. At 21 days of age litter mates were distributed among the various treatments so as to assure equal average weight for all groups. The animals were housed individually in cages with raised screen bottoms, and food and water were supplied ad libitum. Individual weekly records were kept on weight gains of the

animals and the amount of food consumed. In all experiments but one (experiment 5) 6 male and 6 female animals were assigned to each treatment. The studies were carried out during a growth period of 5 weeks. Experiment 5 was

TABLE 2

Improvement of rice diet by lysine supplementation (5-Week rat growth data)

L-LYSINE HCL SUPPLEMENTATION TO 90% RICE DIET	GAIN IN GRAMS		FOOD/GAIN	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
%				
		Males		
0.00	51.1 ± 4.16[1]	62.5 ± 6.68[1]	8.12 ± 0.42[1]	7.41 ± 0.51[1]
0.025	- -	67.8 ± 7.16	- -	6.70 ± 0.36
0.05	70.1[2] ± 3.79	82.5[2] ± 4.41	6.56[2] ± 0.33	5.82[2] ± 0.19
0.10	63.8 ± 6.50	76.6 ± 12.71	7.12 ± 0.70	6.17 ± 0.56
0.20	51.3 ± 4.66	72.0 ± 5.78	7.84 ± 0.74	6.17 ± 0.26
0.30	- -	72.0 ± 4.72	- -	6.05 ± 0.34
0.40	49.6 ± 3.33	- -	7.14 ± 0.42	- -
0.80	52.6 ± 4.50	- -	6.55 ± 0.27	- -
		Females		
0.00	61.2 ± 5.33	71.1 ± 3.47	7.33 ± 0.32	6.62 ± 0.14
0.025	- -	84.1 ± 4.98	- -	5.88 ± 0.07
0.05	70.5[2] ± 3.51	69.6 ± 6.73	6.81 ± 0.28	6.70 ± 0.33
0.10	88.0[2] ± 7.83	90.4 ± 10.31	5.74[2] ± 0.25	5.58[3] ± 0.24
0.20	70.8 ± 8.35	76.3 ± 12.36	6.42 ± 0.33	6.60 ± 0.73
0.30	- -	73.8 ± 6.20	- -	6.40 ± 0.51
0.40	70.0 ± 8.81	- -	6.14 ± 0.36	- -
0.80	57.8 ± 5.11	- -	7.15 ± 0.59	- -

[1] Standard deviation of the mean.
[2] Significant difference (t-test).
[3] Highly significant difference (t-test).

started with 20 male and an equal number of female weanling rats per treatment. In the latter experiment 10 animals of each sex and each treatment were sacrificed after the initial 5 week growth period for determinations of liver fat, liver protein and liver glycogen. The remaining animals were kept on their respective diets for further growth studies until they were mature.

The composition of the basal rice diet used in all experiments is shown in table 1. Precooked, air-dried rice was ground to 20 to 40 mesh and all vitamins and minerals known to be required by rats were added to the ration. In experiments 1, 2 and 3, 1% of diammonium citrate was included in the diet. Substituting graded levels of L-lysine·HCl [1] for the same levels of diammonium citrate resulted in isonitrogenous diets throughout the experiments. In the other three experiments diets containing 91% rice were used and the L-lysine·HCl replaced equal amounts of rice. Since earlier exploratory experiments have indicated that the protein content of rice was increased slightly by cooking, causing an increase in the growth rate of rats and in the dietary protein efficiency, precooked rice was used throughout this study. In the last experiment parboiled rice and raw, long-grain rice were compared with pre-cooked rice.

RESULTS

In experiments 1 and 2 graded levels of L-lysine·HCl were added to the basal diet. The design of these experiments is seen in table 2, which also gives the total gain of the animals and the efficiency of food utilization. Supplementation of the rice diet with 0.05% of L-lysine·HCl improved the growth of the male animals 37 and 32% in the two experiments. Supplementation with a smaller amount, 0.025% of L-lysine·HCl, was considerably less beneficial, and supplementation with larger amounts of lysine did not give further improvements. In fact, the improvements in gain and food efficiency obtained with 0.05% of L-lysine·HCl were not maintained when larger amounts of lysine were supplied. The female rats behaved very similarly, as seen from table 2, maximum benefit being obtained when 0.1% of L-lysine·HCl was added to the diet.

In order to obtain further information on the improvement of the quality of rice protein attainable with lysine supplementation, a diet very similar to that described, but without the

[1] du Pont L-lysine · HCl containing 95% L-lysine · HCl and 5% D-lysine · HCl. D-lysine · HCl.

added diammonium citrate, was used. A preliminary study (experiment 3) was carried out to determine what contribution diammonium citrate might make to the nutritive value of the rice diet. This diet, therefore, was fed to groups of male and female rats and other groups of animals were fed similar diets in which the diammonium citrate was replaced isocalorically with cerelose or glycine. As seen from table 3, there was no difference in the performance of the animals on these three diets. It was concluded, therefore, that under the conditions of this experiment the animals receiving the basal rice diet did not benefit from added diammonium citrate for the purpose of tissue synthesis.

TABLE 3

Five-week rat growth data

SUPPLEMENT TO 90% RICE DIET	Experiment 3			
	MALES		FEMALES	
	Gain	Food/Gain	Gain	Food/Gain
%	*gm*		*gm*	
Diammonium citrate, 1.0	55.9 ± 5.60 [1]	7.27 ± 0.75 [1]	60.1 ± 4.59 [1]	6.75 ± 0.28 [1]
Glycine, 1.0	59.7 ± 6.77	7.31 ± 0.72	62.2 ± 5.01	6.46 ± 0.29
Cerelose, 1.0	60.0 ± 7.52	7.19 ± 0.67	60.5 ± 4.57	6.67 ± 0.31

[1] Standard deviation of the mean.

The next experiments then employed the basal diet containing 91% of precooked rice together with graded levels of L-lysine·HCl as seen in table 4. In experiments 4 and 5 highly significant responses to L-lysine·HCl supplementation were obtained, best gains and feed efficiency being observed at a level of 0.10% of supplementary L-lysine·HCl for both males and females. Ten of the original 20 males from experiment 5 and an equal number of females were kept on the same diets for further studies. The growth curves for the groups of animals on the basal diet and on the diet supplemented with 0.1% of L-lysine·HCl, as described earlier, are seen in figure 1. Although it appears that by reducing the number of animals a slight bias was introduced unintentionally in favor of the ly-

sine-supplemented groups, nevertheless the improvement in growth rate due to lysine supplementation is consistent throughout the entire experiment. The slopes of the response curves of the males as well as of the female rats are very

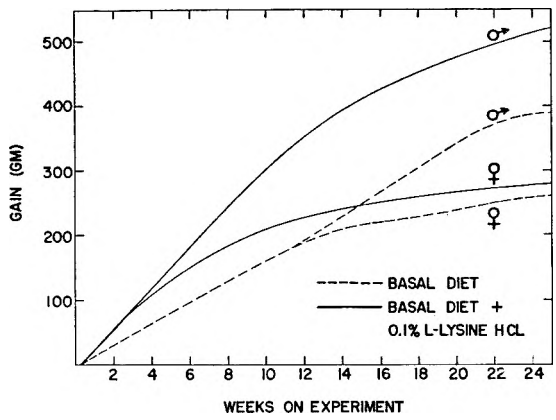

Fig. 1 Growth response of male and female rats on precooked rice diet to supplementation with optimum level of L-lysine·HCl.

TABLE 4

Five-week rat growth data

L-LYSINE HCL SUPPLEMENTATION TO 91% RICE DIET	GAIN IN GRAMS		FOOD/GAIN	
	Experiment 4	Experiment 5	Experiment 4	Experiment 5
%				
		Males		
0.00	85.2 ± 6.74 [1]	82.6 ± 4.05 [1]	6.02 ± 0.264	5.82 ± 0.139 [1]
0.05	100.6 ± 8.64	106.2 [2] ± 3.79	5.30 ± 0.292	4.98 [2] ± 0.112
0.10	121.8 [2] ± 7.60	117.8 [2] ± 9.09	4.79 [2] ± 0.117	4.81 [2] ± 0.176
0.20	- -	92.8 ± 5.84	- -	4.93 [2] ± 0.145
		Females		
0.00	93.0 ± 6.55	81.3 ± 3.11	5.40 ± 0.210	5.79 ± 0.111
0.05	112.8 ± 7.16 [3]	114.0 [3] ± 4.93	4.80 [3] ± 0.087	5.03 [2] ± 0.149
0.10	132.4 [2] ± 8.30	122.7 [2] ± 6.01	4.56 [2] ± 0.121	4.68 [2] ± 0.138
0.20	- -	115.0 [3] ± 7.35	- -	4.61 [3] ± 0.178

[1] Standard deviation of the mean.
[2] Highly significant difference (t-test).
[3] Significant difference (t-test).

similar to those recorded for rats of the same strain maintained on lysine-supplemented bread diets (Rosenberg and Rohdenburg, '52).

The design and results of experiment 6 are seen in table 5. The precooked rice diet (7.75% protein) was compared with a

TABLE 5

Lysine supplementation of diets from precooked rice, parboiled rice and raw long-grain rice

(Experiment 6, 5-week rat growth data)

L-LYSINE HCL SUPPLEMENTATION TO:	MALES		FEMALES	
	Gain	Food/Gain	Gain	Food/Gain
%	*gm*		*gm*	
Precooked rice diet				
0.00	79.5 ± 5.66 [1]	6.02 ± 0.22 [1]	84.6 ± 8.83 [1]	5.81 ± 0.45 [1]
0.10	115.3 ± 13.55 [2]	4.84 ± 0.35 [2]	110.0 ± 12.56	4.69 ± 0.32
0.20	83.7 ± 8.72	5.55 ± 0.37	123.6 ± 10.69	4.32 ± 0.13
Parboiled rice diet				
0.00	68.3 ± 14.71	6.60 ± 0.25	77.7 ± 6.71	5.77 ± 0.23
0.10	102.3 ± 12.08 [3]	4.89 ± 0.23 [3]	117.0 ± 5.38 [3]	4.50 ± 0.08 [3]
0.20	83.4 ± 8.76	5.14 ± 0.35	114.2 ± 20.54	4.66 ± 0.39
Raw long-gain rice diet				
0.00	39.2 ± 3.69	9.80 ± 0.59	43.0 ± 4.54	8.82 ± 0.61
0.10	54.2 ± 5.54 [2]	7.23 ± 0.41 [2]	67.5 ± 10.91	6.09 ± 0.84
0.20	42.2 ± 2.56	8.44 ± 0.75	51.5 ± 6.13	7.95 ± 1.09

[1] Standard deviation of the mean.

[2] Significant difference (t-test).

[3] Highly significant (t-test).

similarly compounded diet of parboiled rice (8.25% protein) and of raw, long-grain rice (6.38% protein) in feeding experiments with male and female weanling rats. Noteworthy differences were found in the nutritive value of the three types of rice studied. All three diets, however, were improved by supplementation with 0.1% of L-lysine·HCl.

Liver fat and liver protein were determined in the animals from experiments 2 and 5. Three male and three female rats per group were killed with pentobarbital sodium with mephenesin at the end of the 5-week growth period. Their livers were removed, frozen with dry ice, lyophilized and ground. Fat and protein in the pooled samples were determined by the AOAC procedures ('55). For the determination of liver glycogen, 10 animals of each sex and each treatment of experiment 5 were killed with pentobarbital sodium with mephenesin. The livers were removed and approximately 1 gm samples taken for glycogen assay by the method of Carroll et al. ('56).

As seen in table 6, lysine supplementation of the rice diets caused a reduction in liver fat and an increase in the liver protein. Although the changes in fat and protein concentration, caused by the amount of supplementary lysine found optimum for growth, are substantial, supplementation with larger quantities of lysine produced further changes in liver fat and liver protein towards the levels obtained with the stock diet. There was no apparent effect of dietary lysine supplementation on liver glycogen concentration.

DISCUSSION

In all 5 experiments a pronounced improvement was observed in the growth rate of rats maintained on 90% rice diets when the first limiting amino acid, lysine, was brought into balance with the second limiting amino acid, threonine, by supplementation with L-lysine·HCl. Best balance was achieved with approximately 0.10% of L-lysine·HCl. As the basal rice diet contained about 0.25% of lysine, (analyzed microbiologically [Rosenberg and Rohdenburg, '51]), a total of 0.33% of lysine produced optimum balance. At the end of the 5-week experimental period the improvement in weight gain due to lysine addition ranged from 32 to 50% for the males (average 41%) and from 27 to 58% for the females (average 44%). This substantial improvement in weight gain was accompanied by an average saving of 20% of the food consumed (range 15 to 27%) per unit of gain. These results suggest that proper sup-

plementation of rice diets with lysine might be of great benefit to humans.

The addition of larger than optimum amounts of lysine to the experimental rice diet did not permit the rats to grow as well as when the optimum amount of L-lysine·HCl, 0.1%, was

TABLE 6

Effect of lysine supplementation on liver fat and liver protein of rats fed rice diets

L-LYSINE HCL SUPPLEMENTATION TO BASAL DIET	LIVER FAT IN % OF DRY WEIGHT			
	Experiment 2 [1]		Experiment 5 [1]	
	Males	Females	Males	Females
%				
0.00	22.04	24.56	31.98	40.83
0.05	19.01	-	-	-
0.10	-	17.87	23.44	36.71
0.20	-	-	18.51	32.51
0.30	14.38	16.70	-	-
Stock diet	9.86	10.08	-	-

L-LYSINE HCL SUPPLEMENTATION TO BASAL DIET	LIVER PROTEIN IN % OF DRY WEIGHT				FRESH WEIGHT IN MG/% OF LIVER GLYCOGEN	
	Experiment 2 [1]		Experiment 5 [1]		Experiment 5 [2]	
	Males	Females	Males	Females	Males	Females
0.00	47.36	44.62	36.24	35.42	3213	2851
0.05	46.44	-	-	-	-	-
0.10	-	53.72	46.19	39.04	3448	2806
0.20	-	-	55.54	42.93	2371	3141
0.30	59.74	56.78	-	-	-	-
Stock diet	67.10	68.01	-	-	-	-

[1] Values determined on pooled livers from three animals per sex and per treatment.

[2] Average values of determinations on ten animals per sex and per treatment.

added. Supplementation with twice the optimum amount, namely 0.20%, resulted in a growth response which in many experiments was very similar to that obtained with the basal rice diet without any lysine supplementation. The latter result confirms similar observations from other laboratories (Harper et al., '55). In view of the highly significant growth response at the 0.1% level of lysine supplementation, it must be con-

cluded that high levels of lysine overbalance the amino acid pattern. The consistency with which higher levels of lysine depressed growth may well explain why other workers did not find a growth response for lysine.

The growth response attainable by supplementation of rice diets with small amounts of lysine was not noted by earlier workers probably because of their different experimental approach. Pecora and Hundley ('51) added to the experimental rice diets various essential amino acids in the amounts suggested by Rose ('37) for the growing rat. Supplementation with 2.0% of DL-lysine·HCl, equivalent to 0.8% of L-lysine did not improve the diet. These investigators, however, made the important observation that the combination of lysine plus threonine produced approximately a threefold increase in growth. They discovered in later experiments that the amounts of added lysine and threonine could be reduced to one-fifth without reducing the benefit obtained from the full supplements. These results have been confirmed and extended by various investigators. Harper et al. ('55) tested not only the effect of the lysine-threonine combination at the reduced level, 0.20% of L-lysine·HCl and 0.24% of DL-threonine, but tested the effect of each amino acid separately. At these levels, no growth response was obtained from either lysine or threonine alone, but the combination gave the results found by the earlier workers. Harper et al. ('55) investigated specifically the deposition of liver fat in rats fed various rice diets. Although a slight reduction in liver fat from 28 to 25.7% was obtained when 0.2% of L-lysine·HCl was added, this was not considered to be meaningful. The reduction in liver fat due to lysine supplementation of rice diets in the present study (table 6) is somewhat more pronounced. Although the results of the two experiments are not identical as far as the absolute amounts of fat found in the livers are concerned, the trends are the same. The observation that liver fat decreases with increasing amounts of lysine supplementation and that this effect does not parallel the effects on growth is a confirmation of the work of Harper et al. ('55).

SUMMARY

The nutritive value of the protein of white polished rice can be improved significantly by supplementation with the first limiting amino acid, lysine, in amounts sufficient to bring this amino acid in balance with the second limiting amino acid.

In 5 experiments, male and female weanling rats were fed diets containing 90% precooked white polished rice supplemented with graded amounts of L-lysine·HCl. Substantial improvements in growth rate and in efficiency of food utilization were obtained with 0.05 to 0.10% of L-lysine·HCl supplementation. This growth response was accompanied by a decrease in the amount of fat and by an increase in the amount of protein deposited in the liver.

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