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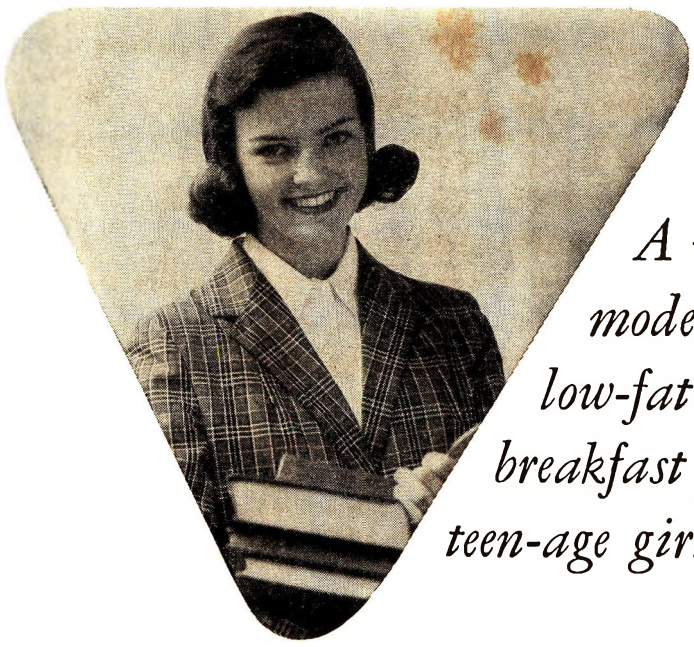

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Nutrients	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Niacin equiv.	Ascorbic Acid
Totals supplied by Basic Breakfast	503	20.9 gm.	0.532 gm.	2.7 mg.	588 I.U.	0.46 mg.	0.80 mg.	7.36 mg.	65.5 mg.
Recommended Dietary Allowances—Girls, 13 to 15 Years (49 kg.—108 lb.)	2600	80 gm.	1.3 gm.	15 mg.	5000 I.U.	1.3 mg.	2.0 mg.	17 mg.	80 mg.
Percentage Contributed by Basic Breakfast	19.3%	26.1%	40.9%	18.0%	11.8%	35.4%	40.0%	43.3%	81.9%

Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1959.
Food & Nutrition Bd.: Recommended Dietary Allowances, Revised 1958. Natl. Acad. Sci.—Natl. Research Council Publication 589, 1958.
Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

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

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

The Nutritional Value of Fats after Use in Commercial Deep-Fat Frying[1]

C. E. POLING, W. D. WARNER, P. E. MONE AND E. E. RICE
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Investigators in several laboratories have demonstrated that the nutritive value of fats may be decreased by severe heating, by oxidation, or by heating and oxidation under laboratory conditions. (Lassen et al., '49; Crampton et al., '53; Raju and Rajagopalan, '55; Kaunitz et al., '55, '56; Johnson et al., '56, '57; Witting et al., '57; and Perkins and Kummerow, '59.) Based on these observations it has been implied or suggested that similar changes may occur in fats when used in commercial frying operations. However, there has been no direct evidence that this is the case except the observation of Witting et al. ('57). These investigators reported significant growth depression for corn oils which had been used in potato chip frying, and for lard which had been used at a drive-in restaurant. No significant growth depression was observed for a hydrogenated vegetable shortening which had been used in doughnut frying.

On the other hand, Chalmers ('54) has reported absence of polymerized products and only slight analytical changes in cottonseed oil which had been used commercially in frying "potato crisps." Also, Melnick et al. ('58) have reported the absence of thermal polymers from an extensive series of oils which had been used commercially in the production of potato chips; they have stated, based on other analytical similarities, that "there are no differences between fresh and heated oil of such magnitude to warrant criticisms of the wholesomeness of the oil being absorbed by the chip." Using animal-feeding criteria, Keane et al. ('59) found no deleterious effects from commercially-used frying oils and actually reported a significantly higher caloric value than that obtained for the unheated controls.

There is no question that changes in fats can be caused by heating and/or oxidation, and that under severe conditions these changes are reflected by impaired growth and probably by tissue changes in animals to which they are fed. However, the conditions of commercial usage differ so widely from those of laboratory testing that extrapolation of the laboratory data to commercially-used fats is of questionable significance. Under conditions of commercial usage, fats are exposed to varying quantities of water, to foodstuffs of various types and to agitation, either intentional or by introduction of foodstuffs; they may be purified by decanting or filtration; and they are often supplemented with fresh fats to an extent which, in some applications, as frying potato chips, results in a rapid turnover of the frying fat. Any or all of these conditions may alter the response of the product to heat and may either increase or decrease the biological effects observed when these fats are fed to animals.

The work reported herein was initiated to secure more direct information with samples of "used" fats and oils which had been obtained directly from commercial establishments.

SAMPLE PROCUREMENT

In order to test whether commercial usage results in damage similar to that reported for laboratory treatment, 34 samples of such "used" fats were secured from a variety of establishments, including restaurants, bakeries, potato chip fryers,

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[1] Most of the data herein have been presented as papers at the meeting of the American Institute of Nutrition, Chicago, 1957. See Poling, C. E., W. D. Warner and E. E. Rice 1957 Detection of changes in nutritive value of frying fats. Federation Proc., *16:* 396 (abstract); and Rice, E. E., P. E. Mone and C. E. Poling 1957 Effect of commercial frying operations on nutritive value of fats. Federation Proc., *16:* 398 (abstract).

TABLE 1

Description of samples of fats procured or prepared for biological evaluation

Sample no.	User	Type of fat	Description of use
1	Large potato chip manufacturer	Corn oil	Continuous replenishment of oil with turnover every two days. Oil filtered daily.
2[1]	Medium-size potato chip manufacturer	Soybean oil	Continuous replenishment of oil with turnover every three days. Oil screened daily.
3[1]	Medium-size potato chip manufacturer	Hydrogenated cottonseed oil	Continuous replenishment of oil with turnover every two days.
4[1]	Medium-size potato chip manufacturer	Hydrogenated vegetable oil	Continuous replenishment of oil with turnover every three days. Filtered every 7 days. Sample taken 7 days after filtration. 185°C.
5[1]	Doughnut fryer	Hydrogenated lard	Continuous replenishment of oil with filtration daily and complete turnover every two days. (Second sample taken later from same source as sample no. 5A.)
5A[1]	Doughnut fryer	Mixture of animal and hydrogenated vegetable fat	Continuous replenishment of oil with filtration weekly. Used 8½ hours daily at 182°C. Fat turnover every three days.
6[1]	Doughnut fryer	Hydrogenated lard	Continuous replenishment of oil with turnover every 1½ days. (Second sample taken later from same source as sample no. 6A.)
6A	Doughnut fryer	Hydrogenated lard	Continuous replenishment of oil with turnover every three to 4 days, used at 182°C, 10 hours/day.
6B	—	Same brand manufactured about same time as sample 6A	Unused.
7	Doughnut fryer	Lard	Continuous replenishment of oil with filtration daily and complete turnover every three days.
8	Doughnut fryer	Lard and hydrogenated vegetable fat blend	Continuous replenishment with filtration weekly. Used at 182°C.
8A	—	Same brand as sample 8 manufactured about the same time	Unused.
9	Retail bakery—doughnut fryer	Mixed animal and vegetable frying fat	Continuous replenishment with daily filtration.
10[1]	Retail bakery—doughnut fryer	Hydrogenated vegetable oil	New oil supply every 10 days. Old oil filtered and added back to restore fat level.
11[1]	Retail bakery—doughnut fryer	Hydrogenated vegetable oil and lard mixture	Turnover every three days.
12[2]	Retail bakery—doughnut fryer	Hydrogenated lard	Strained weekly. Turnover every 7 days.

13	Retail bakery—doughnut fryer	Lard	Filtered weekly Turnover every three days.
14[2]	Retail chicken fryer	Hydrogenated vegetable oil	Daily turnover.
15	Retail chicken fryer	Hydrogenated vegetable oil	Used about 12 hours daily with discard after 4 days use. This sample used two days.
16	Small chicken fryer	Hydrogenated vegetable oils	Continuous replenishment of fat with filtration after each day. Used 8 hours daily, 7 days/week.
17	Large short-order grill	Beef fat and vegetable oil blend	Used 18 hours daily for all-purpose frying. Strained daily, filtered every third day.
18[1]	Short-order grill	Animal fat	Fat used one week for general-purpose frying, then discarded. Sample taken from fat ready for discard.
19[1]	Short-order grill	Hydrogenated meat and vegetable oils	Fat was strained twice a week. This sample had been used 6 days.
20	Short-order grill	Beef fat	Frying with no straining for 10 days. Sample taken after 4 days.
21	Short-order grill	Meat fat and vegetable oil blend	This sample represents fat discarded after 10 days' use without straining.
22	Medium-size restaurant	Meat fat and vegetable oil blend	Fat used 6 days without straining before discard. Sample taken third day.
23	Small restaurant	Beef fat	This sample taken as fat was ready for discard after general frying for one week.
24[1]	Short-order grill	Meat fat and vegetable oil blend	This sample was taken as fat was discarded after being used for general frying 24 hours/day for 7 days with no straining.
25	Large restaurant	Meat fat and vegetable oil blend	This sample represents fat used for general frying for 7 days with occasional straining. (Second sample taken later from same source as sample no. 25A.)
25A	Large restaurant	Hydrogenated vegetable oil	Used for general frying for 10 days (at 180°C) without straining, then discarded. Sample taken on 10th day.
25B	—	Same brand of fat as no. 16 and 25A, manufactured in same plant in same month	Unused.
26	Large restaurant, fish specialty	Lard	Sample represents fat used 7 days without straining and ready for discard.
27[1]	Shrimp fryer	Corn oil	Oil filtered daily and discarded after one week. Sample had been used one day.

TABLE 1 (*Continued*)

Description of samples of fats procured or prepared for biological evaluation

Sample no.	User	Type of fat	Description of use
28[1]	Restaurant	Hydrogenated vegetable	All-purpose fryers. Fats strained daily, with turnover every three days.
29	Medium restaurant	Beef fat and hydrogenated vegetable oil	Fat filtered every week. Turnover every 4th day.
30	Crackling fryer	Pork fat	Cracklings heated at 204°C. Fat discarded daily.
30A[1]	Fried pie manufacturer	Hydrogenated vegetable oil	Fat filtered daily.
31	Laboratory	Winterized cottonseed salad oil, brand A	Unused.
31A			Heated at 182°C for 120 hours without stirring in 28-pound commercial deep fryer.
32	Laboratory	Hydrogenated vegetable oil	Unused.
32A			Heated same as 31A.
33	Laboratory	Blend of hydrogenated vegetable oils (less hydrogenated than no. 32)	Unused.
33A			Heated same as 31A.
34	Laboratory	Hydrogenated lard	Unused.
34A			Heated same as 31A.
35	Laboratory	Pork fat and hydrogenated cottonseed oil blend	Unused.
35A			Heated same as 31A.
36	Laboratory	Crystal modified lard and tallow blend	Unused.
36A			Heated same as 31A.
37	Laboratory	Second sample of winterized cottonseed salad oil, brand A	Unused.
37A			Heated at 182°C for 120 hours without stirring in a three to 5 pound home-type deep fryer.
38	Laboratory	Third sample of winterized cottonseed salad oil, brand A	Unused.
38A			Heated same as 31A.
39	Laboratory	Fourth sample of winterized cottonseed salad oil, brand A	Unused.
39A			Heated same as 37A.
40	Laboratory	Fifth sample of winterized cottonseed salad oil, brand A	Unused.
40A			Heated same as 31A.
41	Laboratory	Winterized cottonseed salad oil, brand B	Unused.
41A			Heated same as 37A.

[1] A sample of unused fat was secured at the same time the used fat sample was taken.
[2] The sample of unused fat secured was heated also in the laboratory at 182°C for 120 hours (see table 5).

doughnut fryers, and after certain miscellaneous uses. These samples have been described briefly in table 1.

The used-fat samples were secured by technical sales representatives who called on the various establishments and asked for samples of the fat then being used, for descriptions of their frying methods, and for samples of their unused fats (the unused fats from any particular establishment did not, for practical reasons, necessarily represent the same lot of fat as the used fat, but they were similar in composition). Some of the used-fat samples had been used to the point of "exhaustion"[2] and had been discarded (see table 1); others had been used for shorter periods of time or were taken from vats where continual replenishment was practiced. Only three of the fats had been in use for less than 24 hours when sampled (nos. 27, 30, and 30A, table 1) and one of these was obtained from an establishment which used fat for only one day before discarding it. Because fried foods retain considerable fat, doughnut and potato chip manufacturers and many restaurants add fat either continuously or at regular intervals to keep the levels in their vats relatively constant. These frequent additions of fresh "make-up" fat minimize the need for complete change of fat and result in a more or less continuous operation. In such cases efforts were made to get an estimate of the "turnover," namely, the number of days required for the addition of an amount of fat equal to that normally contained in the vat. Samples from such processes obviously represent a blend of relatively fresh fats with products that have been used for more than the "average" or "turnover" period.

EXPERIMENTAL PROCEDURE

Ad libitum feeding

An initial group of 6 commercially-used samples (nos. 4, 5A, 6A, 8, 16 and 25A) representing several types of commercial operations was secured and tested biologically by feeding to two replications of 4 rats each as 20% additions to a natural-type basal ration believed to be nutritionally adequate for the rat. Since it was not possible to secure an unused sample known to be from the same lot as the used fat, a sample of the unused fat which had been picked-up at the same time as the used sample was included for "control" purposes. In three instances it was necessary to use for reference purposes the same brand of fat manufactured about the same time as the used fat (nos. 6B, 8A, 25B). Also included in the first experiment were 6 laboratory-heated samples of typical commercial frying fats of varying compositions held at 182°C for 120 hours without frying in a commercial-type deep-fat fryer holding 28 pounds (nos. 31A, 32A, 33A, 34A, 35A, 36A). The unheated controls were samples from the same lot in each instance (nos. 31, 32, 33, 34, 35, 36).

The percentage composition of each diet was as follows: ground whole wheat, 20.5; control or test fat, 20.0; lactalbumin, 15.0; soybean oil meal, 15.0; dried non-fat milk solids, 10.0; meat and bone meal, 10.0; yeast and salt mixture, 2.5; alfalfa meal, 2.0; cold-pressed wheat germ oil containing 1% of added synthetic *dl*-α-tocopherol, 2.0; liver powder (N.F.), 1.0; vitamins A and D oil (2250 I.U. of A and 300 I.U. of D/gram), 1.0; and corn starch, 1.0. The percentage composition of the yeast and salt mixture was as follows: Torula yeast, 78.7; NaCl, 19.8; $MnSO_4 \cdot H_2O$, 1.0; and irradiated yeast (27,000 I.U. of vitamin D/gm, 0.5. The diets were fed for 12 weeks.

The results shown in table 2 demonstrate that weanling male rats[3] fed unused fats grow well on such a diet. When commercially-used fats were compared with unused control fats the differences in growth or food intakes are not consistent and probably are not significant. All of the animals were vigorous and appeared to be normal in every respect. Post-mortem gross examinations of the livers and kidneys revealed no differences between the animals fed used or unused fats. This is in marked contrast with the statistically significant liver enlargements

[2] The use life of a frying fat is limited by the eventual development of undesirable flavors which may carry over into or develop rapidly in the fried food, or by the development of "foaming" characteristics which make further frying difficult or impossible.

[3] From Holtzman Rat Company, Madison, Wisconsin.

TABLE 2

The effect of laboratory-heated or commercially-used fats on 12-week growth and food efficiency of rats fed a natural-type diet

	Weight gains						Food/gain	
	First 3 weeks		Next 9 weeks		12 Weeks		12 Weeks	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Laboratory-heated								
31, 31A	140[1]	114[2]	221[1]	245[2]	361[1]	359[2]	3.00[1]	3.36[2]
32, 32A	138	127	235	225	373	352	3.12	3.43
33, 33A	141	119	228	236	369	355	3.10	3.31
34, 34A	139	134	239	225	378	359	3.07	3.41
35, 35A	146	128	241	221	387	349	3.13	3.53
36, 36A	141	123	230	223	371	346	3.15	3.58
Av.	141[3]	124[3]	232	229	373	353	3.10	3.44
Commercially-used								
4[4]	142	141[5]	221	239[5]	363	380[5]	3.20	3.20[5]
5A[4]	144	139	242	232	386	371	3.23	3.17
6B, 6A	148	134	251	216	399	350	3.02	3.26
8A, 8	144	149	235	237	379	386	3.21	3.13
25B, {25A / 16	140	{140 / 139	224	{225 / 230	364	{365 / 369	3.18	{3.25 / 3.15
Av.	144[3]	140[3]	235	230	378	370	3.17	3.19

[1] One rat had severe respiratory disease. Averages without this rat were 141, 232, 373 and 2.98.

[2] One rat was sacrificed on account of middle ear infection. Values shown are for 7 animals.

[3] The average amounts of food eaten per gram of gain at the end of the first three weeks for the various groups were: unheated laboratory samples, 1.60; laboratory-heated sample, 1.71; unheated commercial samples, 1.61; and commercially-used samples, 1.63.

[4] A sample number was not assigned to the unheated sample. See table 1.

[5] One rat died, probably of respiratory disease. Values shown are for 7 animals.

found in rats fed laboratory-heated fats.[4] When laboratory-heated fats were fed, there was an initial growth depression with gradual recovery extending over approximately three weeks. After this time the average growth rate was approximately equal to that supported by the unused fats; however, the initial growth depression was not made up fully even after 12 weeks at which time the difference was found to be statistically significant and of even greater magnitude when tested by covariance analysis with adjustment of the gains for food intake. Food efficiencies of the rats fed laboratory-heated fats were decreased, and differences among the individual gains of the 6 fats subjected to laboratory-heating were not statistically significant either before or after adjustment for food consumption. The livers of the laboratory-heated groups were larger in size than those of the corresponding controls, and the kidneys were also slightly larger[5] (table 3). The enlargement of both was found to be statistically significant. There was no mortality due to consumption of the laboratory-heated fats (two rats out of 96 fed these samples and the respective controls died of other causes). The animals were in apparent good health throughout the experiment and those fed the laboratory-heated samples could not be distinguished from the control animals in appearance.

Moreover, when one of the laboratory-heated fats, no. 31A, was fed in a syn-

[4] The laboratory-heated samples and their unheated controls constituted a balanced factorial experiment and the results were submitted to statistical analysis. In regard to the commercially-used samples it was virtually impossible to obtain the exact controls and balance needed for statistical treatment. Because the complexity and magnitude of the problem of characterizing a population of commercially-used fats resulted in such shortcomings which could not be overcome, the statistician has expressed an opinion that statistical analysis of such data is not appropriate, and none has been made. Statistical significance, where the test was made, will be clearly indicated throughout the paper as in this instance.

[5] The animals were killed by chloroform anesthesia, after which the livers and both kidneys were removed immediately and weighed.

TABLE 3

The effect of laboratory-heated and commercially-used fats on liver and kidney size

Sample	Liver size[1]				Kidney size[1]			
	Weight		Percentage of body weight		Weight		Percentage of body weight	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
	gm	*gm*	*%*	*%*	*gm*	*gm*	*%*	*%*
Laboratory-heated								
31, 31A	16.15[2]	21.51[3]	3.9[2]	5.1[3]	3.10[2]	3.65[3]	0.75[2]	0.86[3]
32, 32A	18.38	19.32	4.2	4.7	3.20	3.41	0.74	0.82
33, 33A	16.37	18.47	3.8	4.5	2.92	3.18	0.68	0.77
34, 34A	17.22	18.53	3.9	4.4	3.17	3.10	0.72	0.74
35, 35A	17.81	18.21	4.0	4.5	3.16	3.10	0.70	0.77
36, 36A	16.36	17.49	3.8	4.3	3.06	3.24	0.71	0.79
Av.	16.86	18.87	3.9	4.6	3.10	3.27	0.72	0.79
Commercially-used								
4[4]	16.82	17.91[5]	4.0	4.1[5]	2.96	3.33[5]	0.70	0.75[5]
5A[4]	16.37	17.22	3.7	4.0	3.06	3.27	0.68	0.76
6B, 6A	17.24	15.69	3.7	3.8	3.07	2.93	0.66	0.71
8A, 8	17.03	16.93	3.9	3.8	3.18	3.23	0.77	0.72
25B, {25A / 16	15.58	{17.85 / 17.74	3.7	{4.2 / 4.1	2.75	{3.21 / 3.32	0.65	{0.75 / 0.77
Av.	16.61	17.22	3.8	4.0	3.00	3.21	0.68	0.74

[1] Averages for 8 animals/group.

[2] One rat had severe respiratory disease. Without this rat values for liver were 16.62 and 3.9; for kidney they were 3.15 and 0.75.

[3] One rat was sacrificed on account of middle ear infection; averages are for 7 animals/group.

[4] A sample number was not assigned to the unheated sample. See table 1.

[5] One rat died, probably of respiratory disease. Averages are for 7 animals/group.

TABLE 4

The effect of type of diet on growth and food consumption of rats fed unheated or laboratory-heated fat

	Weight gains[1]		Food consumed		Food/gain	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
First three weeks						
Synthetic diet A	144	88	284	202	1.95	2.29
Synthetic diet B[2] and C	—	99	—	208	—	2.10
Natural diet	140	114	218	190	1.56	1.67
Third to 8th week						
Synthetic diet A	196	205	612	678	3.14	3.29
Synthetic diet B[2] and C	—	197	—	645	—	3.27
Natural diet	169	178	500	557	2.96	3.12
Total for 8 weeks						
Synthetic diet A	340	293	896	880	2.63	3.01
Synthetic diet B[2] and C	—	296	—	853	—	2.88
Natural diet	309	292	718	747	2.32	2.56

[1] Cottonseed salad oil unheated (no. 31) and laboratory-heated (no. 31A) were fed. All data for each treatment represent one replication of 4 rats.

[2] One rat was sacrificed after three weeks because of middle ear infection. Averages are for three rats.

thetic diet (modification A)[6] in place of the natural-type diet the growth depression during the first three weeks was more definite but the recovery following the initial slow growth was also more pronounced, thus partially compensating for the slow start (table 4). In another replication the same synthetic diet was fed, modified first (modification B) by including one per cent of DL-threonine, replacing cornstarch, for 17 days, and then by increasing the casein to 30%, replacing sucrose (modification C), beginning on the 22nd day. These changes did not appreciably alter the response to the synthetic diet (table 4).

In addition, the rats fed the laboratory-heated sample in the synthetic diet initially showed enlargement and softness of fecal pellets with considerable tendency to "chain" together. There was considerable oily yellow staining of the hairs around the anus with occasional adherence of feces thereto. Although the degree of involvement varied considerably among the different animals, the feces were mostly formed, with almost no frank diarrhea. These changes gradually improved and had almost disappeared by the end of the third week and were not observable after the 4th week. Barely perceptible changes in feces were observed with careful examination in only three out of 96 rats fed the 6 laboratory-heated fats in the natural-type diet.

Energy value determinations

A biological method for determining available energy in foodstuffs has been developed and reported by Rice et al. ('57). In brief, this technique depends upon supplying adequate quantities of protein, vitamins and minerals to weanling rats, but limiting the energy intake. Under these circumstances growth is proportional to energy intake and the extent to which a food is utilized may be measured by comparing it with materials of known availability. In practice 5 gm of a basal ration supplying 17 to 20 Cal. and sufficient nutrients to provide for the maximum possible needs of the rat were fed to weanling male rats for one week. During this period the test animals gained 5 to 8 gm. At the end of this period some of the diets were supplemented with varying quantities of prime steam lard as a source of energy, usually 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 gm daily. A curve relating gains to the energy supplied by the lard was plotted and used as a reference for the test samples. The gains made by experimental groups were interpolated on the reference curve to determine the energy available to those animals from the respective test fats. This amount of energy divided by the theoretical amount (9 Cal. per gm) in the sample fed and multiplied by 100 gives the percentage utilization of the test fat. Most commercial shortenings are highly digestible and will give available energy equivalent to 90 to 100% of the theoretical value. Lower biological availability of a fat, either because of composition or because of damage from heating or oxidation, will produce less growth which will be reflected in a decreased energy equivalent. For the most accurate comparisons, particularly when fats of different chemical or physical characteristics are being evaluated, it is necessary to compare each treated sample directly with its untreated control. This technique permits rapid evaluation of large numbers of samples at a low cost per sample.

As shown in the last part of table 5, severe laboratory heating results in a substantial reduction in available energy of fats. The excellent availability of the energy in commercially-used fats, shown in the first part of the table, is in distinct contrast with the lower values for the laboratory-heated samples.

In the first energy availability determinations, 2.0 gm of test fats were fed daily, but since it was observed that 2.0 gm of laboratory-heated fat might not be fully

[6] Percentage composition: vitamin-free casein, 15.0; unheated or laboratory-heated fat, 20.0; vitamins in sucrose, 20.0; finely divided cellulose (Cellu Flour), 5.0; salt mixture (Jones and Foster, '42), 4.0; wheat germ oil, 2.0; L-cystine, 0.3; choline chloride, 0.1; liver powder (N.F.), 0.7; corn starch, 32.9. The vitamin mixture provided per 100 gm of diet: thiamine·HCl, riboflavin, pyridoxine·HCl, each, 0.8 mg; Ca pantothenate, 4.4 mg; niacin, 4.0 mg; 2 methyl naphthoquinone, 1.0 mg; folic acid, 25 μg; biotin, 10 μg; *p*-aminobenzoic acid and inositol, each, 10 mg. Each rat was given one drop weekly of oleum percomorphum and one drop weekly of cold-pressed wheat germ oil containing 5 mg of added synthetic *dl*-α-tocopherol, on different days.

TABLE 5

Influence of laboratory or commercial heat treatments of fats on the biological availability of energy of the fats and on liver size of the animals fed two types of fat

Commercially-used fats						
Sample no.	Availability of energy[1]			Liver size		
	Unused	Used	Retention as percentage of unused	Unused as percentage of body weight	Used as percentage of body weight	Used as percentage of unused
	%	%	%	%	%	%
From doughnut manufacturers						
5	86[2]	98	114	4.60	4.92	107
5A	95	91	96	—	—	—
6	93*	94	101	4.71*	5.29	112
6B, 6A	93	96	103	—	—	—
7	—	102	—	—	4.50	—
8A, 8	103	96	93	—	—	—
Av.			101			110
From potato chip manufacturers						
1	—	98	—	—	4.69	—
2	90	96	107	4.52	4.69	104
3	90*	92	102	4.42*	4.82	109
4	96	91	95	—	—	—
Av.			101			106
From bakeries						
9	—	97	—	—	4.57	—
10	98*	96	98	4.68*	4.98	106
11	95*	94	99	4.41*	4.88	111
12	84[3]	93	111[3]	4.39	4.64	106
13	—	94	—	—	4.77	—
Av.			103			108
From restaurants and grills						
22	—	81[4]	—	—	5.26	—
23	—	88	—	—	5.27	—
25	—	83	—	—	5.14	—
25B, 25A	97	78	80	—	—	—
28	86*	88	102	4.60*	5.22	113
29	—	93	—	—	4.59	—
17	—	89	—	—	4.61	—
18	92*	102	111	4.61*	5.29	115
19	87*	84	97	4.53*	5.14	113
20	—	80	—	—	5.07	—
21	—	94	—	—	4.46	—
24	88*	77[5]	88[5]	4.62*	5.08	110
14	97**	101	104	4.59**	4.95	108
15	—	94	—	—	4.92	—
25B, 16	97	96	99	—	—	—
26	—	94	—	—	5.38	—
27	103*	95	92	4.39*	4.69	107
Av.			97			111
Miscellaneous						
30	—	102	—	—	4.44	—
30A	92*	101	110	4.61*	4.76	103
Av. of all commercial samples			100			109

TABLE 5 (*Continued*)
Laboratory-heated fats

Sample no.	Unheated	Heated	Retention as percentage of unheated	Unheated	Heated	Retention as percentage of unheated
	%	%	%	%	%	%
12	84[3]	65	77[3]	4.39	6.17	141
14	97**	77	79	4.59**	5.72	125
31, 31A	88*	75	85	—	—	—
32, 32A	93	77	83	—	—	—
33, 33A	98	65	66	—	—	—
34A	—	74*	—	—	—	—
35, 35A	90	72	80	—	—	—
36, 36A	96	69	71	—	—	—
37, 37A	97	68	70	4.54*	7.55*	166*
38, 38A	88	65*	74	4.38	7.48*	171
39, 39A	97	57*	59	4.42	7.12*	161
40, 40A	102	71	70	4.33	6.97	161
41, 41A	92	58	63	4.77*	7.26*	152
Av.			73			154

[1] Available energy was determined as indicated in the text. Rats fed 1.5 gm of untreated fats typically gained 30 to 35 gm in 7 days and livers weighed 4.1 to 4.6 gm. Those fed 1.5 gm of commercially-used fats gained 28 to 35 gm in 7 days and livers weighed 4.2 to 4.5 gm. Body and liver weights varied slightly with the type of fat used and with the series of samples fed, but since each fat was referred to an appropriate control run simultaneously, these variations were canceled out.

[2] All values represent averages of two replications of 4 rats each, except where noted otherwise by asterisks.

[3] The 84% available energy of the unheated sample represents an average of two replicates averaging 77 and 90%, which differ more than usual. The 90% value is more in line with expected values and, on the basis of this value, the percentage of available energy retained after use would be 103; after laboratory-heating, 72.

[4] The 81% available energy of the used sample represents an average of two replicates averaging 95 and 67%, which differ so much more widely than usual that little doubt exists that one value is aberrant. There is a greater probability that the low value is aberrant.

[5] The 77% available energy of the used sample represents an average of two replicates averaging 87 and 67%, which again differ so much more widely than usual that one value is indicated to be aberrant. There is a greater probability that the low value is the aberrant one. If this should be true, the percentage of available energy retained after use would be 99%.

* Indicates one replication of 4 rats, see footnote 2.

** Indicates three replications of 4 rats each, see footnote 2.

consumed (this was true for no. 31A), 1.5 gm were usually fed in subsequent experiments to assure the complete consumption of the test fats.

Liver weights were not taken in the initial energy availability experiments but they were determined in subsequent tests using this technique. Table 5 records the slight enlargement of livers observed for the commercially-used samples. These differed in marked degree from those of the laboratory-heated samples. Two samples, no. 12 and 14, of the unused commercial fats were also subjected to the laboratory-heating and showed marked reduction in energy value and an increased liver size (table 5), as compared with the same fats after commercial usage. Table 5 summarizes all of the laboratory-heated and commercially-used samples for ready comparison, with summaries by sources for the commercially-used samples. There are no pronounced differences among the averages for the used fats from the various commercial sources.

DISCUSSION

All of the criteria considered—as 12-week growth, feed efficiencies, energy availabilities and liver weights— indicate that fats heated in the laboratory at high temperatures for long periods have been altered in some fashion. The close correlations between the availability of energy

and the other observations have indicated the energy availability technique to be a useful measure of the nutritive quality of fats. Since this is a relatively short-term, precise testing method, the survey of a considerable number of commercial fats was possible.

The nature of the liver enlargement produced consistently by the laboratory-heated fats has not been fully determined. Grossly, the livers are normal in appearance except for the increased size. Preliminary histological examinations reveal no outstanding morphological changes. Analytical values for two sets of livers indicate that the solids and fat content are not changed significantly. The thiamine, riboflavin and niacin concentrations increase substantially. Virtually maximal enlargements appear to develop within two to three days. Other investigators have reported that feeding heated and/or oxidized oils affects organ size. Kaunitz et al. ('55; '56) reported enlargement of livers, kidneys and adrenals and decreased size for spleens and thymuses, all without histological changes. Raju and Rajagopalan ('55) also observed enlarged livers. Johnson et al. ('57) have reported liver enlargement with no change in lipid or solids content and little or no histological change.

When the energy availability technique was applied to a series of commercially-used samples, the energy values were not decreased for most samples and the liver weights were increased slightly in comparison with values obtained for control samples. This is in marked contrast with the substantially decreased energy values and the markedly increased liver weights obtained when laboratory-heated samples were fed, despite the fact that some of the commercial samples had been heated to an extent which seemed to be similar to that for laboratory samples (see samples no. 4, 18, 19, 21, 23–25A and 26).

These differences between laboratory-heated samples and commercially-used fats may be due to any one of a number of factors. The most consistent differences between the two treatments would be the introduction of food into the fat, resulting in stirring of the fat both mechanically and by steam emanating from the food, and the occasional or continual restoration of fat levels with fresh fat. It has been shown by Kaunitz et al. ('55) that the addition of fresh fats to fats which had been heat-treated in the laboratory reverses the tendency of the latter to depress growth. Regardless of the cause, it is apparent from data reported herein that the feeding of samples of commercially-used fats does not result in the adverse biological symptoms which others have predicted on the basis of experiments with laboratory-heated fats. Since even those commercially-used samples which had been used to exhaustion and discarded did not show markedly adverse biological symptoms when fed at substantial levels in the diet of experimental animals, the probability that commercial products need special consideration is greatly reduced. The difference between these commercially-used samples and laboratory-heated samples emphasizes the importance of testing foods produced under regular operating conditions whenever possible.

SUMMARY

A short term (7 day) feeding procedure has been developed to detect changes induced in fats by heating. This involved measurements of biologically-available energy of the fats fed and weight of the livers of the rats. Unsaturated fats which had been damaged by long heating at high temperatures or under oxidative conditions reduced energy values and tended to cause substantial increases in liver weights.

The majority of 34 samples of commercially-used fats obtained from bakeries, restaurants, or manufacturers of potato chips or doughnuts showed no impairment of nutritional value, in marked contrast both with our own findings for laboratory-heated fats and with the predictions of others based on observations for laboratory-heated fats. Only slight, possibly not significant, increases were observed in liver weights when testing commercially-used fats, again in sharp contrast to the marked statistically significant increases observed for laboratory-heated fats.

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Growth Response of the Confused Flour Beetle, Tribolium confusum (Duval) to Six Selected Protein Sources [1,2]

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The need for an ultra-small-scale animal method for assessing overall nutritive quality arose during progress of an investigation of effects of trace element nutrition of plants on nutritive value of the foodstuffs produced. Aside from the obvious effect that a trace-mineral deficiency in the soil might cause a corresponding low level in the plant, it was thought that such a condition might limit the plant in its ability to synthesize important nutrients such as amino acids, proteins and vitamins. This paper, reporting part of the development of such a method, describes application of *Tribolium* larval growth response to the evaluation of 6 protein food sources that had previously been evaluated in tests with the mouse, rat and dog (Allison, '55; Allison et al., '50). The growing body of published information on insect nutrition suggested the potential usefulness of these organisms which, in contrast with microorganisms, fulfill the essential criterion of an animal nutrition system: the ingestion of a relatively dry, solid diet with subsequent digestion and absorption from the gut. Two insect forms, the German cockroach, *Blatella germanica* (L.), and the confused flour beetle, *Tribolium confusum* (Duval), were included in the early phase of the study, but work with the cockroach was discontinued due to the obvious drawbacks of the presence of intracellular symbionts, the reported unfavorable array of amino acids required, marked sexual differences in weight, poor survival on inadequate diets, relative difficulty of rearing and handling, and even the objectionable odors.[5]

Virtually all available information on the nutrition of *T. confusum* is summarized in the review on insect nutrition by Lipke and Fraenkel ('56). Known dietary requirements and data on utilization of foodstuffs by this species have been tabulated.[6]

Feeding brain, liver, cotton seed, soybean, peanut, isolated casein, fibrin, wheat gluten, glycinin and lactalbumin results in good growth and development. Feeding edestin, gliadin, zein and gelatin results in little or no growth of the larvae. Corn starch has been shown to be very effective as a source of carbohydrate.[7] There is no apparent fatty acid requirement.[8] A sterol must be supplied in the diet; several, including cholesterol, are utilized (Fraenkel and Blewett, '43a, b). Known vitamin requirements include biotin, choline, pteroylglutamic acid, pantothenic acid, nicotinic acid, pyridoxine, riboflavin, and thiamine (Rosenthal and Reichstein, '42; Fraenkel

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[7] French, E. W. 1954 The requirements of *Tribolium confusum* for known and unknown vitamins. Ph.D. Thesis, Univ. of Illinois, Urbana.

[8] See footnote 6.

and Blewett, '43b, c, '47, '48).[9,10] The larva requires the 10 amino acids recognized as essential to the growing rat (Lemonde and Bernard, '51 and Fraenkel and Printy, '54). Fraenkel and Printy found, by substituting amino acid mixtures for protein in the diet, that larvae grew and pupated when fed a mixture of the 10 essential amino acids, but that much better growth was supported by Rose's ('48) mixture of 19 amino acids.

Although much progress has been made toward developing diets composed entirely of known chemical constituents, the best of these match the growth-promoting value (to larvae) of the better natural diets only when supplemented with yeast. Besides carnitine, which is required for development of pupae into normal adults (Frŏbrich, '53; French and Fraenkel, '54)[11] the yeast supplies at least two additional factors, one of which is now thought to be zinc, which stimulate larval growth.[12,13] Adequate amounts of these unknown factors are supplied by a quantity of yeast which does not add appreciably (less than 1%) to the protein content of the diet, although some response to the yeast protein would be expected in tests of protein sources deficient in one or more required amino acids. This fact, along with the accumulated nutritional information on *T. confusum*, permits measurement of larval growth under conditions in which (without considering the presence of inhibitory substances), quality of the dietary protein is the determining factor.

MATERIALS AND METHODS

The 6 protein food sources tested were sub-samples of stock materials saved from a cooperative study carried out between 1946 and 1950 (Allison et al., '50). All had been stored dry at room temperature in the intervening time (to 1956–7). The samples comprised defatted whole egg powder,[14] egg albumin,[15] defatted beef muscle,[16] casein,[17] wheat gluten[18] and defatted peanut flour.[19] Methods of preparing these materials and analytical data on them may be found in the report of the cooperative study (Allison et al. ,'50). The diets had the following percentage composition: crude protein, 18; vitamin mixture, 0.63; cholesterol, 1.0; corn oil, 3; mineral mixture, 1.0;[20] and corn starch in amounts dependent upon the protein contents of the test samples. Percentages of protein source material and corn starch, respectively, in the 6 diets were: beef, 19.2 and 75.2; casein, 20.4 and 73.9; egg albumin, 22.5 and 71.8; wheat gluten, 21.6 and 72.8; peanut flour, 29.1 and 65.3, and whole egg, 22.2 and 72.1. A reference diet, consisting solely of whole wheat ground to pass a 40-mesh screen (ca. 0.35 mm openings), was used; it has been found throughout a long series of experiments that the ground whole wheat kernel supports consistent and vigorous larval growth. In general, test diets were milled into a high degree of homogeneity. However, ball-milling was found to decrease nutritional value of the coarsely ground whole wheat, probably by making it difficult for larvae to select germ particles which are relatively high in vitamins, minerals and good quality protein. Also, some actual destruction of B-vitamins may take place during milling.

French ('54)[21] and others have found optimal growth using diets consisting roughly of half protein, with corn starch making up the balance of the diet along with required accessory factors. It was considered, therefore, that the arbitrarily selected 18% level of protein was suboptimal, and thus a suitable level on which to make an evaluation of protein quality

[9] Fraenkel, G. 1949 Unidentified vitamins of the B-complex required by certain insects. Federation Proc. 8, 32 (abstract).

[10] See footnote 7.

[11] See footnote 7.

[12] See footnote 7.

[13] Fraenkel, G. 1957 Zinc and another inorganic growth factor required by *Tenebrio molitor*. Federation Proc. *16*, 386 (abstract).

[14] Viobin Laboratories.

[15] Swift and Company.

[16] Wilson and Company.

[17] Labco, Borden Company.

[18] Huron Milling Company.

[19] Traders Oil Mill Company.

[20] Drackett Mineral Mixture no. 446, modified to contain trace minerals. This mixture, when added to comprise 1% of the diet, provided the following (milligrams per gram of diet): NaCl 6.1; $K_3C_6H_5O_7 \cdot 2H_2O$ (potassium citrate), 13.3; $CaHPO_4$, 20.0; KCl, 3.8; $Ca_3(PO_4)_2$, 13.8; $MgCO_3$, 2.3; $Fe(NH_4)_3(C_6H_5O_7)_2$, 0.9; and (trace elements, in micrograms per gram) $CuSO_4 \cdot 5H_2O$, 11.0; $MnSO_4 \cdot H_2O$, 66.2; $KAl(SO_4)_2 \cdot 12H_2O$, 49.6; KI, 27.6; $CoCl_2 \cdot 6H_2O$, 49.6; $Zn(C_3H_5O_3)_2 \cdot 3H_2O$ (lactate), 49.6; and NaF, 0.055.

[21] See footnote 7.

that would be comparable with results of tests with young rats fed 10% protein diets. Yeast[22] was added to some of the diets and, although the level for addition was selected arbitrarily and is considerably higher than ordinarily used (1 to 2%) in *Tribolium* diets, choice of a 5% level has been supported by subsequent experiments. These showed that yeast had to be supplied in a range of 3 to 5% in an 18% protein diet to achieve maximal growth response to casein, peanut flour protein, and wheat gluten, but not with whole egg powder. Factors in the yeast that probably contributed to this response will be considered in the discussion section of this paper.

Preparation of diets. The protein source, corn starch and mineral mixture were ball-milled together for 10 to 12 hours. Vitamins were then added in a 1:2 ethanol-water solution, 2.0 ml to 10 gm of finished diet. After thorough mixing with a mortar and pestle, the diet was allowed to dry in air. The required quantities of corn oil and cholesterol, in ether solution, were added in the same way. Prepared diets were stored in the cold until used, two to three days. The vitamin solution was made up so that, using 2.0 ml to 10 gm, the prepared diet contained, in micrograms per gram: thiamine·HCl, 12; riboflavin, 18; nicotinic acid, 100; Ca pantothenate, 40; pyridoxine·HCl, 16; inositol, 2000; biotin, 0.6; folic acid, 5; menadione, 1; *p*-aminobenzoic acid, 50; cobalamine, 0.5; ascorbic acid, 10; and choline chloride, 4000. Of this group of substances, inositol, *p*-aminobenzoic acid, menadione, cobalamine, and ascorbic acid are not among the known dietary requirements of this species.[23] The mineral supplement[24] contains all elements now known to be required by animals. At the time this work was carried out, no information was available on quantitative mineral requirements of *T. confusum,* which may well prove to be quite different from those of mammals.

Experimental procedure. Twenty-four hours before starting the experiment, several thousand eggs were collected from the stock culture jars by sifting their contents through a 100-mesh screen. The eggs, in a single beaker, were allowed to hatch, and at the end of the 24-hour period, newly hatched larvae were separated. Ten larvae, checked carefully to insure that all were alive and active, were placed in a 2 by 5-cm shell vial containing a 1 or 2-gm portion of test diet. Each diet was assayed in duplicate or triplicate. All vials were kept at 70 ± 5% relative humidity at 28°C, with continuous air renewal, throughout the experiment. These were shown to be optimum growth conditions by Fraenkel and Blewett ('43a), and were achieved by using a small constant humidity chamber in a constant temperature room. After 18 days, the larvae were recovered from each diet by screening and carefully cleaned of adhering diet material with a soft brush; the survivors were counted, and the group was weighed to ± 0.02 mg on a direct reading balance.[25] Average age to pupation, where measured, was determined by daily observations of the pupae and larvae in each vial. These observations were facilitated by quickly screening the contents of each vial on a 35-mesh sieve.

As a check on the general assay procedure for reproducibility, a positive control of ground whole wheat, the food material in which the insects are most commonly found, was used with each group, run concurrently, of 15 to 20 assay samples. A single sample of whole wheat, stored under refrigeration and ground as needed, was used for this purpose. As shown in figure 1, there was a rather high degree of reproducibility over the period of nearly two years when these and other data were collected. Nevertheless some variation occurred, perhaps due to minor variations in temperature, humidity, initial vigor of the larvae or other causes, so that it seemed advisable for greatest accuracy to make comparisons only within assays run simultaneously.

RESULTS AND DISCUSSION

The principal experimental data on protein quality are presented in table 1, using

[22] Fleischmann, Standard Brands, Inc.

[23] See footnote 6.

[24] See footnote 20.

[25] Roller-Smith, Model G, 50-mg capacity. Special, deep-sided weighing pans confine the larvae satisfactorily. The pans were formed from lightweight aluminum foil and trimmed to counterbalance a 50-mg tare weight.

the basal diets mentioned earlier, with 5% of yeast added and the minerals omitted in a factorial design. Numbers of survivors at 18 days were recorded, and an overall survival of 85% was found. Survival, using the poorest diets was, in general, as good as that with the best diets. It is a noteworthy observation on this type of growth assay that, with no difference in survival and with all larvae remaining very active at 18 days, 50 to 80-fold differences in average weight often occur. The average number of days to pupation is inversely and quite closely correlated ($r = 0.92$) with larval weight at 18 days (fig. 2). Time-to-pupation data thus provide a

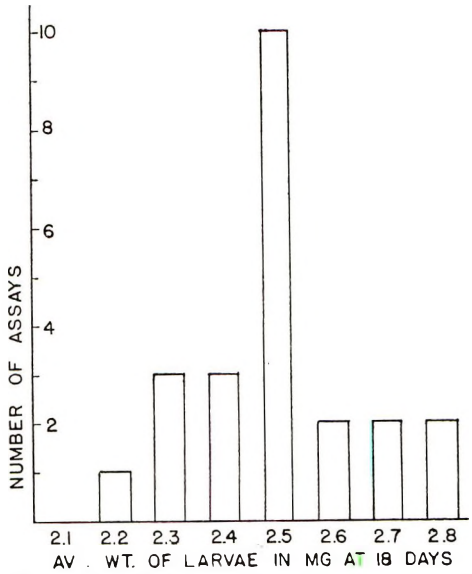

Fig. 1 Whole wheat controls showing variation in response in tests carried out over two years.

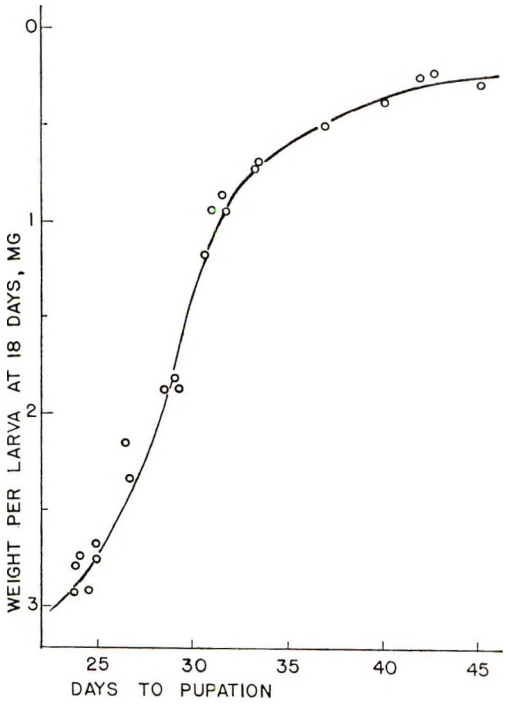

Fig. 2 Relation between weight of larvae at 18 days and time to pupation.

TABLE 1

Effect of yeast and mineral mixture on the protein quality of selected protein sources

Protein source	A Basic diet[1]	B 5% yeast[2] added	C Minerals omitted	D Minerals omitted 5% yeast[2] added
		mg/larva at 18 days		
Whole egg	1.84	2.93	1.86	2.90
Beef	2.70	2.75	2.30	2.74
Casein	0.68	2.68	0.04	0.46
Peanut flour	0.83	2.14	0.92	1.80
Wheat gluten	0.23	1.14	0.06	0.94
Egg albumin	0.28	0.72	0.16	0.36

Control diet, 40-mesh wheat meal, 2.81 mg.
L.S.D. = 0.29 mg at P = 0.05.
L.S.D. = 0.39 mg at P = 0.01.

[1] Basic diet contains the protein food source (to give 18% crude protein), cornstarch, vitamin mixture, cholesterol, corn oil, and Drackett mineral mixture (1%) (see footnote 20 in text).

[2] Fleischmann, dry active.

parallel measure of diet efficiency in support of larval growth, but are somewhat less precise and are more laborious to obtain than the weights.

Quantitative interpretations of the data in table 1 are based on an analysis of variance. The results showed that there were high significant differences in growth attributable to the protein source, the 4 diet variations, and the interaction of these two factors. The data thus afford a measure of overall nutritional value of the protein source materials and also of relative quality of the proteins thus supplied in all instances where the source materials are free of substances inhibitory to protein utilization by the larvae. It is presumed that such an inhibitory substance must have been present in the sample of egg albumin tested; further consideration of this question will follow.

Column A of table 1 shows relative nutritional quality of the protein food sources when fed under conditions assuring an adequate supply of available carbohydrate, minerals, cholesterol and known vitamins required by the larvae. The sources are rated in this order: beef > whole egg > peanut flour = casein > egg albumin = wheat gluten. Additional information on the quality of these foodstuffs is gained from results of feeding the three modifications of the basic diet. The yeast supplement increased protein level of the diets by 1.9% and supplied 0.45% of minerals. On the basis of many experiments run under these same conditions, it appears that attainment of average larval weights of 2.75 to 3.25 mg at 18 days represents the maximum performance to be expected of any diet. It has been observed also that this is a critical weight range which larvae must reach before entering the pupal stage, regardless of the time required.[26] Comparing results from the 4 diet variations, it is concluded that the dried beef supplied adequate amounts of the unknown yeast factors, that it did not provide optimum mineral nutrition without supplementation, but that the small quantity of minerals provided by the yeast overcame this deficiency. This conclusion is supported by results of Fraenkel,[27] which give evidence that the unknown factors are inorganic, with zinc definitely implicated. The casein is deficient in substances provided by both the mineral and yeast supplements, and neither of these components of the diet is utilized effectively without an adequate supply of the other. The peanut flour is likewise deficient in yeast factors, and the mineral supplement and yeast, as with casein, have an additive effect. Growth response attributable to mineral supplement in the wheat gluten diets is not significant statistically, and it is concluded that maximum growth-supporting capacity of this protein is limited primarily by a deficiency of lysine.

In a separate experiment on wheat gluten, supplementation with lysine equivalent to that provided by 5% of yeast increased growth from 0.43 to 1.96 mg; 5% yeast supplement itself increased average larval weight to 2.57 mg; and maximal growth with both 5% of yeast and successive increments of lysine supplementation was 3.13 mg. Results indicate that about two-thirds of the additional growth supported by the 5% yeast supplement to a wheat gluten diet can be accounted for as an effect of the additional lysine thus supplied. Growth, using the whole egg, was not reduced by omission of the 1% mineral supplement, but one or more substances provided by the 5% of yeast addition were necessary for maximal growth. With the possibility that the unknown yeast factors are inorganic, the composition as well as total quantity of mineral supplementation assumes great significance. A significant increase due to yeast and mineral addition, but no increase when the supplements were present singly, was found in the egg albumin diet.

From the results presented in table 1, it is clear that appropriate supplements must be included in certain diets in order to attain a critical evaluation of relative protein quality in a collection of protein food sources that are variable not only in quality of proteins, but also in the nonprotein nutrients which they contain. It is believed that the best approximation of relative protein quality is afforded in this instance by the set of values found with the basic diet supplemented with yeast (column B).

[26] Chirigos, M. A. 1957 Nutritional studies with the insect, *Tribolium confusum* (Duval). Ph.D. Thesis. Rutgers, The State University of New Jersey, New Brunswick.

[27] See footnote 13.

TABLE 2

Evalulation of relative protein quality by tribolium larval growth compared with growth response using standard assay[1]

(Descending order of protein quality from left to right. The numbers are relative values; casein = 100)

Tribolium larval growth: (5% yeast added, data of column B, table 1)						
Whole egg =	beef =	casein >	peanut flour >	wheat gluten >	egg albumin	
109	102	100	80	42	27	
Rat:[2] (a) weight gain: (7 trials)						
Egg albumin =	whole egg =	beef =	casein >	peanut flour >	wheat gluten	
125	119	105	100	39	10	
(b) weight gain/gm N consumed: (7 trials)						
Egg albumin >	whole egg >	casein =	beef >	peanut flour >	wheat gluten	
119	111	100	99	51	16	
Mouse: (a) weight gain (single trial):						
Casein —	egg albumin —	beef —	whole egg —	wheat gluten —	peanut flour	
100	93	85	44	36	29	
(b) weight gain/gm N consumed (two trials):						
Egg albumin —	casein —	beef —	whole egg —	peanut flour —	wheat gluten	
102	100	99	97	64	36	
Puppies: (single trial) weight gain/gm N consumed:						
Whole egg —	beef —	casein —	peanut flour —	wheat gluten —	egg albumin	
108	103	100	58	39	35	

[1] As reported in the collaborative study of Allison et al. ('50).

[2] Results of trials run independently by 7 different laboratories. In the *Tribolium* and rat results, the = sign indicates lack of a statistically significant difference, > indicates a difference significant at P = 0.05 or smaller. No interpretation is made on data of 1 or 2 assays.

These results must be qualified with the knowledge that apparent quality of peanut flour and of wheat gluten may have been enhanced by amino acids from the yeast and, at least in the case of peanut flour (cf. table 3), by a better balance of minerals. The order of protein quality so determined is whole egg, beef, casein, peanut flour, wheat gluten, egg albumin, with no significant differences among the first three. Later data (table 4) will indicate that peanut flour should rate equal to the top three.

In table 2, the data of column B are compared with those of tests with growing young animals in the cooperative study of the Rutgers Bureau of Biological Research (Allison et al., '50). The *Tribolium* test results correspond closely with those of the rat tests in all respects except performance when using an egg albumin diet, and completely parallel results in the growth of young dogs. The mouse is generally a less tractable subject for nutritional study than the rat, but results with the species lead to a conclusion that for it also, casein, beef, whole egg and egg albumin are good protein sources; peanut flour and wheat gluten are of lower value. It is obvious, therefore, that the *Tribolium* larval growth test has considerable potential value for investigation of protein quality in foodstuffs.

In earlier studies on the relative quality of these protein food sources[28] all diets contained 6% of the mineral mixture. Subsequent work, one item of which is illustrated in figure 3, has shown that this supply of minerals can be excessive and in some instances may exert a drastic inhibitory effect on growth. In this case, growth on a diet of unbleached flour plus 10% of yeast was progressively decreased as the added mineral mixture increased from 0 to 5%. Another comparison showing the varied effects of the mineral level is demonstrated in the following data, which give the growth in an early experiment when 6% of minerals were added and the data of column A of table 1 where only 1% of minerals were added. The numbers are average weights per larva (in milligrams) at 18 days.

[28] See footnote 5.

Larval growth, 6% of minerals added					
beef	casein	peanut flour	whole egg	egg albumin	wheat gluten
1.87	1.55	1.19	0.81	0.27	0.07
Larval growth, 1% of minerals added					
beef	whole egg	peanut flour	casein	egg albumin	wheat gluten
2.70	1.84	0.83	0.68	0.28	0.23

There is strong evidence that reversal of the positions of whole egg and casein is an expression of mineral toxicity in the case of whole egg in the first experiment, and mineral deficiency in the case of casein in the second. The whole egg and casein preparations contained 5.35 and 1.46% of ash, respectively, on a moisture-free basis (Allison et al., '50). To test this point further, larvae were grown, using whole egg and casein diets with mineral levels ranging from zero to 6%. Maximal growth with the whole egg diet was obtained with no mineral additions, while 6% of minerals reduced growth by 45%. Maximal growth with the 18% casein diet, on the other hand, required addition of 5% of the mineral supplement and growth was 6.5 times that with the diet containing 1% of minerals.

Since egg albumin is known to be a protein of high nutritional value for the rat and other mammals, its failure to promote good growth of *Tribolium* larvae was thought to be due possibly to a factor specifically toxic to this species and attempts have been made to discover its nature. However, since a fresh sample of egg white, dried in the laboratory, supported excellent growth, it was concluded that the sample from the collaborative study (Allison et al., '50) must have developed the toxicity either during preparation or subsequent storage.

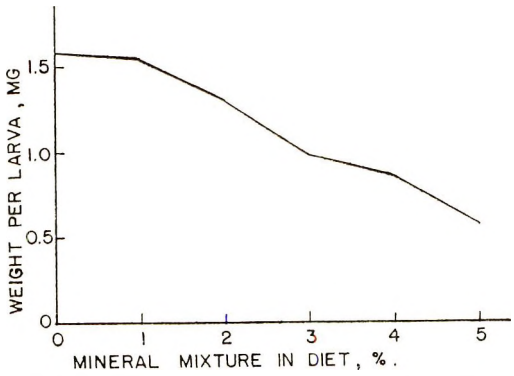

Fig. 3 Effect of increasing mineral mixture (Drackett) on growth of *Tribolium confusum* fed a diet of unbleached flour plus 10% yeast.

The problem of the unknown yeast factors appears to be approaching a solution. French[29] concluded that there were two factors involved, one water soluble, the other insoluble. He failed to produce the yeast effect by supplementation with guanine, adenine, uracil, ribo- and deoxyribonucleic acids, but showed that 0.5 to 1.0% of yeast added to a carbohydrate-free diet was as effective in stimulating growth as 10% of added glucose. It was inferred from this observation that one function of the yeast factors may be to promote utilization of protein as a source of respiratory substrates. From Fraenkel's recent work with *Tenebrio molitor*,[30] it seemed fairly certain that the unknown factors are not organic, as previously believed, but are in reality zinc and a second inorganic factor, possibly potassium. Further development and refinement of the *Tribolium* larval growth assay for evaluation of relative protein quality will obviously require a critical study of the mineral requirements of the species, so that, taking into consideration the mineral nutrients supplied by the protein source, it will be possible to supplement each test diet so as to provide optimal mineral nutrition, qualitatively as well as quantitatively.

The variation in growth with different levels of minerals led to more extended studies of this nature, one of which is illustrated in table 3. Three assays are grouped here but, the growth using the positive control diet was very nearly the same so that comparisons should be valid. The two salt mixtures compared, McCollum Davis no. 185 and Drackett no. 446, differ in that the former is significantly less concentrated in actual mineral con-

[29] See footnote 7.
[30] See footnote 13.

TABLE 3

Effect of different minerals on growth using standard proteins

Protein source	Mineral supplement			
	McCollum Davis no. 185		Drackett no. 446[1]	
	2%	4%	1%	2%
	mg/larva at 18 days			
Whole egg	3.1	—	1.8	2.9
Beef	1.8	—	2.7	3.1
Casein	0.6	1.8	0.7	0.9
Peanut flour	3.1	—	0.8	0.9
Wheat gluten	0.5	0.7	0.2	0.6
Egg albumin	0.8	—	0.3	—
Whole wheat (no minerals added)	2.6		2.8	2.8

[1] For composition see footnote 20 in text.

stituents and contains no added trace minerals. Contrary to previous results, whole egg seemed to give better growth with moderate increments of minerals and did not require the trace minerals supplied by the Drackett mixture. The same was true of peanut meal where the Drackett minerals may have created an actual imbalance. Beef, on the other hand, did not support as good growth without the trace minerals. Casein required a high level of some major mineral.

Since wheat alone gives very good growth of larvae, it would appear that the mineral balance thus furnished is adequate. Therefore, the ash of wheat, as well as of other seemingly suitable natural products, was used as the mineral supplement as shown in table 4. In this experiment, whole egg and beef gave maximal growth regardless of the source of minerals and even in their complete absence. This finding, for beef, differs slightly with the results in table 1. With peanut meal and the ash of various natural products, excellent growth resulted, much better than that with the Drackett minerals which, in this case, showed no growth-stimulating effect. It would appear that peanut meal protein has a high quality for the flour beetle if the proper mineral balance is maintained.

The results with casein are interesting in that growth on the ash of various natural products was no better, and in some cases poorer, than with an equal amount of the Drackett mineral mixture. Previous results with casein have shown that growth will improve with added minerals up to the 5% level. However, wheat contains only 2% or less of ash and this amount, in wheat, is sufficient. Whole egg contained 5.35% of ash and the amount of egg in the diet, 22%, would therefore furnish only 1.18% of ash in the whole diet, yet the whole-egg diet usually did not benefit from mineral additions.

It would appear, then, that the mineral requirements of the confused flour beetle are rather exacting. There is a need for

TABLE 4

The value of the ash of natural products as a mineral supplement for Tribolium confusum

Protein source	Mineral supplement[1]					
	Ash of:				Drackett no. 446	None
	Wheat	Yeast	Liver	Egg		
	mg/larva at 18 days					
Whole egg	3.2	3.0	2.9	2.9	2.9	3.1
Beef	3.2	3.3	2.9	3.0	3.1	2.8
Casein	0.8	0.9	0.4	0.5	0.9	0.1
Peanut flour	3.2	3.1	3.1	3.2	1.7	1.7
Wheat gluten	0.5	0.4	0.6	0.5	0.6	0.1

[1] Added at the 2% level.

both the macro and the minor elements but it is possible to demonstrate a toxicity of excess minerals, as well as a deficiency, and it may well be that imbalance can also reduce growth. In the case of coarsely ground (40-mesh) wheat, it is probable that the larvae can select from the larger particles those of higher nutritive value. It has been shown[31] that ball-milled wheat gives much lower growth than 60-mesh material but that addition of a complete vitamin mixture restores growth to a higher level than when using the 60-mesh sample. In other words, there was no apparent deficiency of minerals even without the presumed selective food consumption.

Huot and associates ('57) have studied the mineral requirements of *T. confusum* using a 20% casein diet, their criteria of adequacy being the length of time to pupation and to emergence as adults. They found that anywhere in the range of 1 to 8% of U.S.P. salt mixture no. 2 gave the best results. In a later paper these same authors ('58) determined the requirements for individual elements to be roughly as follows in mg per 100 gm of diet: Mg, 50; Ca, 600; and K, 450. In each case there was a wide range of tolerance over which little difference was apparent. The requirements for Na and Cl were extremely low. The results reported by these workers might be criticized on the basis that the lowest time to pupation in any of the experiments, 35 days, is twice that reported, namely 18 days, for their positive controls fed whole wheat. It would seem obvious that there is some limiting factor, possibly fatty acids, in their experimental diets and it might be that the tolerances on mineral levels would have been much narrower had more nearly optimal conditions prevailed.

We have found that, as shown in figure 2, there seems to be a very definite relation between larval growth rate, as measured by weight at 18 days, and days to pupation. With the possible exception of carnitine, a similar relation between plane of nutrition and physiological response may hold through the rest of the life cycle. Thus with these insects it should be possible to carry on extended studies on all phases of growth and reproduction on a microscale in an abbreviated time. Morphological examination, chemical analysis and biological experimentation could be applied to groups at each stage to yield developmental patterns associated with diet and studies of several generations could be accomplished relatively quickly.

SUMMARY

An animal growth assay, using larvae of the confused flour beetle, *Tribolium confusum* (Duval), was used to measure the protein quality of a group of 6 selected food sources, whole egg, beef, egg albumin, peanut flour, casein and wheat gluten, which had been assayed previously using the rat, mouse and dog. In general, the order of protein quality was the same for the insect as for the mammals, with peanut flour showing higher values for the insect and albumin an apparent toxicity which had developed during storage. The flour beetle was shown to have a rather exacting requirement for minerals which could influence the apparent protein quality of different food sources. The beetle seems to have great potential value as a subject for fundamental research in nutrition.

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[31] See footnote 26.

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The Cariostatic Effect in White Rats of Phosphorus and Calcium Supplements Added to the Flour of Bread Formulas and to Bread Diets

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A previous publication (McClure and Muller, '59a) presented our evidence of caries inhibition due to $CaHPO_4$ and Na_2HPO_4 when added to bread diets or incorporated in the flour of a bread formula. The experiments of this report extend these results. They evaluate the cariostatic effect of the following compounds: Na_2HPO_4; $(NH_4)_2HPO_4$; $Ca(H_2PO_4)_2 \cdot H_2O$; $Ca_3(PO_4)_2$; sodium phytate, calcium lactate; and calcium gluconate. Previous experiments particularly pertinent to the anticaries action of phosphorus and calcium compounds have been documented by Nizel and Harris ('60), McClure and Muller, ('59a) and McClure and McCann ('60).

EXPERIMENTAL

The data of this report were obtained in 4 experiments similar in design and following the plan of our previous experimental caries studies (McClure, '58; McClure and Muller, '59a; McClure, '59; McClure and McCann, '60). The essential data are presented in tables 1 and 2. Summary data appear in table 3.

The bread in the diets of experiment 1 (table 1) had the following formula (in per cent): bread flour, 86.2; sugar, 2.6; salt, 1.7; lard, 2.6; yeast, 1.7; nonfat, dry milk solids, 5.2. The water absorbed was 0.65 liters per kilogram of dry ingredients. The formula included a yeast food, i.e., 10.9 ml of a 0.1% solution of $KBrO_3$. The minerals added to the flour of this bread which was baked in the research facilities of the U. S. Department of Agriculture, are shown in table 1.

Bread used in experiment 2, table 1 and in experiments 3 and 4, table 2, was prepared by a local baker, according to the following formula (in per cent): bread flour, 78.8; salt, 1.5; sugar, 4.6; nonfat, dry milk solids, 3.0; yeast, 3.0; lard, 9.1. To this mix was added 0.49 liters of water per kilogram. The mineral supplements added to the flour or directly to the bread diets are indicated in the tables. The bread was air dried and ground fine prior to mixing with the other ingredients of the diet.

The ash, calcium, phosphorus, and the Ca/P ratio of the various diets varied widely (tables 1 and 2). The basal cariogenic diets 349, 363, 370 and 374 were uniformly low in phosphorus (0.10 to 0.12%). Calcium content was 0.08, 0.45, 0.58 and 0.61%, and the calcium/phosphorus ratios varied accordingly. All the diets also contained below optimum quantites of protein. They were nutritionally inadequate for normal growth. However, the essential purpose of a control diet in experimental caries studies such as these, is the production of a high incidence of severe dental caries. This was accomplished very successfully.

All the rats were the Sprague Dawley strain from the National Institutes of Health stock colony. Started at weaning age and housed two per cage, the rats consumed food and water ad libitum. Control and supplemented diets were evaluated with littermate rats. In experiments 1, 3 and 4, the diets were fed 98 days, in experiment 2, 84 days.

The lower molar teeth were diagnosed and assigned scores for dental caries as previously described (McClure and Muller, '59a). In addition to the caries diagnosis, bood samples were obtained by decapitation from rats in experiment 4. The blood of 4 rats fed the same diet was

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TABLE 1

The composition and analysis of diets and the effect of phosphate and calcium supplements on rat caries

Diet	Experiment 1				Experiment 2			
	349	350	351	352	363	364	365	366
Diet composition, %								
Bread—nonmineralized flour	79.80	—	—	—	78.67	—	—	—
Bread (flour + 1.0% Na_2HPO_4)	—	79.80	—	—	—	78.67	—	—
Bread (flour + 1.75% $Ca(H_2PO_4)_2 \cdot H_2O$	—	—	79.80	—	—	—	—	—
Bread (flour + 2.15% Ca lactate·$5H_2O$)	—	—	—	79.80	—	—	—	—
Bread (flour + 2.00% Ca gluconate)	—	—	—	—	—	—	79.05	—
Bread (flour + 1.0% $(NH_4)_2HPO_4$)	—	—	—	—	—	—	—	78.69
Glucose[1]	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Vitamin mix[2]	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20
Ca CO_3	—	—	—	—	1.13	1.13	0.75	1.11
Diet analysis, %								
Ash	2.08	2.82	2.59	2.73	2.30	2.98	2.05	2.42
Calcium	0.08	0.08	0.29	0.30	0.45	0.51	0.46	0.48
Phosphorus	0.12	0.31	0.48	0.12	0.12	0.26	0.11	0.29
Calcium-phosphorus ratio	1.50	3.88	1.66	0.40	0.27	0.51	0.24	0.60
Protein (N × 6.25)	11.31	11.88	11.88	11.56	11.56	11.81	11.06	11.83[3]
Dental caries experience								
No. of rats	26	29	27	28	33	37	39	33
Average daily gain, gm	0.81	0.86	1.30	1.09	0.37	0.52	0.36	0.66
Rats with caries, %	76.92	51.72	44.44	53.37	75.75	35.13	74.36	21.21
Carious teeth/rat	3.15	1.24	1.19	1.68	2.91	0.51	2.97	0.56
Carious areas on occlusal surfaces	3.58	0.80	0.70	0.64	1.45	0.11	1.18	0.09
Carious areas on smooth surfaces	3.37	1.45	1.30	2.00	4.08	0.57	4.39	0.79
Severity score/rat	13.73	3.72	3.41	4.11	13.64	0.92	10.38	0.97

[1] Cerelose, Corn Products Refining Company, New York.
[2] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.
[3] Corrected for 1.0% $(NH_4)_2HPO_4$ in bread flour.

pooled to give an analytical sample of serum which was analyzed for calcium by flame spectrophotometry and phosphorus according to Gee and Dietz ('53).

RESULTS

With no exceptions the addition of these phosphate supplements resulted in significant reductions in the dental caries experience. The absence of caries, in one instance due to 1.41% of sodium phytate in the diet (diet 377) and also with 1.17% of Na_2HPO_4 in the diet (diet 375), was indeed quite unusual. In the case of sodium phytate this was an unexpected finding. The very soluble $(NH_4)_2HPO_4$ in two trials was also very cariostatic (diets 366 and 371). Added to these bread diets (diets 372 and 376), insoluble $Ca_3(PO_4)_2$ had a significant anticaries action — a result contrary to a negative effect previously obtained with this phosphate when present in wheat flour diets (McClure, '60). $Ca(H_2PO_4)_2 \cdot H_2O$, a relatively soluble phosphate, reduced the caries severity score 75% and the caries incidence 42%.

In table 3 are summarized the changes in caries experience, brought about by the mineral supplements, expressed as percentage of reduction in incidence and in the severity score. Included in this table are data from a previous study of phosphated flours and phosphated bread-diets (McClure and Muller, '59a). In this previous study, it should be noted, the diets contained liver powder as a source of vita-

TABLE 2

The composition and analysis of diets and the effect of phosphorus supplements on rat caries

	Experiment 3				Experiment 4			
Diet	370	371	372	373	374	375	376	377
Diet composition, %								
Bread	80.43	80.43	80.43	80.43	76.13	76.13	76.13	76.13
Glucose[1]	14.00	14.00	14.00	14.00	18.00	18.00	18.00	18.00
Corn starch	2.00	0.89	1.87	0.59	1.00	0.33	0.87	0.09
L-Lysine	—	—	—	—	1.00	1.00	1.00	1.00
Vitamin mix[2]	2.20	2.20	2.20	2.20	2.00	2.00	2.00	2.00
NaCl	—	—	—	—	0.50	—	0.50	—
$CaCO_3$	1.37	1.37	0.00	1.37	1.37	1.37	0.00	1.37
Na_2HPO_4	—	—	—	—	—	1.17	—	—
$Ca_3(PO_4)_2$	—	—	1.50	—	—	—	1.50	—
$(NH_4)_2HPO_4$	—	1.11	—	—	—	—	—	—
Sodium phytate[3]	—	—	—	1.41	—	—	—	1.41
Diet analysis, %								
Ash	2.34	2.92	3.02	3.67	2.57	3.35	3.21	3.09
Calcium	0.58	0.63	0.65	0.58	0.61	0.64	0.69	0.59
Phosphorus	0.10	0.37	0.37	0.42	0.10	0.34	0.38	0.34
Calcium-phosphorus ratio	0.17	0.59	0.56	0.72	0.16	0.53	0.55	0.58
Protein (N × 6.25)	10.69	11.62[4]	11.00	10.75	10.63	11.56	11.56	10.93
Dental caries experience								
No. of rats	38	37	39	33	35	34	37	37
Average daily gain, gm	0.39	0.49	0.55	0.45	0.72	1.64	1.71	1.61
Rats with caries, %	92.10	21.62	51.28	42.42	85.71	0.0	32.43	0.0
Carious teeth/rat	4.05	0.65	3.35	1.24	3.51	0.0	0.86	0.0
Carious areas on occlusal surfaces	2.13	0.16	0.92	0.27	1.49	0.0	0.62	0.0
Carious areas on smooth surfaces	5.87	0.73	2.03	2.00	5.31	0.0	0.89	0.0
Severity score/rat	18.18	1.49	5.84	3.45	14.17	0.0	2.46	0.0

[1] Cerelose.
[2] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation.
[3] A product of Corn Products Refining Company, containing 21.2% total P, 17.6% phytin P, 3.2% orthophosphorus.
[4] Corrected for 1.11% $(NH_4)_2HPO_4$ in diet.

TABLE 3

Summary data on caries inhibitory effect of phosphorus and calcium supplements in bread flour and bread diets

Diet no.	Mineral supplement in bread diet or added to flour used to prepare bread	Dental caries reduction	
		Incidence	Severity
		%	%
—[1]	2.00% Na_2HPO_4 added to flour	29.7	42.1
350	1.00% Na_2HPO_4 added to flour	49.1	72.9
364	1.00% Na_2HPO_4 added to flour	53.6	93.3
—[1]	1.40% Na_2HPO_4 added to bread diet	37.0	64.8
375	1.17% Na_2HPO_4 added to bread diet	100.0	100.0
366	1.00% $(NH_4)_2HPO_4$ added to flour	72.0	92.9
371	1.11% $(NH_4)_2HPO_4$ added to bread diet	76.5	91.8
351	1.75% $Ca(H_2PO_4)_2 \cdot H_2O$ added to flour	42.2	75.2
—[1]	2.00% $CaHPO_4$ added to flour	37.0	42.6
—[1]	1.40% $CaHPO_4$ added to bread diet	19.8	32.1
372	1.50% $Ca_3(PO_4)_2$ added to bread diet	44.3	67.9
376	1.50% $Ca_3(PO_4)_2$ added to bread diet	62.2	82.6
377	1.41% Sodium phytate added to bread diet	100.0	100.0
373	1.41% Sodium phytate added to bread diet	53.9	81.0
352	2.15% Calcium lactate added to flour	30.6	70.1
365	2.00% Calcium gluconate added to flour	1.8	23.9

[1] From results previously published (McClure and Muller, '59a).

mins and the rats were given orally, vitamins A, D and E. These experiments lasted only 60 days and the control rats had a relatively low average severity score, namely, 7.32, compared with scores averaging 13.64 to 18.18 as developed by the use of these bread diets, over a longer time.

The results of the calcium and phosphorus analysis of the blood serum furnish evidence that the phosphorus supplements increased the serum phosphorus content. The blood serum of the control rats averaged 12.5 ± 0.54 mg/100 ml of phosphorus, whereas rats receiving phosphorus-supplemented diets averaged 14.5 ± 0.54, 14.4 ± 0.59 and 14.4 ± 0.59 mg/100 ml of phosphorus, when Na_2HPO_4, $Ca_3(PO_4)_2$ and the sodium phytate, respectively, were the supplements. The average serum calcium in the three groups of rats receiving phosphorus supplements was respectively as above: 11.4 ± 0.22, 11.5 ± 0.35, and 11.7 ± 0.23 mg/100 ml. The control group of rats averaged 10.8 ± 0.15 mg/100 ml of serum calcium.

DISCUSSION

In two previous studies (McClure, '59) and McClure and Muller ('59a), extensive dental caries developed in white rats when given diets composed essentially of dry white bread and cerelose (commercial glucose). Extensive caries were again developed in this current study by using essentially dry bread and glucose as the diet components. After using this type of diet 84 or 98 days, carious lesions were found on practically all surfaces of the teeth. The distribution of carious areas on occlusal and smooth surfaces is shown in the caries experience data of the tables. The occlusal surface lesions did not appear to have originated in the deep sulci but rather on the upper cuspal areas. The appearance of buccal and lingual surface lesions agrees with our previous observations (McClure and Muller, '59a). Relatively low in sugar, finely powdered, and containing mainly bread and sugar, these experimental diets contain ingredients common to an average human dietary. The type of rat caries observed, closely resembles human dental caries.

Although a low content of phosphorus as well as an insufficient quantity of possibly inadequate protein are characteristic features of the diets, the identity of these deficiencies with the cariogenic property of the diets remains speculative. True, all the phosphate supplements significantly reduced or prevented caries. In one experiment (diet 352), however, a calcium lactate supplement also reduced dental caries significantly, and the phosphorus (0.12%) remained low as in the control diet 349.

A relation of cariogenicity to dietary protein was noted in a previous study (Bavetta and McClure, '57), namely, that "diets containing 13% casein developed a high incidence of severe caries but caries was very limited with 24% casein in the diet." Thus the low protein content of the bread diets might explain their cariogenicity.

The failure to respond to a 1.00% of lysine supplement (diet 374 vs. 370) is in agreement with previous evidence that, in general, only cariogenic diets containing skim-milk powders and cereal foods made lysine-inadquate by heat processing, become significantly less cariogenic by lysine supplementation (McClure, '60; Cremer et al., '60).

With respect to the significance of phosphates as anticaries additives to bread flours, these experiments contribute extensive and significant new evidence. The pronounced cariostatic effect of Na_2HPO_4 and $(NH_4)_2HPO_4$ at a level of 1.00% in the bread flour, is particularly noteworthy. These two phosphates also when added directly to ordinary bread-diets (375) and (371) were strikingly cariostatic.

As previously mentioned the dramatic reduction in caries with sodium phytate in the diet was somewhat unexpected. Selection of this organic phosphate was based on the premise that, although soluble, its nonionic phosphate would be unavailable particularly within the oral cavity. It was desirable also to compare this soluble organic phosphate with insoluble $Ca_3(PO_4)_2$ and soluble Na_2HPO_4 and $(NH_4)_2HPO_4$.

Previously there has appeared in the literature evidence that refined and processed cereal foods are more cariogenic than the raw cereals (Constant et al., '52; Constant et al., '54; Taketa and Phillips, '57; Jenkins et al., '59). Recently

Madsen[1] reported the anticariogenicity of seed hulls (rice, peanuts, cotton seed and barley hulls) in the diets of cotton rats. In these provocative studies the role of organic phosphates, including phytin,[2] have been mentioned and studied to some extent as an anticaries factor—particularly by Jenkins and associates ('59). These later workers have attempted to evaluate speculation, based on limited evidence by Osborn et al. ('37) that a "caries protective factor" is lost in the refining of sugars and cereals. Jenkins et al. ('59) report from *in vitro* solubility studies using teeth and sodium phytate solutions, that phytate and other organic phosphates may be responsible for an anticaries effect of brown flour. A 20 mg/100 ml solution of sodium phytate was quite effective in reducing solubility of teeth in an acetic acid buffer (Jenkins et al., '59).

So far as is known no evidence has appeared prior to these studies, indicating any cariostatic effect of sodium phytate when added to cariogenic experimental rat diets. Explanation of the cause of this significant action is purely speculative. It is known that phytate is hydrolized in the lower intestine and its phosphorus is nutritively available (Mellanby, '50). In our studies it will be noted that sodium phytate, Na_2HPO_4, and $Ca_3(PO_4)_2$ were equal in phosphorus availability as shown both by improved growth and in their effect on the level of serum phosphorus. In this connection it can not be overlooked that the rat is an extremely coprophagic animal (Barnes et al., '57) and its fecal excretion becomes a definite factor in evaluating phenomena in its oral cavity. The very likely recirculation through the oral cavity of fecal phosphates thus complicates the situation.

As mentioned above the anticaries effect of $Ca_3(PO_4)_2$ as observed in this experiment (diets 372 and 376), is contrary to a prevous negative result with $Ca_3(PO_4)_2$ as a supplement in wheat flour diets. These bread diets, however, were more complex and contained NaCl, which in previous studies (Van Reen and Glassford, '59; McClure and Muller, '59b) has been implicated in the cariostatic effect of an insoluble phosphate ($CaHPO_4$).

Our limited studies with calcium lactate (diet 352) and calcium gluconate (diet 365) suggest that not all calcium supplements may be cariostatic. Previously we found $CaCO_3$ to give equivocal results (McClure and McCann, '60). In diets 370 and 374, $CaCO_3$ was present at a level of 1.37% (diet 363 contained 1.13% of $CaCO_3$) and these diets remained highly cariogenic. One other calcium salt, namely, calcium acetate, has been found to be cariostatic.[3] Recently Limbasuta and Johansen[4] also found equivocal results with $CaCO_3$ and an anticaries effect of calcium acetate.

The mechanism by which phosphates are able to inhibit dental caries remains unresolved and has not been enlightened greatly by these studies. Previously, much consideration has been given to the possibility that a localized oral action, perhaps related to the solubility of the phosphate, was a most likely explanation. Thus the cariostatic effect of the very insoluble $Ca_3(PO_4)_2$ was not anticipated although this is our first experience with $Ca_3(PO_4)_2$ in association with NaCl in the diet. We are not inclined to the hypothesis that a dietary nutritive deficiency of phosphorus is an adequate explanation of the cariostatic effect of phosphates.

SUMMARY

A relatively simple diet composed of dry bread, glucose and essential vitamins, resulted in extensive dental caries in white rats.

In two trials, bread prepared with a flour which contained 1.0% of Na_2HPO_4 resulted in a reduced caries incidence averaging 51.0%; the severity score was reduced an average of 83.0%. Use of bread flour containing 1.0% $(NH_4)_2HPO_4$ was responsible for a 72.0% reduction in caries incidence and 93.0% reduction in severity score. These phosphates also significantly inhibited caries when added directly to

[1] Madsen, K. O. 1960 The anticariogenicity of seed hulls in the cotton rat diet. Federation Proc., *19:* 323 (abstract).

[2] It is generally agreed that phytin in cereals is the calcium-magnesium salt of phytic acid (inositol hexaphosphoric acid).

[3] McClure, F. J., unpublished data.

[4] Limbasuta, S., and E. Johansen 1960 The effect of several calcium compounds on rat caries. Internat. A. Dent. Res., Abstracts of 38th General Meeting, p. 57.

bread diets. $Ca(H_2PO_4)_2 \cdot H_2O$ at a level of 1.75% in the bread flour, and 1.5% $Ca_3(PO_4)_2$ in the bread-glucose diet significantly inhibited dental caries.

In two trials 1.41% of sodium phytate added to the bread-glucose diet reduced caries incidence an average 77.0% and caries severity score an average 91.0%.

Calcium lactate added to bread flour was anticariogenic, but calcium gluconate had no anticaries effect.

Three phosphate compounds, namely, Na_2HPO_4, $Ca_3(PO_4)_2$ and sodium phytate, were equally effective as phosphorus supplements as measured by improved rate of growth and increased content of blood-serum phosphorus.

These results add substantially to previous evidence of a significant cariostatic effect of a phosphated bread and a phosphate supplement added to a bread-glucose cariogenic diet.

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Studies on the Vitamin K Requirement of the Chick

I. REQUIREMENTS OF THE CHICK FOR VITAMIN K_1, MENADIONE AND MENADIONE SODIUM BISULFITE[1]

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Almquist ('54) reviewed the requirements of the chick for vitamin K_1 and 2-methyl-1, 4-naphthoquinone (menadione). In much of this work the chick curative method was used. The minimum effective dose per chick in curative assays of 18 hours was found to be approximately 1.0 μg of vitamin K_1 and 0.3 to 0.64 μg of menadione when whole blood clotting time was used to measure activity. Using the same criterion the minimum effective dose in a 6-hour assay was 1.5 μg of vitamin K_1 and 0.5 μg of menadione. The minimum effective dose of menadione per chick was 6 to 8 μg when prothrombin time was used in an 18-hour test period to measure activity. When a 5-day test was used, the daily requirement for menadione was found to be approximately 2 μg and 3.8 μg for vitamin K_1.

Frost and associates ('56) found menadione sodium bisulfite complex (63% menadione sodium bisulfite, U.S.P.) three times as effective as menadione as a source of vitamin K in the modified K-low A.N.R.C. broiler ration (National Research Council, '54). Shelton et al. ('56) reported that the chick's requirement for menadione was 1.20 and 1.39 mg/kg of diet in diets containing 5.7 and 2.7% of fat, respectively, and 0.20 mg/kg for menadione sodium bisulfite regardless of fat level. Frost et al. ('56) showed that 0.40 mg of menadione per kg of diet was insufficient for the chick. In a similar study, Perdue et al. ('57) observed that 0.40 mg of vitamin K_1 per kg of diet also was insufficient.

The results of the investigations reviewed by Almquist ('54) indicated that menadione was approximately 1.5 to 3 times more effective than vitamin K_1 on a weight basis. Shelton et al. ('56) reported that menadione sodium bisulfite furnished by menadione sodium bisulfite complex was approximately 18 times as effective as menadione on a molar basis. Perdue and co-workers ('57) concluded that menadione sodium bisulfite complex was 1.7 times more effective on the basis of equimolar weight than vitamin K_1. Quick and Stefanini ('48) reported that for the chick, vitamin K_1 was slightly more effective than menadione on a molar basis. Griminger ('57) reported menadione sodium bisulfite to be 3 times more effective on a molar equivalent basis than menadione in studies with turkey poults.

Because of insufficient information concerning the dietary need of the chick for vitamin K, experiments were conducted to determine the quantitative requirements of the chick for vitamin K_1, menadione and menadione sodium bisulfite and to compare the relative effectiveness of each form of the vitamin under comparable dietary and environmental conditions.

EXPERIMENTAL

Male White Plymouth Rock chicks obtained from a commercial hatchery were used in these experiments. At the beginning of each experiment they were randomly allotted to the treatments, wing-banded, group-weighed and placed in electrically heated brooders with raised wire mesh floors. The chicks were weighed individually at weekly intervals thereafter

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[2] Present address: International Minerals and Chemical Corporation, Skokie, Illinois.

and weekly feed consumption was recorded, except in experiment 2, when biweekly records were maintained. Feed and water were supplied ad libitum. The waterers were scrubbed twice daily to prevent the production of vitamin K_2 by bacterial fermentation. The feeders were checked regularly and wet feed removed.

The basal diet used in these experiments is given in table 1. Isolated soybean protein was selected as the protein source because of its low fat content which was found to be approximately 0.2% by analysis. A commercial preparation of vitamin E-free lard was used in these experiments. The lard had been molecularly distilled to remove vitamin E and should also be free of vitamin K.[3] The unidentified growth factor source consisted of 6 parts of distillers dried solubles, 3 parts of dried whey product and 3 parts of fish solubles on a wet basis. These three ingredients were mixed and dried in a forced-draft oven at 65°C for 12 hours. The supplement was then ground and extracted continuously for 24 to 48 hours with acetone. Dam and Schønheyder ('36) reported that acetone is a better solvent for vitamin K_1 than ether or alcohol. The mineral mixture contained all required minerals. The vitamin mixture contained all the known vitamins except vitamin K.

TABLE 1

Composition of basal diet

	Amount/ 100 gm diet
	gm
Glucose[1]	55.50
Isolated soybean protein	21.70
Unidentified factor source[2]	10.00
Stripped lard	3.00
Cellulose[3]	3.00
DL-Methionine	0.70
Glycine	0.30
	mg
Choline chloride (70%)	210.00
Vitamin A (10,000 I.U. per gm)	50.00
Vitamin D_3 (3000 I.U. per gm)	33.30
Vitamin E (50,000 I.U. α-tocopheryl acetate per pound)	25.00
Inositol	25.00
Niacin	5.00
Ca pantothenate	2.00
Riboflavin	1.00
Thiamine·HCl	1.00
Pyridoxine·HCl	0.45
Folic acid	0.40
Biotin	0.02
Vitamin B_{12}	0.002
$CaHPO_4$	2151.00
$CaCO_3$ (gr. limestone)	1492.00
KH_2PO_4	867.00
NaCl (iodized)	600.00
$MgSO_4$	250.00
$FeSO_4 \cdot 7H_2O$	33.30
$MnSO_4 \cdot H_2O$	33.30
$CuSO_4 \cdot 5H_2O$	1.67
$ZnCl_2$	1.00
$Na_2MoO_4 \cdot 2H_2O$	0.83
KI	0.26
$CoCl_2 \cdot 6H_2O$	0.17
N,N′-diphenyl-*p*-phenylenediamine (DPPD)	22.0

[1] Cerelose, Corn Products Refining Company, New York.

[2] Contains approximately 55% distillers' dried solubles, 30% dried whey product and 15% dried fish solubles.

[3] Solka-Floc, Brown Company, Berlin, New Hampshire.

Vitamin K_1 and menadione were dissolved in 95% ethyl alcohol for addition to the diet. Pure menadione sodium bisulfite was dissolved in 50% ethyl alcohol and menadione sodium bisulfite complex was dissolved in water. Each form of the vitamin was then diluted to the desired volume and aliquots taken for the individual diets. A series of graded levels of each form of the vitamin to be tested was added to the basal diets. At least one level was known to be adequate. After mixing, the diets were stored in covered feed storage cans.

Prothrombin time determinations were used to measure vitamin K activity. Initially, prothrombin times were determined using a method similar to that of Hoffman and Custer ('42). In later experiments this method was modified by placing the thromboplastin solution in a 1 by 5-cm test tube and incubating it at 41°C in a water bath. A sweep-second-hand stop watch was used to measure the time of clotting. The thromboplastin solution was prepared by adding 10 ml of 0.85% saline solution to a 2-ml vial of Difco EE 100 chick embryo extract. Whole blood was used instead of plasma because of the report by Almquist and Klose ('39) that the prothrombin times were more accurate when whole blood was used.

The vitamin K requirement of the chick was resolved using the method of Almquist

[3] Distillation Products Industries, Rochester, New York.

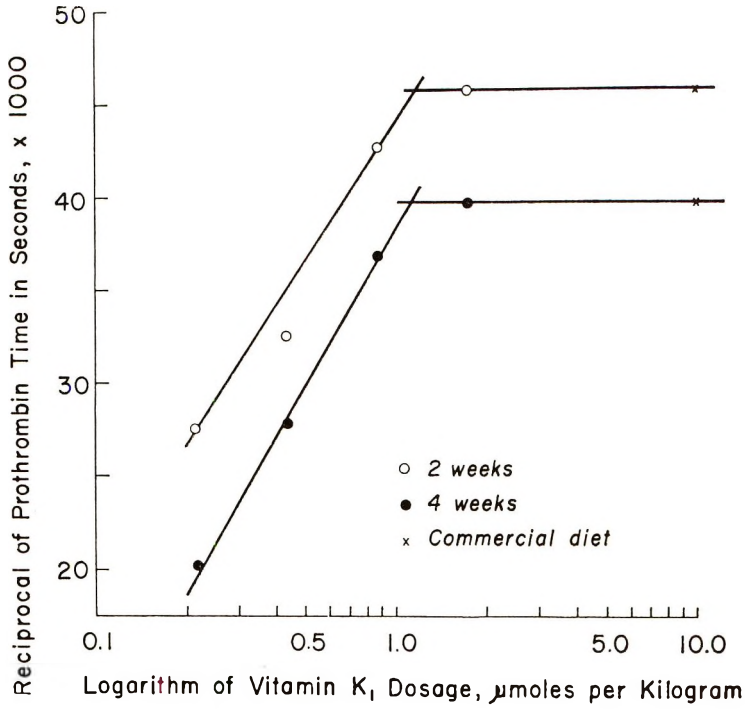

Fig. 1 Relationship of reciprocal of prothrombin time to log-dosage of vitamin K_1 in the diet. The commercial diet contained menadione sodium bisulfite.

and Klose ('39). These investigators reported that the reciprocal of the prothrombin time plotted against the logarithm of the vitamin K dosage gives a sloping straight line as long as the dosage is inadequate. At deficient levels of vitamin K, as the amount in the diet increases the prothrombin time decreases, but, when the chicks are fed adequate amounts of vitamin K, no further decrease in prothrombin time occurs. A plot of these data, therefore, gives a base line which intersects the line formed by plotting the reciprocals of the prothrombin times of chicks fed deficient levels of this vitamin against the logarithm of the dose.

The slope of the regression line was calculated using the method of least squares (Snedecor, '56). The calculated intercept of the regression line with the base line was taken to be the requirement of the chick for vitamin K. This procedure is illustrated in figure 1. Unless otherwise stated, the reciprocals of the individual determinations of the prothrombin time $\times$ 1,000 were used in the calculations. The logarithm of the dosage in moles instead of weight was used, so that a comparison between the various forms of vitamin K could be made on the basis of the active nucleus of vitamin K_1. Prothrombin determinations were made on 10 chicks per treatment at two and 4 weeks of age.

RESULTS AND DISCUSSION

Experiment 1 was conducted to determine the degree of vitamin K deficiency that could be obtained with the basal diet and the response of the chick to graded levels of vitamin K_1. Each lot contained 20 chicks. The levels of vitamin K_1 added to the diet were 100, 200, 400, and 800 μg/kg. A commercial broiler diet containing 2.5% of alfalfa meal and 3300 μg/kg of menadione sodium bisulfite furnished by menadione sodium bisulfite complex, was fed one lot of chicks in order to determine whether the minimum prothrombin time obtained with the basal diet was the same as that obtained with a practical diet. The results of this experiment are presented in table 2. They showed that the

TABLE 2

Effect of graded levels of vitamin K_1 on prothrombin time of chicks (experiment 1)

Treatment		Av. weight 4 weeks	Feed/gain	Prothrombin time	
				2 weeks	4 weeks
	μg/kg diet	*gm*		*seconds*	*seconds*
Basal	0	384	1.80	65.1	77.4
Basal + vitamin K_1	100	387	1.78	37.2	50.6
Basal + vitamin K_1	200	384	1.72	31.6	37.0
Basal + vitamin K_1	400	385	1.80	25.2	27.4
Basal + vitamin K_1	800	406	1.74	22.3	25.3
Commercial diet		500	1.71	22.6	25.1

basal diet was markedly deficient in vitamin K as well as suitable in other respects for use in studies on this vitamin. The results also indicated that the chick's requirement for vitamin K_1 was between 400 and 800 μg/kg of diet.

The regression lines of the two-and 4-week data, along with the base lines, are given in figure 1. These lines were parallel, indicating a similar response of the chick to vitamin K_1 at two and 4 weeks. The requirement, as determined by the calculated intercept of the regression line with the base line, was 532 and 519 μg/kg of diet at two and 4 weeks respectively.

At two weeks 40% of the chicks fed the basal diet bled to death after the prothrombin determinations were made. At the end of the experiment at least 10 chicks from each treatment were examined for hemorrhages. None was found in any of the chicks receiving vitamin K_1 whereas 8 of the surviving 12 chicks fed the basal diet were hemorrhagic.

Experiment 2 was conducted to compare the requirements of the chick for vitamin K_1, menadione and menadione sodium bisulfite. In this experiment, each lot contained 15 chicks. The levels of vitamin K_1 added to the basal diet were 100, 200, 400 and 800 μg/kg. The selection of these levels was based on the results of experiment 1.

Equimolar amounts of menadione and menadione sodium bisulfite were also added to the basal diet to determine the requirement for them. The menadione sodium bisulfite was provided in the form of a complex containing 63% of menadione sodium bisulfite, U.S.P. Since the molar weight of vitamin K_1 is 451, menadione 172 and menadione sodium bisulfite (3 H_2O) 330, then 1 mole of menadione is 38% of the weight of vitamin K_1 and 1

TABLE 3

Comparative effects of vitamin K_1, menadione and menadione sodium bisulfite (experiment 2)

Treatment		Av. weight 4 weeks	Feed/gain	Prothrombin time	
				2 weeks	4 weeks
	μg/kg diet	*gm*		*seconds*	*seconds*
Vitamin K_1	100	507	1.67	40.3	38.1
Vitamin K_1	200	526	1.68	29.8	29.9
Vitamin K_1	400	508	1.69	25.9	26.5
Vitamin K_1	800	509	1.72	22.9	24.7
r[1]				0.87	0.88
Menadione	38	497	1.66	69.9	77.5
Menadione	76	461	1.75	51.8	54.4
Menadione	152	508	1.68	38.9	40.6
Menadione	305	501	1.69	31.2	30.2
r				0.88	0.87
Menadione sodium bisulfite[2]	73	500	1.63	39.7	43.0
Menadione sodium bisulfite	146	500	1.71	28.2	30.1
Menadione sodium bisulfite	292	495	1.64	24.9	25.8
Menadione sodium bisulfite	584	482	1.74	24.0	24.6
r				0.85	0.90

[1] Correlation coefficient.

[2] Menadione sodium bisulfite, U.S.P., furnished by menadione sodium bisulfite complex.

mole of menadione sodium bisulfite is 73% of the weight of vitamin K_1. Theoretically, this plan would result in three points with which to form the regression line for each form and one point each, or a total of three points with which to form the base line.

The results of this experiment are presented in table 3. Insufficient menadione was supplied to produce normal prothrombin time, which left only menadione sodium bisulfite and vitamin K_1 to form the base line. At two weeks the average prothrombin time of the lot of chicks receiving the diet with the highest level of menadione sodium bisulfite was high, due primarily to a prothrombin time of 42 seconds for one chick. This value was omitted in calculating the average shown in table 3. At 4 weeks the prothrombin times of the chicks fed diets with the highest levels of vitamin K_1 and of those with menadione sodium bisulfite were essentially the same.

The requirement for vitamin K_1 at two and 4 weeks was 577 and 523 µg/kg of diet respectively, 970 and 619 µg for menadione and 356 and 320 µg for menadione sodium bisulfite. The requirement for vitamin K_1 was in agreement with that found in experiment 1. Menadione sodium bisulfite showed approximately the same activity as vitamin K_1 on a molar basis whereas menadione was less active.

The correlation coefficients for vitamin K_1, menadione and menadione sodium bisulfite were not statistically different. This indicates that the slopes of the regression lines of these three forms of vitamin K are approximately parallel. These results do not agree with the conclusions of Almquist ('52, '55) that the slope of the regression line of menadione is 35% greater than that of vitamin K_1. Within the range of this experiment, the potency ratios of vitamin K_1, menadione and menadione sodium bisulfite were the same at any deficiency level when compared on a molar basis.

Because of the results obtained with menadione, experiment 3 was conducted to restudy the comparative requirements of vitamin K_1, menadione and menadione sodium bisulfite. Duplicate lots each containing 8 chicks were used for this study. The levels of vitamin K_1 fed in this experiment were 50, 100, 200, 400, 800 and 1600 µg/kg of diet. Equimolar amounts of pure menadione sodium bisulfite starting at 100 µg of vitamin K_1 were also fed. The levels of menadione were equimolar to vitamin K_1 starting at the 200 µg level. They in-

TABLE 4

Comparative response of the chick to vitamin K_1, menadione and menadione sodium bisulfite (experiment 3)

Treatment		Av. weight 4 weeks	Feed/gain	Prothrombin time	
				2 weeks	4 weeks
	µg/kg diet	*gm*		*seconds*	*seconds*
Vitamin K_1	50	496	1.90	44.1	42.4
Vitamin K_1	100	497	1.74	28.6	27.0
Vitamin K_1	200	482	1.74	24.0	24.0
Vitamin K_1	400	503	1.75	19.2	20.6
Vitamin K_1	800	515	1.74	19.2	18.7
Vitamin K_1	1600	531	1.78	19.6	18.9
r[1]				0.95	0.95
Menadione	76	476	1.70	24.6	26.9
Menadione	152	528	1.63	20.2	21.4
Menadione	305	507	1.67	17.9	19.6
Menadione	610	531	1.69	18.6	19.0
Menadione	1220	481	1.70	19.3	19.0
r				0.91	0.96
Menadione sodium bisulfite[2]	73	543	1.72	25.3	26.8
Menadione sodium bisulfite	146	569	1.67	21.2	22.4
Menadione sodium bisulfite	292	521	1.77	18.6	19.8
Menadione sodium bisulfite	584	519	1.64	18.4	19.2
Menadione sodium bisulfite	1168	501	1.74	17.8	18.8
r				0.98	0.95

[1] Correlation coefficient.

[2] Pure menadione sodium bisulfite, U.S.P.

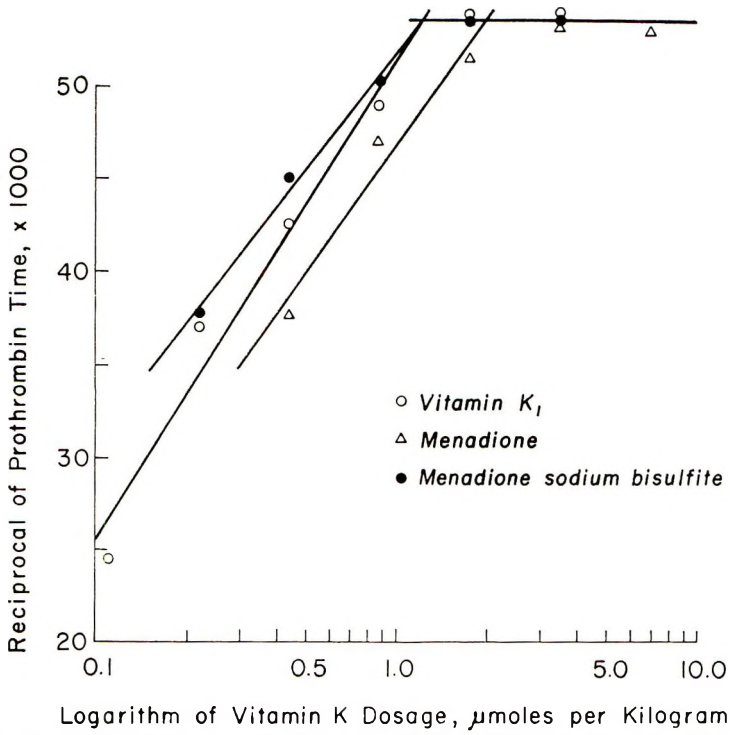

Fig. 2 Graph of log-dosage against reciprocal of prothrombin time showing similar slopes of regression lines for vitamin K_1, menadione and menadione sodium bisulfite at 4 weeks.

cluded one level equimolar to 3200 μg of vitamin K_1. The requirements for each form of vitamin K were determined using the average of the individual prothrombin times of each lot of chicks instead of individual prothrombin times.

The results of experiment 3 are given in table 4. The results obtained at two weeks made it necessary to estimate the chick's requirement for menadione and menadione sodium bisulfite from only two points although the vitamin K_1 group had three points from which to calcuate the regression line. The requirement for vitamin K_1 at two and 4 weeks was 464 and 541 μg/kg of diet, respectively, that for menadione 205 and 339 μg, and that for menadione sodium bisulfite 254 and 393 μg. The correlation coefficients were found not to be statistically different, thus confirming the findings in experiment 2. Regression lines for vitamin K_1, menadione and menadione sodium bisulfite at 4 weeks are given in figure 2.

A summary of the chick's requirement for vitamin K_1, menadione and menadione sodium bisulfite under the experimental conditions described, is given in table 5. Variation in the requirement was observed in all three forms studied, the greatest being with menadione. The requirement for vitamin K_1 at two and 4 weeks was 524 ± 46 and 528 ± 10 μg/kg of diet; 588 ± 284 and 479 ± 140 μg for menadione and 305 ± 51 and 357 ± 36 μg for menadione sodium bisulfite. The requirement, expressed on a weight basis was the greatest for vitamin K_1 and least for menadione sodium bisulfite. The requirements at two and 4 weeks for vitamin K_1 were 1.16 and 1.17 μmoles/kg of diet; 3.42 and 2.79 μmoles for menadione and 0.93 and 1.08 μmoles for menadione sodium bisulfite. On a molar comparison, menadione was approximately 40% as active as either of the other forms at 4 weeks. When the comparisons were made in the same experiment (2 and 3), the results suggested that menadione sodium bisulfite was slightly more active than vitamin K_1.

The results obtained in the experiments on the quantitative requirement of the

TABLE 5

Requirements of the chick for vitamin K_1, menadione and menadione sodium bisulfite

Treatment	Experiment no.	Requirement			
		Micrograms		Micromoles	
		2 weeks	4 weeks	2 weeks	4 weeks
Vitamin K_1	1	532	519	1.18	1.15
Vitamin K_1	2	577	523	1.28	1.16
Vitamin K_1	3	464	541	1.03	1.20
Av. ± S.D.[1]		524±46	528±10	1.16	1.17
Menadione	2	970	619	5.64	3.60
Menadione	3	205	339	1.19	1.97
Av. ± S.D.		588±284	479±140	3.42	2.79
Menadione sodium bisulfite	2	356	320	1.08	0.97
Menadione sodium bisulfite	3	254	393	0.77	1.19
Av. ± S.D.		305±51	357±36	0.93	1.08

[1] Standard deviation.

chick for vitamin K_1 agree with the conclusion of Perdue and associates ('57) that the NAS-NRC ('54) recommendation of 0.18 mg/lb of diet (0.40 mg/kg) is insufficient to meet the requirement of the chick for this vitamin. Likewise the results obtained with menadione are in agreement with the conclusions of Frost et al. ('56) that 0.40 mg/kg of menadione is insufficient for the chick. In contrast, the results with menadione and menadione sodium bisulfite do not agree with those of Shelton et al. ('56). Neither do the results obtained in these experiments agree with the conclusion of Perdue and co-workers ('57) that menadione sodium bisulfite complex was approximately 1.7 times as effective on a molar basis as vitamin K_1. On the other hand the comparison of vitamin K_1 to menadione on a molar basis agreed with the results of Quick and Stefanini ('48) and the results with menadione and menadione sodium bisulfite agree with the conclusions of Griminger ('57).

One of the factors which may account in part for discrepancies in findings and variance in molar biological potency is difference in length of retention in the tissues. Dam et al. ('54) and Taylor et al. ('56) presented evidence showing that vitamin K_1 is retained in the body for a considerable length of time, whereas Richert ('44) found that 31 to 42% of administered menadione was excreted in the urine within 18 hours. Solvonuk et al. ('52) injected menadione C^{14} into mice and dogs and found that it was rapidly absorbed and excreted in the urine.

Another factor which apparently caused discrepancy in the results of some of the early investigations was the method of administering the dose. Dam et al. ('38), Dann ('42), Ansbacher et al. ('40) and Thayer et al. ('39, '40) have all presented evidence to this effect.

However, the primary factor causing the difference in the requirement for the three forms of vitamin K studied in this investigation is probably absorption. Menadione, a fat-soluble crystalline compound, is perhaps less readily absorbed than oil-like vitamin K_1 or water-soluble menadione sodium bisulfite. This may explain the variability observed in these studies and that of other workers when comparing menadione with other forms of vitamin K. Being a crystalline compound, it cannot be assumed that the same proportion goes into solution in the dietary fat and is absorbed in each experiment. However, this view is not supported by the findings of Jaques and associates ('54) that menadione was completely absorbed whereas only about 50% of vitamin K_1 was absorbed.

Fischer et al. ('56) observed that, when injected intravenously, vitamin K_1 and menadione had apporoximately the same molar biological activity. Richert et al. ('40) compared the activity of several synthetic forms of vitamin K and found that, when injected intravenously, all but one (di-potassium-1, 4-dihydroxy-2-methylnaphthelene-sulfate) had the same molar activity as menadione. These findings indicate, in view of the results of this investigation, that under some circumstances

menadione is incompletely absorbed. The degree of absorption is probably to some extent affected by the diet. Shelton et al. ('56), for instance, obtained different requirements for menadione at different dietary fat levels.

CONCLUSIONS

Experiments were conducted to ascertain the quantitative requirement of the chick for vitamin K_1, menadione and menadione sodium bisulfite. Prothrombin determinations were used to measure vitamin K activity.

The requirement for vitamin K_1 at two and 4 weeks was 524 ± 46 and 528 ± 10 μg/kg of diet; 588 ± 284 and 479 ± 140 μg for menadione and 305 ± 51 and 357 ± 36 μg for menadione sodium bisulfite. When expressed on a molar basis, the requirement for vitamin K_1 at two and 4 weeks was 1.16 and 1.17 μmoles, respectively, per kg of diet; 3.42 and 2.79 μmoles for menadione and 0.93; and 1.08 μmoles for menadione sodium bisulfite. Thus on a molar basis, vitamin K_1 and menadione sodium bisulfite were almost equally active and both were 2.5 times more active than menadione.

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Effects of Deficiencies of Pyridoxine, Riboflavin and Thiamine upon the Catecholamine Content of Rat Tissues[1]

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Three catecholamines (CAs) are known to be present in animal tissues: epinephrine (E), norepinephrine (N), and dopamine (3-hydroxytyramine). In addition, the amino acid precursor of the CAs, dopa (3, 4-dihydroxyphenylalanine), occurs in some mammalian tissues. Since Green et al. ('45) showed that pyridoxal phosphoric acid is the coenzyme of dopa decarboxylase, there has been sporadic interest in the consequences for CA metabolism of a deficiency of vitamin B_6. The findings, however, have not been definitive. Despite diminished activity of dopa decarboxylase in tissues of pyridoxine-deficient rats[3] there is no detectable change in the E and N content of the adrenal gland[4] (West, '53). According to Blaschko et al.[4] there is a decrease in the rate at which the vitamin B_6-deficient rat replenishes the store of E and N in the adrenal medulla following a period of insulin hypoglycemia; but it is difficult to assess this result inasmuch as the degree of deficiency of these rats was not described nor were quantitative data provided in this report.

Since 1956, experiments have been conducted in this laboratory on the role of various nutritional factors, including pyridoxine, riboflavin, thiamine, and some amino acids, in the biosynthesis of CAs in the rat.[5] Our earlier experiments with pyridoxine-deficient rats were concerned with the E and N content of various tissues during the course of the vitamin deficiencies. When it became possible to measure dopamine also (Carlsson and Waldeck, '58; Drujan et al., '59; Murphy and Sourkes, '59) the problem of pyridoxine deficiency was re-investigated to take this CA into account as well.

Riboflavin has been investigated in the present research for two reasons: (1) in its phosphorylated forms it serves as coenzyme in oxidation reactions and may play a role, albeit an indirect one, in the oxidative steps in CA biosynthesis from phenylalanine and tyrosine and (2) riboflavin deficiency leads to a decrease in the monoamine oxidase content of rat liver, as determined manometrically (Hawkins, '52; Sourkes, '58) and the change in the concentration of this enzyme may affect the balance of amines in the tissues.

The effect of thiamine deficiency upon the CA content of the adrenals was first investigated by Goodall ('51). He obtained some evidence that the deficiency reduces the E content of the rat adrenal, with but little effect on the N content. Although diphosphothiamine-linked enzymes are not known to act in any of the reactions by which the CAs are synthesized, the deficiency state produces marked physiological changes in the organism which might very well be reflected in modified amounts of these amines in the organs. It was therefore deemed important to investigate this problem under carefully controlled conditions of diet.

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EXPERIMENTAL

Male rats of the Sprague-Dawley strain were used for the experimental work. Upon attaining body weights of 90 to 100 gm the rats were distributed at random among the diets fed. In experimental series 1, riboflavin and pyridoxine deficiencies were studied simultaneously in a multifactorial design, using one group of rats as controls on a fully supplemented diet, another group fed the control diet with riboflavin omitted, a third group fed the control diet with pyridoxine omitted, and a fourth group receiving a diet lacking both riboflavin and pyridoxine. This arrangement was designed to detect interactions between the vitamin deficiences. The control diet had the following percentage composition: alcohol-extracted casein, 20; hydrogenated vegetable fat,[6] 13; powdered sucrose, 52; salt mixture (U.S.P. 14), 4; U.S.P. cod liver oil, 5; cellulose, 2; and vitamin mixture, 4. The vitamin mixture consisted of the following in milligrams: thiamine·HCl, 150; riboflavin, 150; biotin, 6; Ca pantothenate, 400; pyridoxine·HCl, 150; menadione, 20; folic acid, 30; 0.1% dilution of vitamin B_{12} in mannitol, 5; nicotinamide, 1000; inositol, 10,000; *p*-aminobenzoic acid, 2000; choline chloride, 4000; and powdered sucrose, to make 200 gm of mixture. The appropriate vitamins were omitted from the mixture when prepared for the deficiency diets. The control diet thus contained 30 mg of pyridoxine·HCl and 30 mg of riboflavin per kg. These values are much above the animals' requirement for growth (Brown and Sturtevant, '49). The animals consumed the diet ad libitum. A rat from each dietary group was killed by guillotining at 19, 31, 32, 38, 46 and 54 days after being placed on the experiment. Adrenal glands, liver, brain, spleen and heart were removed for analysis for CAs.

In experimental series 2, the same pyridoxine-deficient diet as above was used, but the vitamin mixture was prepared according to the method of Waisman and Boldt ('57). The control diet was supplemented with 5 mg of pyridoxine·HCl per kg of diet. This amount represents about 5 times the growing rat's requirement for pyridoxine (Brown and Sturtevant, '49). In this series animals were killed in groups, for analysis of tissues, at 50, 52, 63, 64, 66, 72, 78, 97 and 114 days after starting to receive the diets.

Experimental series 3 consisted of rats consuming a commercial, synthetic, pyridoxide-deficient diet[7] ad libitum. Control rats were matched with the deficient ones by weight at the start of the experiment. Their food allotment was adjusted daily in order to maintain their body weights and those of the deficient rats at the same level. The animals were fed the diets soon after weaning when their body weights were between 60 and 70 gm. These rats were used in metabolism tests at various stages of the deficiency as described below.

Series 4 included three groups in which the effect of thiamine deficiency upon the adrenal CAs was measured. The diets for the first two of these experiments contained acid-hydrolyzed casein as the source of amino acids, with supplements of phenylalanine, tyrosine and tryptophan (Townsend and Sourkes, '58). In a third experiment a similar diet was used but this one was based upon alcohol-extracted casein. Two thiamine-deficient and two control rats were killed after receiving the diets for 21 days; the remaining animals were killed on the 23rd day.

The CAs and dopa were determined by spectrophotofluorometric methods. Specific directions have been given elsewhere for measuring total CAs, as the norepinephrine equivalent (Sourkes and Drujan, '57); for E and N (Drujan et al., '59); and for dopamine and dopa (Murphy and Sourkes, '59). The analysis of variance and the calculations of correlation coefficients were performed according to standard statistical techniques (Snedecor, '46). Where the mean CA contents of treatment groups differed significantly the analysis of covariance was carried out to determine whether the observed differences persist after statistical adjustment of organ weights to the same value by accounting for the regression of CA content on organ weight.

Effect of pyridoxine deficiency. The data obtained with 24 rats in series 1 (6 replicates, 4 diets; one rat fed each diet per

[6] Crisco.

[7] Nutritional Biochemicals Corporation, Cleveland.

replicate) were subjected to the analysis of variance. In certain cases the deficiency of pyridoxine or of riboflavin influenced the CA content of the organs, but in no case did the interaction of the two deficiencies attain statistical significance. In the second experimental series 33 rats were used: 15 full-fed controls and 18 deficient rats. In both series the following parameters were measured: body weights, organ weights and CA content of the organs. The mean values are set out in table 1. It may be seen that the pyridoxine-deficient rats gained appreciably less than the controls. In the course of the deficiency the weight of certain organs increased, relative to body weight. This was the case for the adrenal glands and heart. The relative weight of the brain also showed an increase but this was statistically significant only for the more seriously deprived rats (series 2). The relative weights of livers and spleens of the deficient animals were not affected. Olsen and Martindale ('54) observed that the ratios of the weight of the adrenals, heart, liver and kidney, respectively, to body weight were increased in rats fed a pyridoxine-deficient diet for 38 weeks; their results are thus partially confirmed by the present series 2. In comparing the data of table 1 with those of Olsen and Martindale ('54) it may be noted that the diet used here provided considerably larger amounts of vitamins (except choline) and, moreover, included menadione, folic acid, and vitamin B_{12}. Olsen and Martindale added deoxypyridoxine to their diet during the first 4 weeks of the experiment.

There were no significant differences in the E and N content of the adrenals, brain, liver or spleen in the rats of series 1. In the rats of series 2 the E and N content of the adrenals was lower in the deficient animals than in the controls by about 20%; this apparent effect may be related to body weight because the difference no longer attained statistical significance after adjustment of the CA values for regression on body weight or adrenal weight. The correlation between body weight and adrenal CA levels has been noted by Hökfelt ('51) as well as in this laboratory.[5] The E and N content of the liver was significantly reduced in the deficient rats; these differences were no longer statistically significant after adjustment of the values for regression of liver weight. A lower N content of the pyridoxine-deficient rat hearts was not significant.

It thus appears that even with prolonged periods on the deficiency diet, significant effects of pyridoxine deficiency upon the formation of tissue E and N are not readily detected. It was considered possible that the concentration of dopamine, the immediate product of the reaction in which vitamin B_6 is involved, might be a more sensitive indicator of the extent of the deficiency and, in line with this, the problem was studied from another aspect as soon as satisfactory methods for measuring dopamine and dopa had been worked out. In the next series of animals (series 3) rats received the experimental diets shortly after weaning, using inanition controls along with a deficient group. Instead of measuring dopamine in the tissues as for E and N, the stock of animals was drawn upon once to three times each week for 5 to 12 weeks after starting to feed the experimental diets, for metabolic studies. In these studies each rat was injected with L- or D-dopa intraperitoneally and placed in a metabolism cage for the collection of urine. Five rats were housed together in each cage for the collection of the pooled urine. Water, but not food, was available during the 24-hour collection period.

The results of giving dopa to such animals are set out in table 2. The proportions of the two dopa isomers, respectively, excreted as dopamine varied from run to run. Nevertheless, in each test the deficient rats excreted substantially less of the administered dopa as the amine. Dopa was regularly present in the urine of the animals given D-dopa, and again the deficient animals excreted less of the amine. The urinary amino acid is considered to be the unchanged D-isomer, for incubation of these urines with bacterial tyrosine decarboxylase[8] resulted in no change in the analyses for dopamine and dopa.

In two trials both deficient and control groups were injected with α-methyl-*meta*-

[5] See footnote 5, page 145.

[8] Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE 1

Effect of pyridoxine deficiency on the fresh weight and catecholamine (CA) content of rat organs

	Series 1: (fed diet 19 to 54 days)			Series 2: (fed diet 50 to 114 days)		
	+ B_6	− B_6	P	+ B_6	− B_6	P
Initial body weight, gm	100 ± 2[1]	94 ± 8	> 0.05	91 ± 5	87 ± 4	> 0.05
Final body weight, gm	211 ± 26	152 ± 10	< 0.01	364 ± 24	133 ± 7	< 0.01
Adrenal glands						
Weight, mg/100 gm body weight	18.1 ± 2.7	21.5 ± 1.6	> 0.05	9.7 ± 0.8	26.1 ± 3.3	< 0.01
Epinephrine, μg	17.49 ± 0.28	16.79 ± 0.28	> 0.05	24.32 ± 1.48	17.92 ± 2.14	< 0.05[2]
Norepinephrine, μg	5.70 ± 0.14	6.20 ± 0.14	> 0.05	10.47 ± 5.29	8.42 ± 1.21	> 0.05
Epinephrine + norepinephrine, μg				34.79 ± 5.64	26.34 ± 2.45	< 0.05[3]
Liver						
Weight, gm/100 gm body weight	4.08 ± 0.14	3.75 ± 0.14	> 0.05	3.38 ± 0.27	4.03 ± 0.18	> 0.05
Epinephrine, μg	1.94 ± 0.17	1.64 ± 0.17	> 0.05	0.49 ± 0.05	0.28 ± 0.04	< 0.05[4]
Norepinephrine, μg	4.63 ± 0.35	4.04 ± 0.35	> 0.05	2.01 ± 0.12	1.20 ± 0.11	< 0.05[4]
Brain						
Weight, gm/100 gm body weight	0.95 ± 0.11	1.17 ± 0.08	> 0.05	0.54 ± 0.02	1.38 ± 0.07	< 0.01
Norepinephrine, μg	0.93 ± 0.02	0.92 ± 0.02	> 0.05	0.69 ± 0.08	0.73 ± 0.10	> 0.05
Spleen						
Weight, gm/100 gm body weight	0.24 ± 0.03	0.23 ± 0.02	> 0.05			
Norepinephrine, μg	1.62 ± 0.03	1.59 ± 0.03	> 0.05			
Heart						
Weight, gm/100 gm body weight				0.28 ± 0.01	0.45 ± 0.03	< 0.01
Norepinephrine, μg				0.70 ± 0.32	0.51 ± 0.11	> 0.05

[1] Mean ± standard error.
[2] After recalculation of data as ratio of adrenal E to adrenal weight, $P > 0.05$.
[3] After adjustment by covariance analysis for regression of body weight on adrenal CAs, $P > 0.05$.
[4] After adjustment by covariance analysis for regression of liver weight on its CA content, $P > 0.05$.

TABLE 2

Percentage of administered dopa recovered in urine

No. of days receiving diet	Vitamin B_6 group	Mean body weight	Dopa	Other treatments	Compound administered			
					L-Dopa		D-Dopa	
					Dopamine	Dopa	Dopamine	Dopa
		gm	*mg/kg*		%	%	%	%
38	+	83	10	none	12.5	0	25.4	6.4
	−	82	10	none	6.5	0	7.3	6.2
45	+	85	10	none	8.2	0	15.3	7.1
	−	80	10	none	4.9	0	4.6	2.4
51	+	79	5	AMMT[1]	8.6	0	11.9	9.1
	−	75	5	AMMT	0	0	0.7	2.4
53	+	74	5	none	6.7	0	24.8	5.2
	−	77	5	none	4.4	0	7.1	4.0
56	+	74	10	AMMT	5.8	0.4	14.7	18.3
	−	80	10	AMMT	0.4	0.4	0.8	4.9
60	+R[2]	82	10	pyridoxine-injected[2]	7.0	0	12.9	9.4
	−R	85	10	pyridoxine-injected	11.0	0	14.9	6.4
65	+R	100	10	none	5.7	0	13.5	6.6
	−R	96	10	none	13.4	0	14.8	8.4
66	+	96	10	none	not done		17.6	7.4
	−	76	10	none	not done		5.0	6.9
82	+	92	10	none	not done		18.1	2.8
	−	71	10	none	not done		3.3	6.2
85	+R	124	10	none	7.5	0	24.0	3.8
	−R	135	10	none	3.4	0	11.4	6.1

[1] AMMT (*a*-methyl-*meta*-DL-tyrosine monohydrate), 100 mg/kg, was injected (in 0.9% saline) intraperitoneally into each rat 0.5 hours before the dopa was given and, again, 0.5 hours after.

[2] Rats signified by "R" in column 2 each received 0.5 mg of pyridoxine·HCl on days 59 and 61. They continued to receive the respective deficiency diets.

DL-tyrosine monohydrate[9] (AMMT), a compound previously shown to be a competitive inhibitor of dopa decarboxylase *in vitro* (Sourkes, '54), and *in vivo*.[10,11] In these tests (at 51 and 56 days, table 2) the excretion of dopamine by the deficient rats, already well below control levels, was even further reduced. In the intervening test in which AMMT was not given (at 53 days) the percentage of injected dopa excreted as the amine was in the "non-inhibited" range. The excretion of dopa following adminstration of D-dopa was high in control groups receiving AMMT but this change was not evident in the deficient animals. In the experiment carried out at 56 days L-dopa gave rise to a small amount of urinary dopa, the only occasion on which some of the amino acid appeared in the urine and presumably the result of the action of AMMT in limiting the decarboxylation.

On the 59th and 61st days some of the deficient and supplemented rats were each injected subcutaneously with 0.5 mg of pyridoxine·HCl dissolved in dilute neutral phosphate buffer. The dopa-administration test was performed on days 60, 65 and 85. From the data in table 2 it is clear that the first vitamin injection rapidly brought about repletion of the tissues. On the 60th and 65th days the animals which had previously been deficient (and which had continued all the while to receive the deficient

[9] A gift of Merck Sharp and Dohme Research Laboratories, Rahway, N. J. (Lot L523766-1-3).

[10] Sourkes, T. L., and G. F. Murphy 1960 Effects of catecholamino acids on the catecholamine content of rat organs. Federation Proc., *19:* 295 (abstract).

[11] Sourkes, T. L., and G. F. Murphy 1960 Effects of amino acid decarboxylase inhibitors and pyridoxine deficiency on catecholamine metabolism. Proceedings Third Annual Meeting, Canad. Fed. Biol. Societies, Winnipeg, 1960 (abstract).

diet) excreted a higher proportion of injected dopa as dopamine than did the controls. By the 24th day following the second injection of vitamin (day 85) the deficiency had reappeared as suggested by the urinary excretion of dopamine and dopa, particularly after D-dopa had been administered. A comparable set of rats, not given any vitamin injections, was tested on the 66th and 82nd days. They showed the deficient excretion of dopamine after administration of D-dopa, previously noted at other stages of deficiency as a result of pyridoxine lack. These animals were killed on the 84th day and their brains were analyzed for N and dopamine. The deficient rats' brains contained 0.31 ± 0.03 μg N and 0.74 ± 0.02 μg of dopamine per gm; corresponding figures for the controls were 0.34 ± 0.02 and 0.84 ± 0.08 μg per gm.

Effect of riboflavin deficiency. The same types of statistical analyses were performed for the riboflavin data as for the pyridoxine data of series 1, the experiments in which the two deficiences were studied in a multifactorial arrangement. The riboflavin deficiency affected growth even more severely than the pyridoxine deficiency (table 3). The adrenals, brain and spleen of the deficient animals weighed more, relative to body weight, than those of the corresponding organs of the full-fed controls; the ratio of liver weight to

TABLE 3

Effect of deficiency of riboflavin upon the fresh weight and catecholamine (CA) content of rat[1] organs

	+ Vitamin B_2	− Vitamin B_2	Probability
Initial body weight, gm	101 ± 5.6[2]	103 ± 5.6	> 0.05
Final body weight, gm	252 ± 5.6	129 ± 5.6	< 0.01
Adrenal glands			
Weight, mg/100 gm body weight	13.12 ± 1.60	25.40 ± 1.60	< 0.01
Norepinephrine, μg	6.16 ± 0.53	5.74 ± 0.53	> 0.05
Epinephrine, μg	18.61 ± 1.10	15.67 ± 1.10	> 0.05
Liver			
Weight, gm/100 gm body weight	3.89 ± 0.15	3.96 ± 0.15	> 0.05
Norepinephrine, μg	6.27 ± 1.12	2.41 ± 1.12	< 0.01[3]
Epinephrine, μg	2.69 ± 0.16	0.90 ± 0.16	< 0.01[3]
Brain			
Weight, gm/100 gm body weight	0.42 ± 0.04	0.68 ± 0.04	< 0.01
Norepinephrine, μg	0.98 ± 0.02	0.87 ± 0.02	< 0.01[3]
Spleen			
Weight, gm/100 gm body weight	0.20 ± 0.02	0.26 ± 0.02	= 0.05
Norepinephrine, μg	1.64 ± 0.03	1.57 ± 0.03	> 0.05

[1] Received test diets for 19 to 54 days.
[2] Mean ± standard error.
[3] After adjustment for regression of organ weight on CA content, the probability was > 0.01, but < 0.05.

TABLE 4

Influence of thiamine deficiency on the catecholamine content of rat adrenal glands

Exp.	Days fed deficient diet	No. rats/ group	Catecholamines/pair of adrenals					
			Vitamin B_1-deficient			Controls		
			Norepinephrine	Epinephrine	Total	Norepinephrine	Epinephrine	Total
					μg			μg
A	11–13	6			15.2 ± 1.3[1]			17.6 ± 1.3
B	13–28	6			23.4 ± 3.5			22.8 ± 3.0
C	21–23	5	5.2 ± 0.4	14.6 ± 1.0		6.6 ± 0.6	16.1 ± 0.6	

[1] Mean ± standard error.

body weight remained constant. Only a few differences in CA levels of organs were found. Those which could not be attributed to differences in organ weight (by applying covariance analysis) included liver N and E, and brain N. In each of these cases the CA content was significantly lower ($P < 0.05$) in the organs of the riboflavin-deficient rats.

Effect of thiamine deficiency. The effect of thiamine deficiency on the adrenal content of the CAs was explored in two studies in which only total CAs were measured and one in which N and E were separately estimated (table 4). The differences in adrenal content of these amines were not statistically significant.

DISCUSSION

Several investigators have studied the CA content of rat tissues as a function of pyridoxine deficiency, without demonstrating an unequivocal effect. The experiments described in this paper show that limited changes occur with chronic vitamin deprivation, as in series 2, but that these changes are readily accounted for by variations in organ weight or body weight resulting from the deficiency. In series 3 the brains of one group of rats were analyzed for dopamine, the only organ in which this CA occurs in relatively high concentrations. The dopamine was lower than in the brains of control rats, but the high degree of variation from animal to animal precluded assigning statistical significance to this difference. Similar studies in vitamin B_6-deficient chicks (Weissbach et al., '57) have proven more successful in demonstrating an effect of vitamin deprivation upon the 5-hydroxytryptamine levels in the brain, intestine, liver and blood. This may stem from species differences in the binding of coenzyme by pyridoxine-requiring enzymes under conditions of dietary deficiency. Another explanation may be that the chicks were on the deficient diet from the time of hatching, whereas the rats used here began to receive the diet only some time after weaning. The matter should by no means be closed until similar experiments have been conducted on pyridoxine-deficient progeny of deficient mothers.

Thus, the *lack of vitamin B_6 alone*, other nutrients being present in the diet in sufficient amount, does not lead in rats in the post-weaning period to decreases in tissue CAs. It is entirely possible, of course, that the use of dietary adjuvants such as carbonyl reagents and deoxypyridoxine will favor such decreases. Experiments along these lines are now in progress in this laboratory.

Despite the above findings with CAs of the tissues, an effect of pyridoxine-deficiency can be detected in the urinary content of dopamine in the 24 hours following the administration of dopa intraperitoneally. Control rats limited in food intake to match the mean body weight of the deficients, excrete 6 to 12% of L-dopa and 15 to 25% of D-dopa as dopamine; the corresponding figures for the deficient animals are 4 to 7% and 3 to 7%, respectively. Stated in another way, the deficient rats convert dopa to urinary dopamine at only 52 to 66% of the rate of the controls in the case of L-dopa, and 18 to 30% in the case of D-dopa. The lower relative figures for urinary dopamine after giving D-dopa may be accounted for by the different metabolism of the two dopa isomers. Thus, the decarboxylation of L-dopa leads directly to dopamine and the change in the rate of conversion of this isomer during pyridoxine deficiency may be taken as an indication of the effect of the deficiency upon dopa decarboxylase. However, dopamine can be derived from D-dopa probably only after (1) oxidative deamination of this amino acid; (2) asymmetric transamination of dihydroxyphenylpyruvic acid to L-dopa; and (3) decarboxylation of the L-dopa to dopamine. It has been shown[12] (Clark, '59) that 3-hydroxyphenylpyruvic and 3, 4-dihydroxyphenylpyruvic acids are transaminated *in vivo* to the corresponding amino acids; these are then subject to the action of decarboxylating enzymes. This means that the transformation of D-dopa reflects the action of two pyridoxal phosphoric acid-requiring enzymes: transaminase and amino acid decarboxylase, besides D-amino acid oxidase. Further experiments are necessary to decide whether

[12] Pogrund, R. S., W. Drell and W. G. Clark 1955 Transamination of 3-hydroxyphenylpyruvic acid *in vivo*. Federation Proc., *14*: 116 (abstract).

one or both of the vitamin B_6-linked enzymes are affected under the test conditions described above.

The rats fed riboflavin-deficient diets for 19 to 54 days suffered a decrease in the CAs of the liver and in the N of the brain. The significance of these changes is not easy to interpret at present. Neurological defects do not appear to be important in ariboflavinosis in the rat, although in clinical riboflavin deficiency such changes do appear, all on the sensory side (Stannus, '44). There is no information available which would permit linking these symptoms to an alteration in the concentration of N in the brain.

Thiamine deficiency led to no alteration in the CA content of the adrenal glands. Thus, the three vitamin deficiences exerted no detectable influence upon the adrenal E and N. This attests to the remarkable stability of the adrenal medullary component of the sympathetic nervous system during serious dietary insult.

SUMMARY

The effect of deficiency of three vitamins upon the catecholamine content of several tissues has been studied in the rat. Riboflavin deficiency induced decreases in liver epinephrine and norepinephrine, and in brain norepinephrine, but not in the adrenal glands or spleen. The adrenal glands of thiamine-deficient animals did not differ significantly in epinephrine or norepinephrine concentration from those of their controls. Pyridoxine-deficient rats showed no changes in the catecholamine content of the adrenal glands, liver, brain, spleen and heart which were not attributable to concomitant changes in body weight or organ weight as a result of the deficiency. Pyridoxine deficient rats showed a decreased ability to form urinary dopamine from administered dopa. α-Methyl-*m*-tyrosine, an inhibitor of dopa decarboxylase, interfered with this conversion of dopa to dopamine in both deficient and vitamin B_6-supplemented rats receiving either D- or L-dopa.

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The Nutritional Requirements of the Protein-Depleted Chicken

I. EFFECT OF DIFFERENT PROTEIN DEPLETION REGIMES ON BODY COMPOSITION DURING DEPLETION, REPLETION AND REDEPLETION[1]

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The effects of starvation and malnutrition on the nitrogen composition of various body components have been studied by several workers (Addis et al., '36; Ju and Nasset, '59). Such studies have demonstrated that during depletion various body organs lose nitrogen at different rates. Again, on repletion, the rate of nitrogen accretion varies inversely with the rate of nitrogen loss during depletion of the organ.

The ability of an animal to conserve its body nitrogen under certain conditions has been reported by French et al. ('41) and by Kosterlitz and Campbell ('45). These workers found smaller body nitrogen losses in rats fed a nitrogen-free diet after previous depletion followed by repletion.

The extent to which animals will catabolize collagen when receiving a nitrogen-free or a poor-quality, low-protein diet has also received attention by several investigators. Mendes and Waterlow ('58) reported that rats fed a poor-quality, low-protein diet continued to build collagen even though body weight did not increase. Harkness et al. ('58), demonstrated with mice that, using a nitrogen-free diet, over a 20-day period, during which time the animals were losing body weight, 10% of the collagen nitrogen was lost from the body, compared with a loss of 35% of the noncollagen nitrogen.

The influence of low-protein diets on other body components, notably liver fat, has been reported by Litwack et al. ('52) and Benton et al., ('55). These workers pointed out that the percentage of liver fat increased by feeding low levels of protein and that the extent of fat accumulation was influenced by the type and quality of the protein source.

The present study was designed to compare the effects of three initial depletion regimes, followed by repletion and then redepletion by starvation, on the water, fat, nitrogen and collagen content of the carcass and liver of growing chickens. Whereas much of the previous work on protein depletion has been concerned with comparisons of different treatments for an equal depletion time, in the present study the regimes were evaluated in animals that were depleted and repleted to the same body weight.

EXPERIMENTAL PROCEDURE

Day-old male New Hampshire ♂ × Columbian ♀ chicks were fed a standard 23% protein ration (for composition see table 1) until they attained an average body weight of 130 gm (12 days of age). At that time the animals were distributed on a weight basis into 4 groups of 58. Three depletion regimes, consisting of (1) complete starvation, (2) an essentially nitrogen-free diet and (3) an unbalanced protein diet, containing 15% of gelatin as the only source of nitrogen, made up three of the treatment groups. Chicks in the 4th group served as controls and received the standard ration until the final period of depletion by starvation when they were starved along with chicks in the other three

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treatment groups. The composition of all rations used is given in table 1. The "essentially nitrogen-free" diet contained, by analysis, 0.08% of nitrogen. For the purpose of this experiment it can be considered to be nitrogen-free, and will be designated as such in this discussion. The 4 groups continued to receive the standard starting ration from the time of separation at a body weight of 130 gm until a body weight of 190 gm was attained (16 days of age). At this weight (190 gm) three groups were started on their respective treatments. As soon as the chicks in a treatment group had lost approximately one third of starting body weight (i.e., had decreased from 190 to 130 gm), they were repleted by returning them to the standard ration. Repletion was continued until the animals reached twice their initial starting weight (390 gm) at which time feed was removed and the birds were redepleted by starvation for 6 days. Throughout the experiment, animals of all groups had free access to water.

Five animals each, with body weights approximating the group average were removed (a) after the initial depletion period, (b) upon depletion to the starting weight (190 gm), (c) upon reaching twice the starting weight (390 gm), and (d) after a final 6-day starvation period. Table 2 shows the time intervals between sampling periods together with the average body weights for the same period. The animals, upon removal, were starved for 4 hours to complete passage of food from the digestive tract and then were killed with chloroform. From the control lot, birds were also removed and killed at identical body weights, except that those in the control group corresponding with the initial depletion period were not depleted; instead they were removed when all groups were first separated at body weights of 130 gm.

An explanation for the experimental periods chosen is in order. The first depletion which involved a loss of one third of the initial body weight represents the point in depletion beyond which heavy mortality occurs. Repletion to initial body weight was believed to be of interest for comparison with results of a control group under-

TABLE 1

Composition of experimental rations[1]

Standard diet		Depletion diets		
Ingredient	Amount	Ingredient	Amount	
			N-free	Gelatin
	%		%	%
Corn meal	53.1	Fiber, non-nutritive	3.00	3.00
Soybean meal (50% protein)	34.0	Corn oil	3.00	3.00
Alfalfa meal, dehydr. (17% protein)	3.0	Mineral mix[2]	4.95	4.95
Corn distillers' solubles	4.0	B vitamins[3]	0.15	0.15
Butyl fermentation product	1.0	Vitamins A, D and E[3]	0.10	0.10
Dicalcium phosphate	2.2	Choline chloride	0.20	0.20
Limestone	0.8	Dextrin	44.10	44.10
Sodium chloride	0.5	Glucose	44.50	29.50
Mineral concentrate[4]	0.2	Gelatin		15.00
Vitamin A and D concentrate (1,000,000 I.U. vit. A/pound; 250,000 I.U. vit. D_3/pound)	0.2			
Antibiotic supplement[5]	0.5			
Vitamin B_{12} supplement (20 mg/pound)	0.5			
	gm/100 pounds			
Choline chloride	105			
Niacin	5			

[1] Protein content of ration (N × 6.25): standard 22.9%; N-free 0.5%; gelatin 14.6%.
[2] For composition see Fisher et al. ('60).
[3] For composition see Fisher and Johnson ('56).
[4] Delamix, Limestone Corporation of America, Newton, New Jersey.
[5] Supplying 10 mg Aureomycin/pound of feed.

TABLE 2

Average weight of chickens removed for body composition analysis after 4 hours' starvation

Treatment	Average weight for three periods[1]							
	Depletion		Repletion				Redepletion (by starvation)	
			To initial weight		To twice initial weight			
	Time interval	Body weight[2]	Time interval	Body weight	Time interval	Body weight	Time interval	Body weight
	days	*gm*	*days*	*gm*	*days*	*gm*	*days*	*gm*
Starved	6	132	4	190	7	394	6	243
N-free	14	131	3	190	6	390	6	268
Gelatin-fed	14	133	3	189	6	389	6	260
Control[3]		131	4	190	8	392	6	249

[1] The time intervals correspond to the following periods: *depletion*—from initial body weight of 190 gm to body weight of 130 gm; *repletion to initial weight*—from body weight of 130 gm back to 190 gm; *repletion to twice body weight*—from body weight of 190 gm to body weight of 390 gm; *redepletion* (starvation)—this followed the repletion to twice initial body weight, from 390 gm to approximately 260 gm.

[2] With the exception of the starved and control groups for the final 6-day starvation period, no group of 5 birds ever varied more than 5 gm in body weight from one another.

[3] The control birds received the standard 23% protein starting ration throughout and birds killed for analysis from this group were removed when they had attained an average body weight corresponding to that of the treatment groups. The values shown under depletion corresponds to the weight attained with the standard ration and is not due to any depletion; for the final starvation period, however, the controls were also starved for 6 days along with the three treatment groups.

going the same body weight changes without prior depletion; also because it was expected that changes leading to this body weight would represent a very different pattern of "growth." The continuation to twice the initial body weight was designed to provide enough time for all animals to again reach an essentially normal growth pattern. The final depletion-by-starvation period was expected to provide useful information on adaptation and the effects of previous dietary history.

After the birds were killed the liver was excised and both carcass and liver dried to constant weight at 100°C. Moisture was calculated from the difference between wet and dry weights. The livers were ground with a mortar and pestle until a homogenous mass was obtained. The carcasses, containing everything but the livers, were twice forced through a meat grinder using first a set of coarse blades, and then a set of fine ones.

Nitrogen analyses on carcass and liver samples were carried out by a semi-micro-Kjeldahl procedure. Fat was determined by extracting the carcass and liver samples for two separate 24-hour periods on a shaker with a chloroform-methanol mixture (2:1). The solvent from the supernatant was then evaporated and the residue extracted with petroleum ether. After filtering, the petroleum ether was evaporated and the fat residue weighed. As an estimation of the collagen content of the carcass, hydroxyproline was determined according to the method of Neuman and Logan ('50). The hydroxyproline determinations were carried out in duplicate on pooled samples from all 5 birds removed from each of the groups at each sampling period. All other determinations were made in duplicate or triplicate on individual animals.

RESULTS

The moisture and fat content of carcass and liver are shown in tables 3 and 4, respectively. The greatest difference in carcass moisture content was noted during depletion and repletion to initial weight with the nitrogen-free diet. The lower moisture content in this group correlates well with the increased fat content in the same group (table 4). During the final 6-day starvation period a 2 to 3% increase in carcass moisture over the previous period was observed in all treatment groups. Little difference was noticeable between any of the treatments or periods

TABLE 3

Percentage of moisture in carcass and liver of birds during depletion, repletion and redepletion

Treatment	Moisture content for three periods[1]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver
Starved	73.8 ± 0.5[2]	75.8 ± 0.2	73.7 ± 0.3	71.5 ± 2.7	71.4 ± 0.4	77.2 ± 0.2	74.6 ± 0.3	76.4 ± 0.4
N-free	65.7 ± 0.6	74.4 ± 1.3	68.7 ± 1.7	77.4 ± 0.3	71.4 ± 0.8	76.6 ± 0.4	75.3 ± 0.3	77.1 ± 0.2
Gelatin-fed	72.1 ± 0.4	77.4 ± 0.2	73.9 ± 0.6	77.1 ± 0.7	72.6 ± 0.5	77.6 ± 0.1	74.7 ± 0.3	77.4 ± 0.3
Control[1]	72.0 ± 0.2	78.3 ± 0.1	71.7 ± 0.4	77.8 ± 0.3	70.3 ± 0.4	75.8 ± 0.2	72.3 ± 0.2	75.5 ± 0.4

[1] See footnotes 1 and 3, table 2.
[2] Mean value ± standard error.

TABLE 4

Percentage of fat (dry-weight basis) of the carcass and liver of birds during depletion, repletion and redepletion

Treatment	Fat content for three periods[1]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver
Starved	11.0 ± 1.4[2]	11.4 ± 0.6	17.8 ± 0.7	37.6 ± 6.4	24.6 ± 1.2	14.2 ± 0.5	6.1 ± 0.3	10.8 ± 0.7
N-free	30.7 ± 1.1	24.0 ± 3.8	31.8 ± 1.6	15.7 ± 0.5	26.4 ± 1.5	14.3 ± 0.5	8.6 ± 0.2	12.9 ± 0.7
Gelatin-fed	13.3 ± 1.7	13.5 ± 0.8	16.3 ± 1.7	20.8 ± 3.2	22.4 ± 1.2	12.5 ± 0.2	6.2 ± 1.0	12.6 ± 0.9
Control[1]	24.0 ± 1.0	12.8 ± 0.4	23.9 ± 0.9	12.4 ± 0.3	22.1 ± 0.9	13.1 ± 0.3	5.8 ± 0.5	12.7 ± 0.7

[1] See footnotes 1 and 3, table 2.
[2] Mean value ± standard error.

with regard to liver moisture, despite marked changes in liver fat (table 4) and liver nitrogen (table 5).

The fat composition for carcass and liver showed important differences between treatment groups. Whereas the carcass showed a loss of approximately one-half its fat content upon depletion on the starvation and gelatin regimes compared with the controls, carcasses in the nitrogen-free group actually increased in fat content. Upon repletion to initial body weight the starved group showed an excessive accumulation of liver fat compared either with the controls or with the values during the depletion period. The liver fat changes for the groups starved and fed gelatin also are much greater at repletion to initial body weight than are the corresponding changes for carcass fat. At repletion to twice the original weight both carcass and liver fat values were essentially normal compared with those of the control group. During the redepletion-by-starvation period chicks in all treatment groups lost approximately 70% of the carcass fat whereas the liver fat decreased only slightly when compared with the previous period.

Table 5 shows the nitrogen content of liver and carcass. When expressed on a dry-weight basis, the three depletion regimes induced marked differences during the depletion to a similar body weight. Thus, values for the nitrogen-free group showed approximately 20% less nitrogen than those for either of the other two treatment groups or the controls. Since the change in percentage of nitrogen content during depletion with the nitrogen-free diet reflects not only changes in nitrogen but also of fat and moisture, the magnitude of the differences shown in the upper part of table 5 would be decreased if the results were expressed on an absolute (dry-fat-free) basis. When the nitrogen values are given on a wet-fat-free basis as in the lower half of table 5 they illustrate little variability between groups and periods (with the exception of those with extreme fat content), confirming also in the chicken the close relationship between body nitrogen and water previously shown in the rat (Bender and Miller, '53).

The results presented in tables 4 and 5 may be further clarified by comparing the

TABLE 5

Percentage of nitrogen content of carcass and liver of birds during depletion, repletion and redepletion

Treatment	Nitrogen content for three periods[1]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver
	Dry-weight basis							
Starved	11.6 ± 0.2[2]	13.4 ± 0.0	10.4 ± 0.1	9.2 ± 1.0	10.2 ± 0.2	11.8 ± 0.1	12.8 ± 0.2	13.5 ± 0.1
N-free	8.0 ± 0.1	8.7 ± 0.7	8.4 ± 0.4	12.1 ± 0.1	9.3 ± 0.3	12.2 ± 0.2	12.7 ± 0.7	12.6 ± 0.2
Gelatin-fed	10.5 ± 0.2	12.5 ± 0.1	10.6 ± 0.3	11.9 ± 0.4	10.4 ± 0.2	12.9 ± 0.1	13.1 ± 0.2	13.1 ± 0.4
Control[1]	9.6 ± 0.1	12.7 ± 0.1	9.4 ± 0.2	12.8 ± 0.0	10.1 ± 0.1	12.6 ± 0.1	12.6 ± 0.1	13.0 ± 0.1
	Wet-fat-free basis							
Starved	3.4	3.7	3.3	4.2	3.9	3.2	3.5	3.6
N-free	4.0	2.9	3.8	3.2	3.6	3.3	3.4	3.3
Gelatin-fed	3.1	3.3	3.3	3.5	3.7	3.3	3.5	3.4
Control[1]	3.5	3.2	3.5	3.3	3.8	3.2	3.7	3.6

[1] See footnotes 1 and 3, table 2.
[2] Mean value ± standard error.

TABLE 6

Percentage change in carcass and liver nitrogen between experimental periods[1]

Treatment	Nitrogen changes during three periods[2]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver
Starved	−20.7	−43.6	25.6	135.7	128.8	53.5	−30.9	−46.6
N-free	−29.6	−31.4	34.1	88.6	117.4	53.0	−17.0	−38.4
Gelatin-fed	−23.1	−36.8	32.6	103.1	113.9	66.0	−22.1	−41.4
Control[2]			44.8	36.9	133.0	75.5	−25.7	−37.7

[1] For detailed explanation of changes represented by values in table, see text.
[2] See footnotes 1 and 3, table 2.

TABLE 7

Percentage change in carcass and liver fat between experimental periods[1]

Treatment	Fat changes during three periods[2]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver
Starved	−70.5	−49.5	126.9	1010.0	222.2	−55.1	−86.3	−64.3
N-free	+6.3	95.5	32.0	−11.4	63.1	39.7	−80.1	−46.6
Gelatin-fed	−61.7	−29.3	60.9	225.7	201.7	−7.5	−83.1	−41.9
Control[2]			46.7	30.3	100.6	87.9	−84.4	−41.7

[1] For detailed explanation of changes represented by values in table, see text.
[2] See footnotes 1 and 3, table 2.

relative changes which occurred between time periods. Tables 6 and 7 show these changes for nitrogen and fat for each of the three treatment groups. The values shown for the depletion period represent the change that occurred when the animals were depleted from their original weight of 190 gm to approximately 130 gm. All groups were compared with the control birds analyzed at 190 gm of body weight. The other changes shown represent the increase or decrease from the preceding period. For example, the nitrogen value of 25.6% shown for the starved group on repletion (table 6) represents the increase in total nitrogen between the depletion period and the repletion period to the original starting weight.

On depletion, chicks in the nitrogen-free group lost a much greater percentage of carcass nitrogen than those receiving either of the other two treatments (table 6). The reverse was true, however, for the liver nitrogen loss, also shown in table 6. Compared with the controls, all groups showed a distinct lag in nitrogen accretion in the carcass upon repletion, even though no such lag was evident in total weight gain. Similarly, no lag was evident during repletion to initial body weight for the liver nitrogen; in fact, this organ accumulated three times as much nitrogen as the livers of the control group.

The nitrogen-free group lost less nitrogen during the final starvation period than any of the other treatment groups. The behavior of the gelatin-fed group was similar to that of the controls during the final starvation period, while the initially-starved group lost considerably more nitrogen than the controls and almost twice as much as the nitrogen-free group.

The relative changes in fat content on the various depletion regimes are shown in table 7. Chicks both in the starved groups and the gelatin-fed groups lost large amounts of their carcass and liver fat during depletion. By contrast, the nitrogen-free group increased its carcass fat slightly while showing virtually a doubling of its liver fat.

TABLE 8

Percentage change and absolute amount (in mg) of hydroxyproline in carcass of chickens during depletion, repletion and redepletion[1]

Treatment	Hydroxyproline content and changes during three periods[2]							
	Depletion		Repletion				Redepletion (6-day starvation)	
			To initial weight		To twice initial weight			
	%[2]	mg[3]	%[2]	mg[3]	%[2]	mg[3]	%[2]	mg[3]
Starved	11.2	597	7.5	643	111	1361	−21.5	1079
N-free	−0.6	535	20.4	644	107.3	1335	−21.5	1038
Gelatin-fed	4.5	562	12.1	647	99.2	1289	−18.1	1056
Control[4]		361	32.9	538	169.0	1447	−25.6	1077

[1] For detailed explanation of changes between periods, see text.
[2] Percentage of change between periods.
[3] Absolute amount of change in milligrams.
[4] See footnotes 1 and 3, table 2.

On repletion there was a marked increase in fat deposition of the carcass and liver for the starvation and gelatin-fed groups in comparison with the controls. This increase continued until all groups reached a constant fat content during the repletion period represented by twice the starting weight (table 4).

The fat loss during the final starvation period was essentially the same for all groups. For total carcass there was a suggestion that the previously-starved group lost more and the nitrogen-free group less, as was the case also for the nitrogen losses. A greater fat loss by the initially-starved group during the final starvation was very pronounced in the liver fat changes.

Determinations of hydroxyproline as an estimate of the collagen content of the carcass are shown in table 8. On depletion, the amount of collagen in the carcass of all groups showed a relatively constant value, indicating that during this period the animals did not catabolize collagen protein even though nitrogen losses from the carcass during this period ranged from 20 to 29% (table 6). Upon repletion a distinct lag in collagen formation was observed with all treatments when compared with the controls (table 8) or with the increase in total carcass nitrogen (table 6). During the final starvation period all animals lost a substantial amount of their collagen nitrogen. This marks an important difference in collagen loss during the final starvation period in comparison with the initial depletion period.

DISCUSSION

The present study in the chicken confirms findings on protein depletion in other animal species. It extends previously obtained information to the simultaneously determined body composition changes resulting from three different depletion regimes and, furthermore, permits a comparison of these depletion regimes in animals depleted and repleted to the same body weight.

The comparative fat changes in the carcass and liver of the animals fed the nitrogen-free and the gelatin diet are particularly noteworthy. On an absolute basis, the nitrogen-free birds, upon depletion, showed a slight increase in carcass fat and an enormous increase in liver fat, whereas the gelatin-fed birds lost 60 and 29%, respectively, of their carcass and liver fat (table 7). Since both groups received the respective diets for an equal period (14 days) and caloric intake did not differ greatly, it was unexpected, in view of the work of Litwack et al. ('52), to find no fatty liver condition in the gelatin-fed group. In Litwack's study the animals fed a low casein diet, however, were growing slowly, whereas in the present study the gelatin did not support growth, but instead the animals lost weight at the same rate as the nitrogen-free birds. Litwack et al. ('52) showed that the fatty liver condition when using the low casein diet was completely relieved when gelatin was added to the diet. It is, of course, not known whether the gelatin effect was due to a better amino

acid balance or whether gelatin possesses special lipotropic properties, which would also explain the present findings. The latter explanation seems more probable since the growth rate when using the gelatin-supplemented-casein diet of Litwack was actually depressed despite the fact that liver fat returned to normal.

In addition to the large difference in fat content of carcass and liver between the nitrogen-free and gelatin-fed groups, large differences also occurred in the nitrogen content between these two groups. Chicks in the nitrogen-free group lost considerably more carcass nitrogen than those in the gelatin-fed group, which suggests a partial utilization of gelatin nitrogen for the synthesis of nonspecific nitrogenous compounds, thus sparing body protein and reducing nitrogen losses. It is interesting to note that Garrow ('59) reported recently that dogs receiving a nitrogen-free diet lost about 30% of their body nitrogen but only approximately 15% of their body weight. While no analyses were given for water or fat content, Garrow suggested that the differences might be due to edema fluids. The present observations on the high fat content of the birds fed the nitrogen-free diet would suggest that fat accumulation is a more likely explanation for his findings.

The important role of the liver in intermediary metabolism is well illustrated by the large increase in fat and nitrogen content on repletion to initial body weight (tables 6 and 7). Among all three treatment groups there appears to be at least a qualitative relationship between the fat and nitrogen increase in the liver during initial repletion. The extent of these liver changes may well represent liver function and its impairment.

The smaller loss of carcass nitrogen by chicks in the nitrogen-free group during the final starvation period (compared with the other treatments as well as with the control group, table 6) is in full agreement with the observations reported for the rat by French et al. ('41) and by Kosterlitz ('45). In sharp contrast with the greater nitrogen retention by the nitrogen-free group, was the large nitrogen loss incurred by the group previously depleted by starvation. By comparison with the control and gelatin-fed groups which lost 22 to 26% of carcass nitrogen during the final starvation period, animals in the previously-starved group lost 31% of carcass nitrogen during the second period. This represents a loss of almost twice as much nitrogen when compared with the nitrogen-free group (31 vs 17%).

Bender and Miller ('53) have in recent years called attention to the relatively constant nitrogen-to-water ratio in the growing rat and have applied this relationship to a modified biological value determination procedure. The values in the lower half of table 5 illustrate that, with certain exceptions, the relationship between nitrogen and water also applies to the growing chicken. The exceptions to this relationship, however, namely those instances in which large fat accumulation occurs such as with the nitrogen-free diet, should not be overlooked. Attention is drawn to the proposal of Bender and Doell ('57) that protein evaluation by the growth method can be improved in accuracy by the feeding to littermates of rats the protein-containing ration and a nitrogen-free ration and considering the algebraic difference in body weights as the true gain corrected for maintenance. Our studies suggest this approach to be hazardous in the chicken in view of the large differences in body composition between normal birds and those maintained with a nitrogen-free diet.

The collagen changes observed during the initial depletion (table 8) confirm the observations of Mendes and Waterlow ('58) and those of Harkness et al. ('58). Although there were no losses in collagen (expressed as hydroxyproline) during the initial depletion (in fact an increase was observed in two of the three treatment groups) all groups showed a loss of approximately 20% during the final starvation period. In comparison with the control group the collagen losses in the three treatment groups were somewhat smaller. Since the collagen losses which occurred during the final starvation period cannot be due to the initial depletion, the difference in the stage of growth at the later period must have influenced this collagen loss.

It is interesting to compare the changes in total nitrogen and collagen accretion during the initial repletion period. While the liver showed a three-fold increase in

total nitrogen during initial repletion in comparison with the control group, the carcass nitrogen and collagen showed a decided lag in comparison with the controls. The collagen lag furthermore was much more pronounced than the lag for total carcass nitrogen shown. Mendes and Waterlow ('58) have also shown a distinct lag in the formation of new muscle protein upon repletion of depleted rats.

It is clear from the work of others as well as from the present study that important differences in body composition result from different depletion regimes. In our work, emphasis was placed on a comparison of such treatments when the animals reached similar body weights. Since nutritional requirements are usually expressed directly, or indirectly on a body weight basis, this information seemed of importance for future investigations into the changes in requirements of animals having different dietary histories but similar body weights.

SUMMARY

Three groups of growing chickens were depleted to two thirds of their starting body weight by (a) complete starvation, (b) feeding a nitrogen-free diet, and (c) feeding a gelatin-containing diet. They were then repleted to their starting body weight and to twice the initial body weight. Finally they were starved for a 6-day period. Carcass and liver composition analyses were carried out at each interval for each of the three treatment groups as well as for a control group of chicks that received a standard ration throughout except for the final period when they too were starved.

1. During initial depletion chicks in the nitrogen-free group lost the greatest amount of carcass nitrogen but simultaneously increased in carcass and liver fat compared with the other treatments. Collagen content did not decrease during initial depletion on any regime.

2. On repletion to the starting weight, there was a lag in carcass nitrogen accretion in all treatment groups compared with the nondepleted controls. A similar but more pronounced lag occurred in collagen growth during the repletion period. By contrast, the liver nitrogen increased over threefold compared with that of the control animals for the same period.

3. On repletion to twice the starting weight the body composition of all 4 groups was essentially the same.

4. During the redepletion-by-starvation period, chicks in the previously-starved group lost more carcass nitrogen than those in either the control or the other two treatment groups. On the other hand, the nitrogen-free group conserved body nitrogen, as shown by a loss in carcass nitrogen that was only half that of the previously-starved group. In contrast with the unchanged collagen content of the carcass during the initial depletion period on all regimes, there was a loss of 20% of collagen during final starvation.

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A Growth Response of Rats to Glutamic Acid when Fed an Amino Acid Diet[1]

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The amino acid diet employed in the determination of the availability of lysine in wheat and wheat products (Calhoun et al., '60) provided a growth rate of 40.9 gm/week when fed to weanling rats of the Sprague-Dawley strain. This diet contained 18 amino acids in the amounts found in 20% of wheat gluten (77% protein) supplemented with histidine, lysine, methionine, threonine and tryptophan. Because this diet supported a greater rate of growth than any reported previously for rats fed an amino acid diet, studies have been undertaken in an attempt to explain this observation.

In the previous communication it was suggested that the amino acid pattern used was possibly better balanced for maximum growth rate, especially with respect to the nonessential amino acids. The use of wheat starch as the source of carbohydrate was also mentioned as a possible factor. It is the purpose of this paper to present the results of a series of experiments whereby the nonessential amino acids (except cystine and tyrosine) were eliminated from the diet singly and in certain combinations and one experiment in which the effect of the substitution of sucrose for wheat starch was determined.

EXPERIMENTAL

Diets. The composition of the basal diets (table 1) was essentially the same as previously reported (Calhoun et al., '60) except that the level of proline was reduced to 20% of the original concentration. Preliminary trials showed that this change did not alter the high rate of growth.

The experiments reported here represent a series of separate studies conducted concomitantly with amino acid availability studies. The basal diets were therefore composed according to the amino acid under study. The group receiving each of the basal diets supplemented with an adequate level of the amino acid under study served as the amino acid control group for each experiment. When a nonessential amino acid was omitted from the diet the amounts of the remaining nonessential amino acids (except cystine and tyrosine) were increased in proportion to their nitrogen content so as to maintain the total nitrogen level equal to that contained in the amino acid control diet. In formulating the diets used for determining the response to increments of glutamic acid, proline was held constant and the diets were kept isonitrogenous by varying the amounts of alanine, aspartic acid, glycine and serine. A gluten control diet was fed in each of series 1–3.

Experimental procedure for series 1–3. Weanling male rats (Holtzman) were adapted to the amino acid diets as previously described (Calhoun et al., '60). The gluten diet was fed on the first day. On each subsequent day increments of 25% of the amino acid diet (containing a low level of either threonine, tryptophan or valine) were subsituted so that 100% of amino acid diet was attained by the 5th day. On the 6th day the animals were divided into groups of 5 and were assigned at random to the various diets. The use of the control diets was started the first day with groups 6 and 8 in order to determine the effect on growth rate of the adaptation procedure.

The animals were housed individually in suspended wire-bottom cages in an environmental temperature maintained at approximately 25°C. The diets and distilled

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TABLE 1

Composition of control diets

Ingredient	%
Amino acid diets	
DL-Alanine	0.346
L-Arginine·HCl	0.816
DL-Aspartic acid	0.558
L-Cystine	0.334
L-Glutamic acid	5.664
Glycine	1.588
L-Histidine·HCl·H_2O	0.676
DL-Isoleucine	1.420
L-Leucine	1.158
L-Lysine·HCl	1.368
DL-Methionine	0.468
DL-Phenylalanine	0.840
L-Proline	0.392
DL-Serine	0.732
L-Tyrosine	0.502
Threonine	[1]
Tryptophan	[2]
Valine	[3]
Urea	[4]
Salts 4[5]	4.00
Corn oil[6]	5.00
Vitamins[7]	+
Starch[8] to total 100	
Gluten diet	
Gluten[9]	22.515
L-Histidine·HCl·H_2O	0.20
L-Lysine·HCl	1.00
DL-Methionine	0.20
DL-Threonine	0.20
DL-Tryptophan	0.05
Salts 4[5]	4.00
Corn oil[6]	5.00
Vitamins[7]	+
Starch[8] to total 100	

[1] Diets 2–4 contained 0.35% of DL-threonine and 0.50% of L-threonine. Remaining amino acid diets contained 1.020% of DL-threonine.

[2] Diets 7–10 contained 0.20% of L-tryptophan. Remaining amino acid diets contained 0.216% of L-tryptophan.

[3] Diets 12–17 contained 0.28% of DL-valine and 0.56% of L-valine. Remaining amino acid diets contained 1.464% of DL-valine.

[4] Varied to maintain the nitrogen level of amino acid control diets equal to that of gluten diet (2.702% nitrogen).

[5] Hegsted et al. ('41).

[6] Mazola, Corn Products Refining Company, New York.

[7] Vitamins were supplied in a portion of the starch in mg/100 gm of diet as follows: thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; nicotinic acid, 10; *i*-inositol, 20; *p*-aminobenzoic acid, 20; folic acid, 0.1; biotin, 0.1; menadione, 2.0; Ca pantothenate, 4; choline chloride, 150; vitamin B_{12}, 0.004. Each rat received weekly 2 mg α-tocopheryl acetate dissolved in 2 drops of corn oil. Vitamins A and D were supplied by a drop of halibut liver oil given to each rat weekly.

[8] Aytex P, General Mills.

[9] Contained 12.0% of nitrogen.

water were fed ad libitum for three weeks. Food intakes and body weights were recorded weekly.

Experimental procedure for glutamic acid growth curve. This experiment was conducted as described above except that the groups were assigned to the diets without the usual adaptation period. At the end of the third week, the animals were sacrificed in random order using chloroform. The intestinal contents were removed and empty body weights were recorded. The carcasses were frozen at −29°C, chopped into small pieces and ground in a Hobart grinder with the aid of dry ice snow. The ground carcasses were pooled by groups and were stored at −29°C until analyzed. Nitrogen was determined in triplicate on each group by the Kjeldahl-Gunning procedure. Fat was determined in duplicate on each group by acid hydrolysis using a modified procedure of the Association of Official Agricultural Chemists ('55). Two-gram portions were hydrolyzed with 5 ml of concentrated hydrochloric acid in 50-ml beakers. After transferring to Mojonnier flasks with the aid of alcohol, the hydrolysates were extracted three times with an equal volume mixture of ether and petroleum ether.

RESULTS

The average weight gains and food intakes for series 1–3 are shown in table 3. The data reveal that the omission of glutamic acid from the diet resulted in greatly reduced rates of gain whether it was omitted alone (series 3, group 15) or in combination with aspartic acid (series 3, group 16); aspartic acid and serine (series 3, group 17); or with aspartic acid, serine, proline and alanine (series 2, group 10). The omission of proline (series 1, group 4), proline and alanine (series 2, group 9), aspartic acid (series 3, group 13), or serine (series 3, group 14) had no demonstrable effect on growth rate.

Growth conditions were comparable between the three series as shown by the constancy of growth rate obtained with the gluten control diet (50.2, 49.8, and 47.8 gm/week) and with the amino acid control diets (36.6, 37.8, and 34.5 gm/week).

The substitution of sucrose for wheat starch as the source of carbohydrate (series

TABLE 2

Nonessential amino acid concentrations in experimental diets of series 1–3

Series	Group[1]	Alanine	Aspartic acid	Glutamic acid	Glycine	Proline	Serine
		%	%	%	%	%	%
1	4	0.360	0.580	5.886	1.656	none	0.760
2	9	none	0.610	6.186	1.740	none	0.799
	10	none	none	none	5.850	none	none
3	13	0.366	none	5.966	1.678	0.414	0.770
	14	0.379	0.611	6.197	1.743	0.431	none
	15	0.681	1.096	none	3.128	0.773	1.437
	16	0.762	none	none	3.497	0.864	1.606
	17	0.947	none	none	4.351	1.075	none

[1] Groups 1, 5, 6, and 11 were fed the gluten control diet. Groups 2, 3, 7, 8, and 12 were fed the amino acid control diet, except that sucrose was substituted for starch in diet for group 3.

TABLE 3

Average food intake, weight gain and food efficiency at the end of the three-week period in series 1–3

Series	Diet	Diet description[1]	Food intake	Weight gain	Gm gain/ gm food
			gm/week	*gm/week*	
1	1	Gluten control	98.8	50.2	0.51
	2	Amino acid control	81.7	36.6	0.45
	3	Diet 2, starch replaced with sucrose	75.7	36.2	0.48
	4	Diet 2 minus (P)	84.9	40.8	0.48
2	5	Gluten control	96.8	49.8	0.51
	6	Diet 5[2]	87.9	45.7	0.52
	7	Amino acid control	81.3	37.8	0.46
	8	Diet 7[2]	75.4	34.8	0.46
	9	Diet 7 minus (P + A)	83.3	38.8	0.47
	10	Diet 7 minus (P + A + GA + AsA + S)	53.0	18.7	0.35
3	11	Gluten control	95.3	47.8	0.50
	12	Amino acid control	80.3	34.5	0.43
	13	Diet 12 minus (AsA)	81.3	36.0	0.44
	14	Diet 12 minus (S)	77.3	35.7	0.46
	15	Diet 12 minus (GA)	56.0	21.7	0.39
	16	Diet 12 minus (AsA + GA)	59.0	22.2	0.38
	17	Diet 12 minus (AsA + S + GA)	60.3	24.4	0.40

[1] See table 1 for full description. Proline (P), alanine (A), glutamic acid (GA), aspartic acid (AsA), serine (S).
[2] Fed without adaptation period.

TABLE 4

Average food intake, weight gain, food efficiency and percentage of carcass nitrogen and fat after feeding graded levels of glutamic acid for a three-week period

Group	Level of glutamic acid	Food intake	Weight gain	Gm gain/ gm food	Carcass	
					Nitrogen	Fat
	%	*gm/week*	*gm/week*		%	%
1	0.0	34.3	14.9	0.43	2.805	7.12
2	1.42	47.0	19.9	0.42	2.686	8.33
3	2.83	53.0	23.5	0.44	2.763	8.44
4	4.25	62.2	28.9	0.46	2.834	9.24
5	5.66	69.3	34.9	0.50	2.861	8.58
6	7.08	71.9	34.8	0.48	2.919	8.58
7	8.50	72.9	35.5	0.49	2.864	9.70
8	10.97	64.9	29.8	0.46	2.908	10.0

1, groups 1 and 2) did not alter growth rate.

The adaptation procedure resulted in approximately 8% greater gains per week with both the gluten diet (series 2, group 5 versus group 6) and with the amino acid diet (series 2, group 7 versus group 8).

The results shown in table 4 indicate a definite response to increments of glutamic acid. When glutamic acid was omitted from the diet (group 1) the rate of gain was only 14.9 gm/week. Each increment of added glutamic acid produced greater gains in weight up to the level of 5.66% of glutamic acid. Higher levels produced no further increase in growth rate.

Changes in glutamic acid level in the diet were not accompanied by great changes in body composition as measured by carcass nitrogen and fat (table 4).

DISCUSSION

The stimulation of growth rate of rats by glutamic acid has been studied by other workers. Womack and Rose ('47) showed that glutamic acid and, to a lesser extent, proline led to increased gains when fed with an amino acid diet devoid of arginine. They concluded that arginine, proline, and glutamic acid are mutually interconvertible in the rat but at different rates. Rose et al. ('48) found that when the 10 essential amino acids were supplied, glutamic acid was effective as the sole source of nonspecific nitrogen, but its omission from a mixture of 19 amino acids was without deleterious effect upon rat growth. Accordingly, these workers classified glutamic acid as a nonessential amino acid for the rat. The results presented in this paper are in contrast with those of the above study in that glutamic acid was shown to be necessary for maximum growth rate and the omission of it from a mixture of 18 amino acids resulted in a much lower rate of growth.

In further studies, Rose et al. ('49) showed that diammonium citrate was as effective as glutamic acid in supplying nonspecific nitrogen to diets otherwise adequate in the amino acids essential for the rat. Similarly, Lardy and Feldott ('50) found diammonium citrate to be as effective as a mixture of nonessential amino acids containing an equal amount of nitrogen. More recently, Rechcigl et al. ('57) tested various compounds individually as sole sources of nonessential nitrogen in a diet containing all L-isomers of the essential amino acids (plus cystine and tyrosine). Glutamic acid was stated to be superior to any of the compounds tested, although alanine, glutamine, aspartic acid, asparagine and an additional amount of the essential amino acid mixture nearly equaled its effectiveness. The authors postulated that the poor performance found for diammonium citrate could have been caused by the greater concentration used (1.18 gm of nitrogen/100 gm of diet) as compared with the level tested by Rose et al. (0.574 gm of nitrogen/100 gm of diet). However, this laboratory has found diammonium citrate at a level providing 0.539 gm of nitrogen/100 gm of diet to be only 15% as effective as glutamic acid at an isonitrogenous level.

Ramasarma et al. ('49) fed weanling rats an amino acid diet containing the 10 essential amino acids in the amounts proposed by Rose and co-workers (Rose, '37; Womack and Kade, '44; Rose and Womack, '46). They found that increasing the arginine hydochloride level from 0.25 to 0.75% of the diet had no effect on the growth rate when the diet contained 14.3% of glutamic acid but when all but 2% of the glutamic acid was replaced by a mixture of other nonessential amino acids (including proline) the addition of arginine improved the growth rate. Greater amounts of glutamic acid had no stimulatory effect at the 0.75% level of arginine hydrochloride. The authors interpreted their findings as evidence for a higher requirement for arginine than that reported by Borman et al. ('46). They postulated that this increased requirement was due to the more rapid growth rate that their diet permitted. The possiblity must be considered that with the diet used in the experiments reported here, which permits an even greater rate of gain, the arginine requirement may be elevated to a still greater extent. In these experiments it was demonstrated that maximum growth rate was attained at a glutamic acid level of 5.66% of the diet but not at 4.25% even though the arginine hydrochloride content of the diet was 0.81%.

Lardy and Feldott ('50) found the omission of arginine from the diet did not reduce the growth rate to the extent reported by Borman et al. ('46). They ascribed their observation to their use of diammonium citrate in place of the nonessential amino acids. This hypothesis may be extended to the present study in which the feeding of large amounts of nonessential amino acids may have increased the requirement for arginine to unusually high levels. This possibility is not easily accepted because the level of 0.81% exceeds the estimate of Williams et al. ('54) who reported the arginine requirement of the rat to be 0.77% of the diet by using the carcass analysis method proposed by Mitchell ('50) and at the same time concluded that 75% of this requirement may be synthesized.

It is probable that the apparent requirement for glutamic acid in these studies is concerned with appetite. Inspection of the efficiency data in tables 3 and 4 shows, that while growth was greatly diminished at low levels of glutamic acid, the food intake was also decreased so that the food efficiency ratio was altered only slightly. This is in contrast with results obtained with the essential amino acids where it has been demonstrated that a deficiency results in a greatly reduced efficiency of food utilization. Although the possibility cannot yet be ruled out that the diminution of food intake reflects a loss of palatibility in the absence of glutamic acid, it seems more probable that the effect is related to a biologically-induced loss of appetite, such as reported by Deshpande et al. ('58) in the case of amino acid imbalances.

It may be concluded that the amino acid pattern used in this study either (1) increased the arginine requirement in excess of any level proposed to date (which could be met in part by glutamic acid) or, (2) permitted a requirement for glutamic acid to be demonstrated. It is not known whether the nature of this requirement is concerned solely with the requirement for growth or whether glutamic acid and/or arginine are acting to counter an amino acid imbalance which affects appetite. These possibilities are being investigated.

SUMMARY

A purified diet containing an amino acid mixture patterned after wheat gluten and supplemented with histidine, lysine, methionine, threonine and tryptophan provided an unusually high rate of gain in rats. When the nonessential amino acids (except cystine and tyrosine) were eliminated from the diet, either singly or in certain combinations, it was found that the omission of glutamic acid in each instance resulted in a greatly reduced growth rate. The omission of other nonessential amino acids had no such effect.

Progressively greater weight gains were obtained when increments of glutamic acid were added to a glutamic acid-free diet. The glutamic acid-free diet permitted a gain of only 14.9 gm/week. Maximum growth rate (34.9 gm/week) was obtained with the 5.66% level of glutamic acid.

These findings are contrasted with the results of others which did not show glutamic acid to be superior to other sources of nonspecific nitrogen in synthetic amino acid diets.

ACKNOWLEDGMENTS

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The Biological Activity of 6-Chloro-7-Methyl-9-(1′-D-Ribityl)-Isoalloxazine[1,2,3]

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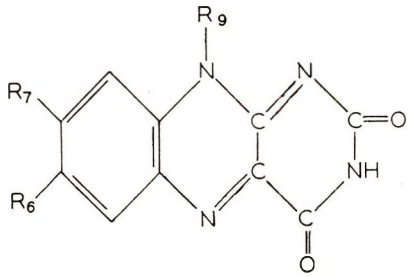
Compounds which have been synthesized for structural resemblance to riboflavin and which have been subjected to biological evaluation fall into three groups. Those which possess "riboflavin-like" activity have been found to be homologs of riboflavin with the naturally occurring polyhydric alcohol D-ribityl as R_9, and with



R_6 and R_7 as certain combinations of H—, CH_3— or C_2H_5—. Compounds of this group have recently been discussed in some detail (Lambooy and Aposhian, '60). Those compounds which possess neither "riboflavin-like" nor "anti-riboflavin-like" activity attract limited attention, while the discovery of a compound whose biological action is that of a "riboflavin inhibitor" is of interest because of the progress being made in chemotherapy today.

The first reasonably potent inhibitor of riboflavin was 6,7-dichloro-9-(1′-D-ribityl)-isoalloxazine prepared by R. Kuhn and coworkers (Kuhn et al., '43). This compound was synthesized to possess two chlorine atoms where the methyl groups are found in riboflavin. Dichlororiboflavin was found to be a reversible inhibitor of several strains of bacteria. The inhibition was temporary; the inhibition index for the compound with *Streptobacterium plantarum*, for example, increased from 25 to 162 depending on whether the incubation was carried out for two or 6 days. Snell and co-workers ('53) have reported that dichloro riboflavin neither promoted growth nor was inhibitory for *Lactobacillus casei*.

Weygand and associates ('51) prepared the mono-halogen analogs, 6-chloro-9-(1′-D-ribityl)-isoalloxazine and 6-fluoro-9-(1′-D-ribityl)-isoalloxazine. These, while having some inhibitory activity for *S. plantarum* P 32, were found to be less effective than dichlororiboflavin.

Others have been interested in halogenated analogs of riboflavin due to the activity of some to enhance regression of lymphosarcoma implants in mice (Holly et al., '50, '52; Shunk et al., '52). Most active in causing such regression is 6,7-dichloro-9-(1′-D-sorbityl)-isoalloxazine. This analog and a few others possessing the same 6,7-dichloro groups in which the polyhdric alcohol (R_9) is other than D-ribityl (such as L-arabityl or D-dulcityl) were found to possess no riboflavin activity or inhibitory properties in growth studies with rats and *L. casei*. The absence of inhibitory properties suggested that these compounds possessed structures which were too unlike that of riboflavin.

The incorporation of a foreign atom into the molecular structure of riboflavin is not essential to riboflavin-inhibitor properties.

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[1] This work was supported in part by grants from the United States Public Health Service. The most recent, Grant no. CY-2940, is from the Cancer Institute.
[2] Brief reference to this work was made at the Symposium on the Anitmetabolites, Their Modes of Action and Therapeutic Implications, 10th Annual Meeting, The National Vitamin Foundation, Inc., New York, 1955.
[3] This compound has been screened for anticancer activity by the Cancer Chemotherapy National Service Center.

Isoriboflavin (Emerson and Tishler, '44) is an isomer of riboflavin in which a methyl group is located at position 5 instead of position 7; 6-ethyl-flavin (Lambooy and Aposhian, '60) is an isomer of riboflavin in which no methyl groups are found but an ethyl group is attached to position 6; L-lyxoflavin (Emerson and Folkers, '51a, b; Snell et al., '53) is an isomer in which the L-lyxityl group has replaced the D-ribityl group at position 9; diethyl-riboflavin (Lambooy and Aposhian, '52) is a homolog in which two ethyl groups have replaced the two methyl groups; D-galactoflavin (Emerson et al., '45; Snell et al., '53) is a homolog of riboflavin in which the D-dulcityl group has replaced the D-ribityl group; 6,7-dimethyl-9-(2'-hydroxy ethyl)- isoalloxazine and dimethyl-9-formylmethylisoalloxazine (Fall and Petering, '56) are homologs in which the ribityl group has been replaced by an ethyl alcohol group and a formylmethyl group respectively. All of the above compounds have been found to interfere with the utilization of riboflavin under certain circumstances. Even compounds of considerably less resemblance to riboflavin, a dinitrophenazine and a diaminophenazine (Woolley, '44), have been found to be mild inhibitors of riboflavin in mice and *L. casei* respectively.

Returning to the basic structure of the isoallozaxines, we may hypothesize that inhibitors of riboflavin might be found among isoalloxazines which possessed a D-ribityl group for R_9 and a chlorine atom at R_6 or R_7 and a methyl group in the alternate position; a structure which might be classified as a combination of the two groups. A compound satisfying these structural requirements, 6-chloro-7-methyl-9-(1'-D-ribityl)-isoalloxazine, has been synthesized in our laboratories (Haley and Lambooy, '54). It has been found to be a potent antagonist of riboflavin for *L. casei*. It has also been found to be the most potent antagonist of riboflavin in the nutrition of the rat to have been described to date. In both test systems there are some features worthy of special comment because they are unusual. These matters will be dealt with more thoroughly in reporting the results and in the discussion, so will be merely stated at this time. During the growth of *L. casei* in media containing both riboflavin and the 6-chloro-7-methyl-flavin a "modified" form of *L. casei* emerges. This modified form is able to utilize either riboflavin or the analog as its sole flavin. This fact is responsible for the unusual form of the curve relating acid production to flavin content of a medium.

Although rats are killed by unusually small amounts of the 6-chloro-7-methyl-flavin, they can be protected against this toxic substance by riboflavin. A remarkable feature of the response of the riboflavin-deficient rat to the administration of the analog is that it grows. The only criterion which can be used to express the inhibitory properties of this material is survival.

METHODS

Acid production by L. casei. The quantity of lactic acid produced by *L. casei* 7469[4] was measured by titration with 0.1 N sodium hydroxide. The observed values were plotted against the appropriate flavin concentrations. One set of duplicate tubes was prepared by the routine procedure (Association of Vitamin Chemists, '51) using graded increments of riboflavin (USP reference standard) from zero to 100 μg per tube. All incubations were for 72 hours at 37°C. The inhibition index was determined from the ratio of the mixture of the two flavins which supported the formation of one-half the amount of lactic acid formed in the presence of the same amount of riboflavin alone.[5] This method for the determination of the inhibition index has been used by Woolley ('44) for the evaluation of the activity of a phenazine compound.

Rat growth and survival. Weanling male rats of the Wistar strain were used throughout this study. The conditions under which the animals were maintained and the riboflavin-deficient diet which they were fed were the same as those described before (Lambooy and Aposhian, '52). When the animals had become satisfactorily riboflavin-deficient, the various flavin

[4] American Type Culture Collection, Washington, D. C.

[5] Inhibition index (I.I.) $= \frac{\mu g \text{ analog at half max. growth}}{0.3\ \mu g \text{ of riboflavin}} \times \frac{\text{mol. wt. of riboflavin}}{\text{mol. wt. of analog}}$.

supplements were administered by stomach tube as a solution in 0.5 ml of water or as a solution or suspension in 0.5 ml of 6% gum acacia solution each day immediately before they were fed. The deficient control animals were given the corresponding vehicle without the flavin. All tests were continued for 28 days from the time the animals had become riboflavin-deficient.

The first study undertaken was for the purpose of discovering whether the analog had riboflavin-like activity or was an inhibitor of riboflavin. Mixtures of the analog and riboflavin were also used; if the analog proved to be an inhibitor, this would give us information about its reversibility by the simultaneous administration of riboflavin. Deficient animals (5 or 6 per group) were fed the basal diet supplemented daily by flavin preparations for groups 1 through 6 consisting of no flavin, 50 μg of analog, 500 μg of analog, 2 mg of analog, 500 μg of analog *plus* 40 μg of riboflavin, 500 μg of analog *plus* 200 μg of riboflavin respectively, and the growth response observed.

The results of the first study compelled us to employ very low levels of the material to study growth and survival of the riboflavin-deficient rat. A group of weanling male Wistar rats was prepared as before, and when the members had become riboflavin-deficient, they were divided by the use of a random numbers table into 9 groups of from 5 to 9 animals each. The average weights of the animals in the 9 groups varied between 42 and 49 gm at the beginning of the test period. The flavin supplements were given by stomach tube as above, and the particulars of the regimens are tabulated in the legend of figure 4.

RESULTS

Acid production by L. casei. Figure 1 shows the acid production by the organism in response to graded levels (zero to 0.30 μg per tube) of riboflavin and to mixtures of riboflavin (0.3 μg per tube) plus graded levels (zero to 100 μg per tube) of the analog. The ratio of the mixture of analog to riboflavin which supported 50% of the acid production by the same quantity of riboflavin alone can be determined from the graph as 24.2 μg/0.3 μg. The use of this ratio for the calculation of the inhibition index gives a value of 76.[6]

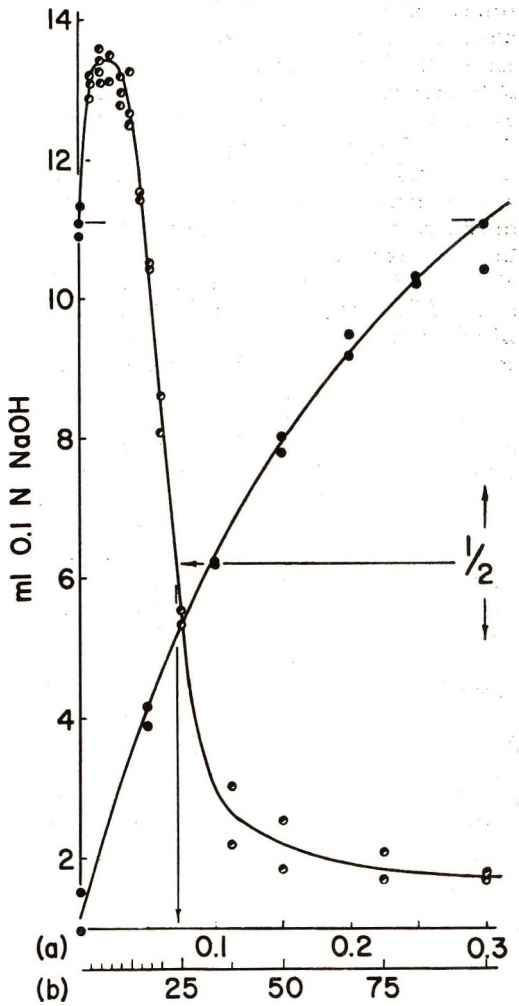

Fig. 1 Lactic acid production by *L. casei* grown in a culture medium containing riboflavin (—●—) and in a culture medium containing mixtures of riboflavin and 6-chloro-7-methyl-flavin (—◓—); (a) riboflavin in μg per 10 ml of culture medium. (b) 6-chloro-7-methyl-flavin in μg per 10 ml of culture medium; each tube contained 0.3 μg of riboflavin.

The production of more acid than could be produced by the utilization of riboflavin alone in tubes containing from 2.5 μg to 15 μg of the analog in addition to the 0.30 μg of riboflavin, is due to the appearance and culturing of a modified form of *L. casei* which is able to utilize the analog for its flavin requirements. The blank

[6] The reference, Haley et al. ('54), gave a value (I. I. = 85) which had been determined from a pH plot of acid production, a less precise procedure.

tubes for the "standard" riboflavin curve contain no riboflavin except for the trace amounts derived from the medium constituents and the inoculum. These blank tubes contained less acid than the mixed flavin tubes containing 0.30 μg of riboflavin and 100 μg of the analog. Since one would expect the latter tubes to be the most severely inhibited, one must conclude that the excess acid which they contain is also due to the presence of the modified form of *L. casei*, although the total number which can utilize the analog is relatively small. This tells us that while it is not immediately obvious from the graph, at no point is the acid production in the tubes making up the mixed flavin curve due solely to the utilization of riboflavin.

That the extra acid production was caused by the modified form of *L. casei* was emphasized in the following way. Inocula were taken from tubes representing three parts of the mixed flavin curve; a point where extensive cell production (5 μg of analog and 0.3 μg of riboflavin), where there had been intermediate cell production (20 μg of analog and 0.3 μg of riboflavin) and where there had been little cell production (75 μg of analog and 0.3 μg of riboflavin). All the cells grown in each of the tubes containing these mixtures were resuspended in equal volumes of saline and used to inoculate a separate series of tubes, each containing 0.3 μg of the analog alone (no riboflavin present). The acid production was initiated in time as shown in figure 2a. Again, the same sources of inocula were used except that all of the cells grown in each of the tubes containing the designated flavin mixtures were resuspended in an appropriate volume of saline to give equal bacterial counts per milliliter. These preparations were used to inoculate a separate series of tubes each contaning 0.3 μg of the analog alone (no riboflavin present). The acid production was initiated in time as shown in figure 2b.

Rat growth and survival. The results of the first study are shown in figure 3, but because of some novel and unexpected observations brief comment is indicated. All animals in the group receiving 2 mg of the analog were dead in 4 days, and all those receiving 500 μg were dead in 8 days. The average survival time of the groups

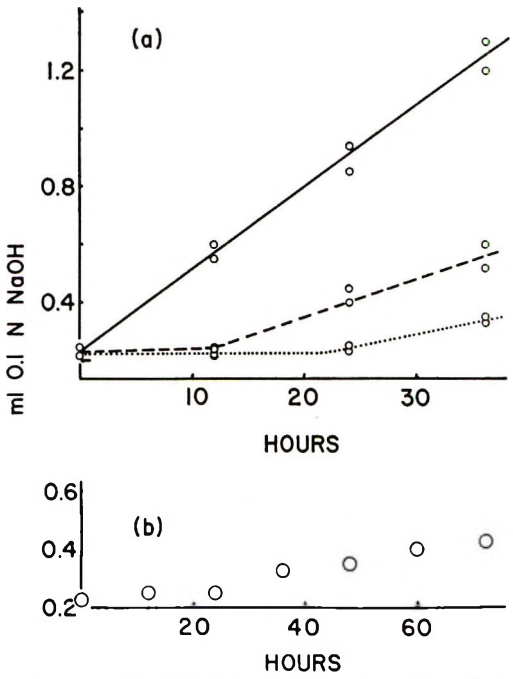

Fig. 2 The time course of lactic acid production by *L. casei* cells obtained from various portions of the mixed flavin curve in figure 1 and grown in a culture medium containing 0.3 μg of 6-chloro-7-methyl-flavin per 10 ml. (a) All the cells from mixtures: 0.3 μg riboflavin *plus* 5 μg chloro-methyl-flavin (—○—), 0.3 μg riboflavin *plus* 20 μg of chloro-methyl-flavin (- - ○ - -) and 0.3 μg riboflavin *plus* 75 μg of chloro-methyl-flavin (· · · ○ · · ·), resuspended in 5 ml of saline and used to inoculate (equal volume additions) separate series of tubes. (b) When cells obtained as in (a) above were resuspended in different volumes of saline to obtain equal cell counts per milliliter of inoculum and used to inoculate (equal volume additions) separate series of tubes. All points for each time interval for the three curves fall within the limits of the ordinate inscribed by the circles.

receiving 500 μg of analog *plus* 40 μg of riboflavin was 11 days, one animal living until the 21st day. All animals receiving 200 μg of riboflavin in addition to 500 μg of the analog survived the test period. Three of 5 animals in the group receiving 50 μg of analog died before the end of the test period, but two survived without additional dietary riboflavin. All animals grew at the same maximal rate and all those cited above showed remarkable recovery from the various manifestations of riboflavin deficiency.

The deficient control group failed to grow for the first 14 days. The remarkable

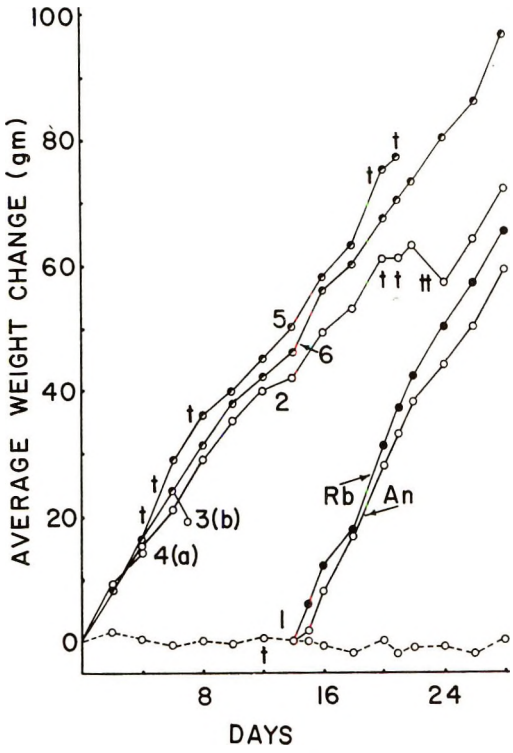

Fig. 3. Average weight changes for animals in groups receiving each day riboflavin (—●—) or 6-chloro-7-methyl-flavin (—○—) or mixtures of these (—◓—). Curves: 1, flavin-free; 2, 50 μg of analog; 3, 500 μg of analog; 4, 2 mg of analog; 5, 500 μg of analog *plus* 40 μg of riboflavin; 6, 500 μg of analog *plus* 200 μg of riboflavin. (a) Animals died on days 2, 3, 4, 4, 4. (b) Animals died on days 4, 4, 5, 6, 8. Death of other animals indicated by †. Rb, riboflavin; An, analog.

growth of the animals receiving the analog alone prompted us to administer 50 μg of riboflavin to two of this group, 50 μg of the analog to two more and continue to give the remaining one the deficient diet. The effect was immediate; there was no difference in the response to the two flavins until the 16th day when one of the analog-supplemented animals died. At the end of the 28th day of the test period the one remaining deficient control rat showed a net loss of 2 gm; another of the original deficient control group had died on the 12th day of the test period. The two animals receiving the 50 μg of analog (group 2) and which survived the 28-day test period were continued for an additional 28 days. They were sacrificed at this time; they had gained 147 and 136 gm. The one deficient control rat which ultimately received the 50 μg of analog was continued for a total of 42 days and was sacrificed after gaining 134 gm.

It is noteworthy that in this first study any animal receiving either flavin, riboflavin or the analog grew as though it had access to nearly optimal amounts of riboflavin (3.5 gm weight increase per day). If the flavin which the animal received was the analog, the animal died.[7] Animals could be protected from the lethal effect of the analog by the simultaneous administration of enough riboflavin. When the level of the 6-chloro-7-methyl-flavin was reduced to very low levels, it became possible to study the potency of the material for growth free of the complication of death. The results of the use of low levels of riboflavin and the analog are shown in figure 4. The differences between the average weight gains of animals in all groups, except the 3 μgEq of analog group, and that of the flavin-deficient group are highly significant (all P values less than 0.001). The similarity in response between the groups receiving 3 μg of riboflavin and 6 μgEq of analog ($P = 0.70$), 6 μg of riboflavin and 12 μgEq of analog ($P = 0.22$) and 12 μg of riboflavin and 24 μgEq of analog ($P = 0.20$) indicates that the analog has about 50% of the potency of riboflavin for the stimulation of growth. The weight gain for the group receiving 3 μgEq of analog is also about half that of the group receiving 3 μg of riboflavin. The differences in weight gains between each of the groups receiving the 3, 6, 12 and 24 μgEq of analog were significant; the P values for successive pairs being 0.006, 0.001 and 0.001 respectively. As observed in the first study, 60% of the animals in the group receiving 50 μgEq of analog failed to survive the 28-day test period as did one of the animals receiving 24 μgEq of analog per day.[7]

[7] Two years later Dr. R. A. Scala found 500 μg of analog to kill all animals in 11 days, and 37.5% of animals severely deficient in riboflavin, receiving 24 μg, failed to survive the 28-day test period.

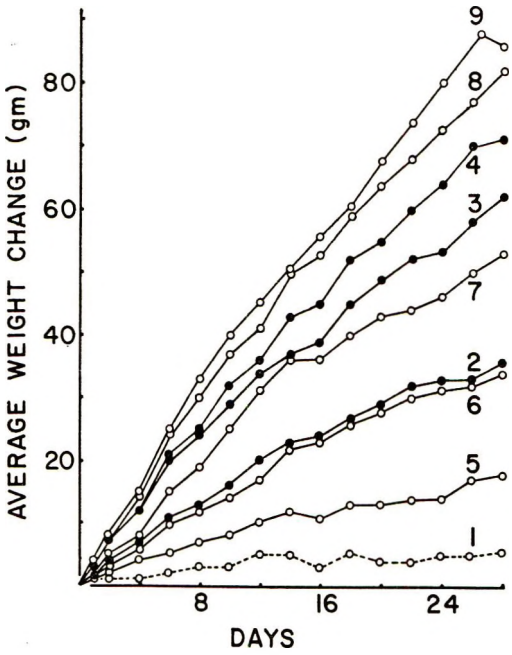

Fig. 4. Average weight changes for animals in groups receiving each day riboflavin or 6-chloro-7-methyl-flavin.

Group no.	No. of animals	Flavin supplement
1	5	Flavin-deficient
5	9	3 μgEq[1] analog
6	9	6 μgEq analog
2	6	3 μg riboflavin
7	9	12 μgEq analog
3	6	6 μg riboflavin
4	6	12 μg riboflavin
8	9	24 μgEq analog[2]
9	5	50 μgEq analog[3]

[1] μgEq, microgram equivalent: a correction for the molecular weights of the two flavins amounting to 1.054 times the corresponding micrograms of riboflavin. For example, 6 μgEq of analog is equal to 6.3 μg. [2] One animal died day 19. [3] Animals died on days 11, 15, 16. Riboflavin (—●—); 6-chloro-7-methyl-flavin (—○—).

DISCUSSION

An earlier observation that *L. casei* appeared to escape from the inhibitory influence of the analog, prompted us to undertake a detailed study of this phenomenon (Scala and Lambooy, '58), and, indeed, a form of *L. casei* was isolated which produced acid in a linear response to graded amounts of either riboflavin or the 6-chloro-7-methyl-flavin. It was found that the inhibition of the normal *L. casei* by the analog was due to the inability of the microorganism to phosphorylate it.

If the production of more than the expected amount of acid in the mixed flavin curve shown in figure 1 is due to the metabolism of this modified form of *L. casei*, one would expect that the larger the total number of cells produced, the larger would be the number of the modified form to appear. Clearly, the larger number of cells is present in the tubes containing 5 μg of analog *plus* 0.3 μg of riboflavin. If inocula of equal volumes prepared from all of the cells of tubes from this series containing a large, (5 μg of analog *plus* 0.3 μg of riboflavin) an intermediate, (20 μg of analog *plus* 0.3 μg of riboflavin) and a small (75 μg of analog *plus* 0.3 μg of riboflavin) number of cells are used to inoculate media containing only the analog, there should be different rates of initiation of acid production dependent on the absolute number of modified cells present. The results as shown in figure 2a are in agreement with expectations. Similarly, if inocula are made from identical culture tubes as those used for the preparation of the inocula above but all the cells are resuspended in sufficient saline so that each inoculum contained the same number of cells per milliliter, there should be no difference in rate of initiation of acid production in media containing the analog. The number of modified cells is dependent on the total number of cells, and we find that the results shown in figure 2b are in agreement with expectations. This means that in any test making use of a mixture of these two flavins the acid produced is the sum of that produced by the normal *L. casei* utilizing riboflavin and the modified *L. casei* utilizing riboflavin or the analog. The small distortion of the curve of acid production due to the presence of modified cells has very little influence on the determination of the inhibition index.

While it is difficult to draw comparisons between compounds which are studied under different conditions, it is possible to recognize qualitative relationships. Snell and associates ('53) used turbidimetry to determine the "growth" produced in cultures of *L. casei* in the presence of the individual flavins or of mixtures of riboflavin and isoriboflavin or galactoflavin or

lyxoflavin. Turbidimetry gives a measure of the number of cells present and is related to the adequacy or the quantity of a flavin in a somewhat different way than is characteristic of the quantity of lactic acid produced. These workers found that within certain quantitative limits of the range of the mixtures of the flavins, that isoriboflavin, lyxoflavin, and galactoflavin all stimulated the growth of *L. casei* if riboflavin were also present. They concluded that the stimulatory effect of these three flavins was due to their causing *L. casei* cells to make more efficient utilization of the riboflavin available to them rather than to the use of the abnormal flavin *per se*. The stimulatory effect of the 6-chloro-7-methyl-flavin is due to the utilization of the flavin *per se*, not by stock *L. casei* 7469, but by an invariably produced "modified" form.

The growth of the riboflavin-deficient rat when it is given 6-chloro-7-methyl-flavin is a striking result. This phenomenon had been observed to a much smaller degree before (Lambooy and Aposhian, '52) in the case of diethyl-riboflavin. In both cases it is due to the displacement of otherwise bound riboflavin from the tissues of the deficient animal. The liberated riboflavin, which formerly had been immobilized in an animal destined to die from riboflavin deficiency, has been made available for critical metabolic requirements. A detailed presentation of the mechanism of actions of the flavin inhibitors which can accomplish this result has been published before (Lambooy, '55). The 6-chloro-7-methyl-flavin has almost unbelievable potency as a displacing agent and we have direct evidence that the stimulus for growth of the riboflavin-deficient rat by this analog is due to this displacement mechanism. The details of this study will be the subject of a future publication.

Since there is no depression of growth, which is only one of the ways in which riboflavin antagonistic action can be displayed, we cannot use this criterion for the determination of the inhibition index. A measure of the potency of the material can be expressed only in terms of its toxicity at very low levels and the reversibility of its effects by riboflavin.

Snell and co-workers ('53) found that the growth of chicks could be stimulated by mixtures of lyxoflavin and riboflavin of 10:1 or below, to weights greater than those produced by the marginal amount of riboflavin alone. At rations greater than these, the growth of the chicks was depressed. These results are somewhat reminiscent of our observations on diethyl-riboflavin which could depress growth of the riboflavin-deficient rat when given in sufficiently large amounts. The 6-chloro-7-methyl-flavin, however, stimulated growth at all levels tested.

If the study of the influence of this analog on the growth of the riboflavin-deficient rat had been limited to low levels of the material (3 to 12 μgEq per day) for periods of test up to 28 days, not only would we have concluded that it was a riboflavin replacement agent but that it was the most potent riboflavin-like compound known short of the vitamin itself. This remark is made to emphasize the rapid recovery of the deficient animals receiving this material. Almost without exception every manifestation of riboflavin deficiency disappeared.[8] The state of their improved health was due to the riboflavin in their tissues becoming available to them for use by, perhaps, more critical enzymes. Ultimately the tissue levels fell to a point where they were no longer compatible with the life of the animal and the paradoxical situation developed in which animals appearing to be in excellent health died of riboflavin deficiency.

It is difficult to imagine the lethal properties of the analog as being due solely to displacement of riboflavin. At all levels, but particularly at the higher levels, it must compete with riboflavin at either the substrate or coenzyme level.

SUMMARY

1. It has been found that 6-chloro-7-methyl-9-(1′-D-ribityl)-isoalloxazine is a reversible antagonist of riboflavin in the nutrition of *Lactobacillus casei* with an inhibition index of 76.

[8] Only a few animals receiving low levels of the analog (24 and 50 μgEq per day) for long periods showed a slight return of the unkempt appearance associated wth this deficiency.

2. This analog of riboflavin has also been found to be a reversible antagonist in the nutrition of the rat. The growth which is stimulated by low levels of this material is approximately equal to the growth stimulated by one-half the quantity of riboflavin. High levels of the analog are lethal to the animal if adequate amounts of riboflavin are not available.

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A Statistical Study of Apparent Digestibility Coefficients of the Energy-Yielding Components of a Nutritionally Adequate Mixed Diet Consumed by 103 Young Human Adults

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Studies of the apparent digestibility of foods or food mixtures consumed by human subjects are not numerous. Those that have been reported involve so few subjects for any one diet or food that generalizations from them are often unwarranted. In particular there is a lack of data from which to judge the extent to which the digestibility of a nutritionally adequate diet is affected by the size and/or sex of the individuals; or by the level of daily diet intake. No data appear to have been reported on interrelationships between digestion coefficients for the several proximate fractions of the human diet.

EXPERIMENTAL

During each of the years 1957 to 1959 inclusive we conducted as a part of a senior nutrition laboratory course, a study of the digestibility of a diet (in the form of muffins and/or biscuits) made from a selection of ingredients that together furnished nutrients meeting the Canadian Dietary Standard requirements. The collaborators were senior college students averaging 22 years of age. During the tests they subsisted entirely on the diet, supplemented only with water or unsweetened coffee or tea taken without milk or cream. Description of the groups starting the tests are given in tabular form in table 1.

TABLE 1

Groups of collaborators involved in the digestion trials

Year of trial	Sex	No. of subjects	Weight	Metabolic size
			kg	$W^{0.75}$
1957	M	15	74 ± 7	25.1
	F	15	59 ± 7	21.3
1958	M	15	76 ± 9	25.7
	F	20	58 ± 5	20.9
1959	M	15	76 ± 9	25.8
	F	26	58 ± 8	21.0

TABLE 2

Ingredient recipe for muffins and/or biscuit diet used[1]

Ingredient	Air dry weight
	%
Whole wheat flour	16
Rolled oats	21
White sugar	4
Brown sugar	15
Butter[2]	22
Dried whole egg	10
Skim milk powder	8
Wheat germ	1
Table salt	0.75
Vanilla	0.50
Baking powder	1.50
Chromic oxide (Cr_2O_3)	0.25

[1] To each 100 gm of dry mix, 11 mg of coated ascorbic acid was added.
[2] See text.

The diet was prepared from the same recipe in each of the three years. Its percentage ingredient composition is given in table 2, and its nutrient content per 100 gm of dry weight in table 3.

For the 1957 trials only, the recipe was prepared in two forms. Muffin batter was prepared by adding 30 cm^3 (2 tbsp.) of water to 100 gm of dry mix, the latter modified from the formula of table 2 by removal of half the butter. The muffins were baked for 15 minutes at 400°F, and

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TABLE 3

Nutrient content of 100 gm of dry matter of muffin mix[1]

Gross energy, Cal.	557
Crude protein, gm	13
Ether extract, gm	24
Calcium, gm	0.16
Iron, mg	2.9
Vitamin A, I.U.	1100
Thiamine, mg	0.31
Riboflavin, mg	0.33
Niacin, mg	1.35
Vitamin C, mg	11

[1] Calories, protein, ether extract by analysis; other values by computation.

eaten with an obligatory allowance of butter as a spread such that the combination represented the original formula.

Biscuit dough was prepared for baking by adding 5 cm^3 (½ tsp.) of water to each 100 gm of mix, forming into rolls, and refrigerating at 0°C (32°F) for 24 hours. The chilled rolls were sliced into individual biscuits and baked at 325°F for 10 minutes.

By offering some choice of these two forms of the same mixture, it was hoped to introduce an apparent variety into the diets. A minimum of 4 muffins was required at the noon and supper meals, with unlimited allowances of biscuits to make up the day's food. However a marked preference was voiced for the muffins, perhaps partly because they were served at the noon meal freshly baked and warm; and partly because they seemed to satisfy appetite better. In any case relatively few biscuits were eaten, especially by the girls. Accordingly in the 1958 and 1959 trials only the muffin form of the diet was used. The change resulted in an improvement in daily food intake.

Individual collaborators and their data were identified for record purposes by assigned numbers. After the first day, food allocation was based roughly on the previous day's consumption, though additional quantities were available on request. The muffins were baked each morning in the required quantities for 24 hours. Just before the noon meal, individual 24-hour diet allowances were assembled into numbered cellophane bags. Each collaborator on arrival at the dining hall, "weighed in," returned in his numbered bag the uneaten biscuits and/or muffins (and butter) from the previous day, and collected in a new bag his next 24-hour allocation. Plain coffee and tea were available at the dining hall at each regular meal time. Most of the collaborators took the noon meal in the hall, but ate their remaining food when and where they individually chose.

The period during which the collaborators subsisted entirely on the test diet was 4 days in the 1957, and 5 days in the 1958 and 1959 trials. All stools passed during the third, fourth, and (excepting 1957) fifth days of the tests were recovered and returned daily to the Nutrition Laboratory in numbered bags provided for the purpose. Since the index method (using Cr_2O_3) was employed (Irwin and Crampton, '51), a quantitatively accurate record of food intake or of stool collection was not necessary for computing digestibility. However, as nearly a complete collection of stool as possible was necessary to provide sufficient sample for analysis, and record of dry matter intake was made for the statistical study of factors affecting digestibility.

TABLE 4

Daily voluntary caloric intake during 1958 and 1959 tests

Sex	Year	No.	Weight of subject		Daily intake		Use of calories for	
					Dry matter	Gross calories	Maintenance	Extra activity
			kg	$W_{kg}^{0.75}$	*gm*			
M	1958	15	76 ± 9	25.7	503	2804 ± 597	2570	234
	1959	15	76 ± 9	25.8	583	3198 ± 1024	2580	608
	Both	30	76 ± 9	25.8	543	3001 ± 26%	2580	421
F	1958	20	58 ± 5	20.9	367	2027 ± 738	2090	−63
	1959	26	58 ± 8	21.0	374	2151 ± 588	2100	−51
	Both	46	58 ± 6	21.0	371	2090 ± 34%	2100	−10

For each fraction studied, the coefficients of apparent digestibility were computed according to the relationship:

$$\% \text{ digestibility} = 100 \frac{(a - b)}{a}$$

where *a* and *b* are the ratios of nutrient to chromic oxide in the food and feces respectively.

Of the 106 collaborators who started in these tests, three were obliged to withdraw, —one because of failure to adjust to the diet, and two because of sickness not related to diet. In general those completing the feeding periods maintained their weights plus or minus one pound.

RESULTS

Voluntary intake of diet

It is a generally accepted premise that animals (including the human) will voluntarily eat nutritionally adequate diets in amounts to satisfy caloric need. The comparisons of the caloric intake from this diet with the Canadian Dietary Standard for adult maintenance is therefore of some interest. The diet used in 1957 (trial 1) was offered in two forms as already noted, and the collection period was of 4 instead of 5 days duration. Whether these conditions were responsible for the wider variations in food consumption, especially with some of the girls, is not clear; but food intake in the trials of 1958 and 1959 was much more uniform than in 1957. Consequently, the tests of these two years were used for a direct comparison of voluntary caloric intake with computed maintenance "requirements." The gross energy maintenance requirements[1] were computed as 100 ($W_{kg}^{0.75}$). The difference between this quantity and the observed consumption is recorded as being used for "extra activity." The results are summarized in table 4.

The average of about 400 gross calories eaten per day by the males in excess of computed maintenance needs may be indicative of more active participation by the boys in sports.

In the 1957 trials only three of the 12 girls consumed sufficient to meet their theoretical maintenance energy requirements. Most of them just about replaced

[1] That is, 93 ($W^{0.75}$) ÷ 93% digestibility = 100 ($W_{kg}^{0.75}$).

TABLE 5

Analysis of variance and covariance of weight of subject and diet dry-matter intake

Source	D.F.	Weight, x_1		Dry matter intake, x_2		Covariance	Coefficients	
		s^2x_1	s	s^2x_2	s	Cov (x_1x_2)	b	rx_1x_2
		kg	*kg*	*gm*	*gm*		*gm*	
Total	102	128	11.3	31336	177.0	1176	92	0.5871[1]
Subgroups	5							
Sex	1	7530[1]		1169480[1]				
Years	2	35		7153				
Sex × years	2	4		117499				
Remainder	97	56	7.5	18325	135.4	287	51	0.2826[1]

[1] Statistically significant at P = 0.01.

their basal metabolism energy losses. The reason for the low food intake appeared to be related to the biscuit form of the diet which was not acceptable after the first few days. The collaborators did not appear to tire of the muffins. The chief obvious consequence of the abnormally low intake was constipation, presumably from restricted volume of ingesta, and consequent increased proportion of metabolic residue in the total fecal excretion.

Apparent digestibility

While it is interesting to speculate on the causes of the differences in food intake between the sexes, and between trials, our primary concern in these studies was the possible effect of differences in intake on the digestibility of the diets. To this end the data for the 103 collaborators who completed the feeding tests were examined statistically. Standard deviations, and subsequently correlations were determined from the "total" and from the "remainder" portions of the analysis of variance, the latter representing the variation between individuals of like sex within tests. Table 5 illustrates the partition of variance and covariance used in all cases.

Table 6 gives in summary the coefficients of apparent digestibility, by years and by sexes, for the 5 diet components considered in these studies. In 1957 the coefficients were averaged in the one case for the 15 males and 12 females; and in the second case for the 14 males and 6 females that were considered to be free of any possible complications from "abnormal" intake or residue excretion. Figure 1 shows the daily dry matter intake of the subjects, relative to their computed maintenance requirement, plotted against the resulting coefficient of digestibility of its calories of gross energy. On this graph there are three aberrant values that lie at least 4 times the standard deviation from the mean, and these we consider as values that might represent errors in recorded intake by these three subjects. Of the remaining 100 observations the 4 girls that consumed less than 50% of their "requirement" and whose digestibility coefficients for energy fall below ± 1 S. D. from the mean, all reported severe constipation. Other individuals who ate no more than these 4, but who were free of constipation, showed digestibility within a range of ± 1

TABLE 6

Summary of coefficients of apparent digestibility (%)

Fraction	Sex	1957	1958	1959	3 years by sexes	3-year totals
Dry matter	M	(15)[1]93 ± 1.9	(15)91 ± 0.8	(15)89 ± 6.5	(45)91 ± 4.3	
		(14) 94 ± 1.2			(44)91 ± 4.2	(103)90 ± 5.4
	F	(12) 84 ± 10.4	(20)91 ± 1.3	(26)89 ± 4.1	(58)89 ± 6.0	(96)91 ± 3.8
		(6) 91 ± 1.2			(52)90 ± 3.2	
Calories	M	(15) 94 ± 2.4	(15)92 ± 1.0	(15)89 ± 7.4	(45)92 ± 4.8	
		(14) 94 ± 1.7			(44)92 ± 4.8	(103)91 ± 5.7
	F	(12) 85 ± 10.8	(20)93 ± 1.4	(26)90 ± 4.1	(58)90 ± 6.2	(96)91 ± 4.1
		(6) 92 ± 1.2			(52)91 ± 3.4	
Protein	M	(15) 87 ± 4.0	(15)82 ± 3.5	(15)79 ± 11.7	(45)83 ± 8.0	
		(14) 88 ± 3.0			(44)83 ± 8.1	(103)81 ± 10.0
	F	(12) 71 ± 18.5	(20)84 ± 3.0	(26)80 ± 8.4	(58)79 ± 11.2	(96)82 ± 7.3
		(6) 83 ± 3.9			(52)82 ± 6.6	
Ether extract	M	(15) 96 ± 1.4	(15)94 ± 1.7	(15)92 ± 7.0	(45)94 ± 4.5	
		(14) 96 ± 1.0			(44)94 ± 4.5	(103)93 ± 5.2
	F	(12) 87 ± 9.3	(20)94 ± 1.4	(26)92 ± 4.2	(58)92 ± 5.7	(96)93 ± 3.9
		(6) 94 ± 1.0			(52)93 ± 3.3	
Carbohydrate (by difference)	M	(15) 96 ± 1.4	(15)95 ± 0.5	(15)94 ± 3.6	(45)95 ± 2.3	
		(14) 96 ± 0.8			(44)95 ± 2.3	(103)94 ± 3.2
	F	(12) 90 ± 6.3	(20)95 ± 0.7	(26)94 ± 2.2	(58)94 ± 3.6	(96)95 ± 2.0
		(6) 95 ± 1.0			(52)94 ± 1.7	

[1] Figures within parentheses indicate number of subjects.

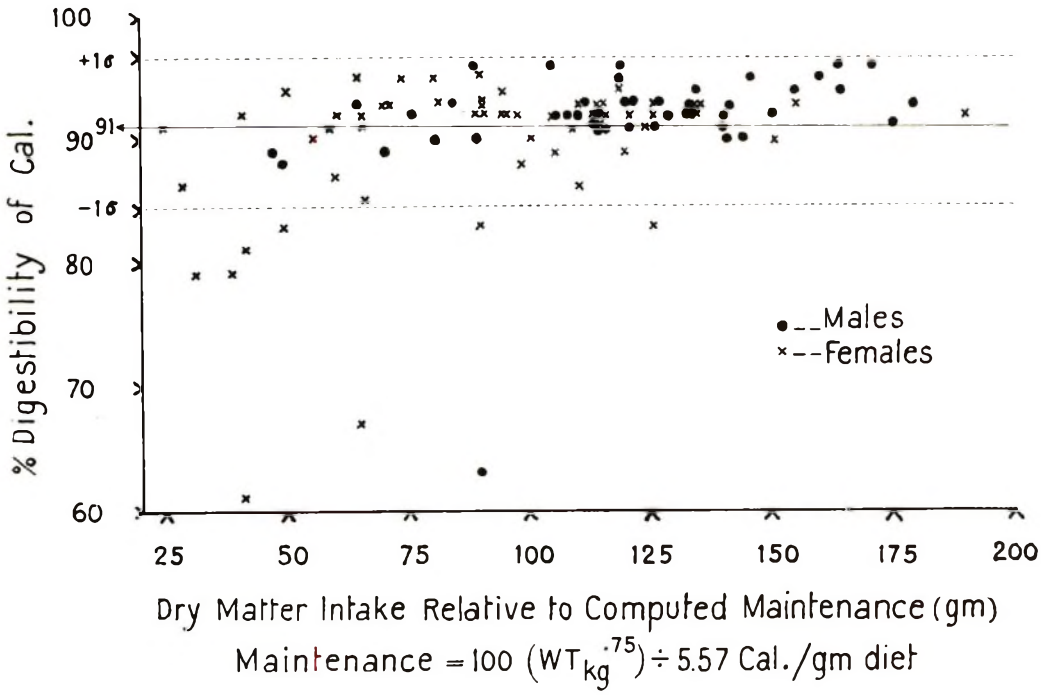

Figure 1

S. D. from the mean, with as many observations above as below the general average.

It is clearly evident that only in the case of the girls in the 1957 test was there any appreciable effect on the extent of the digestibility of the diet components that was related to the level of intake. We conclude, therefore, that low food dry matter intake *per se* does not necessarily alter its normal digestibility.

The standard deviation of the digestion coefficients in this test is larger than many might believe to be typical. The inclusion of all data contributes to this variability, and had the 9 values that were outside the standard deviation been deleted, the mean digestion coefficients would have been raised about one percentage unit and the standard deviation about halved.

The data also suggest that intakes in excess of maintenance may be slightly more completely utilized than lesser amounts. Intakes 50% or more above maintenance, for example, all show digestibility above the general mean, though the differences are statistically insignificant. These data lend no support to the contention that meager intakes are more thoroughly digested than luxus consumption.

Correlation studies

It is evident from table 6 that the significant statistical effect of the inclusion of the "low" (1957) digestibility figures in the three-year data was to increase slightly their variability. The mean values were changed only by one percentage unit, if at all. Consequently in the correlation studies all digestibility figures were included.

The simple correlations, calculated in the one case from "total" and in the second from "remainder" variance,[2] are shown in table 7; and in table 8 the values of 100 r^2 are presented as an indicator of the degree (in per cent) to which the variation in the digestion coefficients is dependent on variations in weight of subject or in diet intake.

The correlations based on remainder variance are indicative of the relationships to be expected of individuals of the same sex in the same test. Since there is a high correlation between weight and sex, the correlations based on total variance are numerically larger than those computed on remainder. However, there is no change in the statistical significance of the (r) co-

[2] See table 5.

TABLE 7

Simple correlation coefficients, r, between variates indicated

		Weight	Intake	Apparent digestibility of				
				Dry matter	Calories	Protein	Fat	CH_2O
Apparent digestibility of:	Weight		0.5871	0.1690	0.1446	0.1475	—	0.1957
	Intake	0.2826		0.3888	0.3746	0.3861	—	0.4347
	Dry matter	0.0532	0.2700		—	—	—	—
	Calories	0.0423	0.2833	—		—	—	—
	Protein	0.0853	0.3049	0.9559	0.9510		—	—
	Fat	—	—	—	0.9490	0.9148		—
	CH_2O	0.0637	0.2884	—	0.9516	0.9290	0.9016	

Based on "remainder" variance.
r required at P 0.05 = 0.195
P 0.01 = 0.254

Based on "total" variance.
r required at P 0.05 = 0.195
P 0.01 = 0.254

TABLE 8

Values of 100 r^2, showing the probable percentage of reduction in variation in "dependent" variable with removal of variation in the "independent" variable (based on remainder variance)

		Weight	Intake	Digestibility of			
				Dry matter	Cal.	Protein	Fat
Dry matter intake		8					
Digestibility of:	Dry matter	0.3	9				
	Calories	0.2	9				
	Protein	0.6	9	90	90		
	Fat	—	–	—	90	83	
	CH_2O	0.04	8	—	90	86	81

efficients whether based on total versus remainder variance.

It is clearly evident from tables 7 and 8 that weight of subject *per se* is not a cause of variation in the completeness of digestion of any of the proximate food fractions. However an effect of level of intake of dry matter on the apparent digestibility of all energy-yielding fractions of the diet is statistically detectable.

That the extent of the digestion of one component markedly affects that of the other fractions is also indicated in tables 7 and 8. This is to be expected, since all fractions are parts of the same food whose exposure in a low-fiber diet to digestive enzymes can be assumed to be comparable.

Multiple correlation and partial regression

Because of the interrelation of body size, food intake and its digestiblity, it seemed worthwhile to measure the relative importance of weight and intake on the degree of digestibility. Table 9 presents the results of this analysis.

The interpretation of the data of table 9 is clear-cut. Body weight and level of food intake together account for only 8% of the variability in the digestibility of the diet, and 90% or more of the effect is traceable to intake level. However, the partial regressions, statistically and practically, are insignificant. Thus we conclude that for purposes of low fiber ration

TABLE 9

Relative effects of size of individual and daily dry-matter intake on the digestibility of certain food components

Variables correlated		Relative beta values	Partial regressions b	R	100 (R^2)
Independent	Dependent				
		%			%
Live weight	Digestibility of dry matter	10	−0.0169	0.271	7.4
Dry matter intake		90	0.0100		
Live weight	Digestibility of calories	12	−0.0117	0.286	8.2
Dry matter intake		88	0.0047		
Live weight	Digestibility of protein	1	−0.0013	0.269	7.2
Dry matter intake		99	0.0160		
Live weight	Digestibility of CH_2O	6	−0.0072	0.289	8.4
Dry matter intake		94	0.0061		

description, the average apparent digestibility coefficients of the energy-yielding fractions are, in the practical sense, independent of sex and intake, and the means may be taken as constants with standard deviations of individual observations ranging from 4 to 10% depending on the diet fraction.

Physiological fuel values

The practical usefulness of coefficients of apparent digestibility is to compute the usable energy of a food or diet. This may be expressed in terms of either digestible or metabolizable calories. These measures differ only that in the latter there is an accounting for urinary energy loss arising from incomplete metabolism of protein.

It is interesting to compare the coefficients of digestion and the physiological fuel values obtained in this study with the so-called Atwater values often used to compute metabolizable calories of foods or of human diets (table below).

The physiological fuel values for the protein and fat of the Macdonald College diet are appreciably lower than the often-used figures. The protein digestibility in particular is only 88% of that on which the Atwater fuel values are based. According to the Atwater scheme, the metabolizable energy yield per gram of a diet is computed as the sum of the digestible carbohydrate, plus digestible "fat," plus digestible protein, minus 1.25 Cal. for each gram of digestible protein in the food. Correction of the urinary loss in this way makes it necessary to employ separately both chemical and biologically determined figures for carbohydrate, fat and protein in order to arrive at the energy value of a food or diet.

Greater precision in arriving at the digestible energy is possible by determining the digestible energy directly by bomb calorimeter, values for food and resulting feces, since this avoids assumptions as to appropriate heats of combustion, and the uncertainties in both food and feces of "carbohydrate by difference." The estimate of metabolizable energy can be made with as much accuracy as is attainable from the Atwater scheme by deducting from the bomb-determined digestible calories, the product of 1.25 times the percentage of digestible protein of the food.

For example, the diet involved in these studies yielded $(5.57 \times 0.91) = 5.07$ Cal. of digestible energy per gram from which we must deduct approximately 1.25 Cal. for each gram of digestible protein or (0.13 gm of protein × 80% of digestibility × 1.25 Cal./gm) = 0.13 Cal.; leaving a

	Coefficients of apparent digestibility		Physiological fuel values for mixed diet	
	Atwater	Macdonald	Atwater	Macdonald
Protein	92	81	4	3.56
Ether extract	95	93	9	8.65
Carbohydrate	98	94	4	4.09

metabolizable energy value of the diet of (5.07 − 0.13) = 4.94 Cal./gm.

In equation form we would write: Metabolizable cal./gm = (gross cal. × % digestibility) − (1.25 × crude protein × % digestibility of crude protein). Since the apparent digestibility of the protein is approximately 80%, the last term in this equation turns out to be numerically equal to the percentage of protein in the diet, since multiplying 0.80 × 1.25 = 1.0. Thus we can write for the diet of this study:

$$\begin{aligned}\text{cal./gm} &= (\text{gross cal.} \times \%\ \text{digestibility}) - \%\ \text{crude protein} \\ &= (5.57 \times 0.91) - 0.13 \\ &= 5.069 - 0.13 \\ &= 4.939\ \text{Cal.}\end{aligned}$$

A deviation of each 5 percentage units from the average 80% digestibility of protein modifies the correction only one Calorie in each 100 Calories of metabolizable energy as computed above.

It seems therefore that for practical purposes deducting from the digestible calories per gram of a diet one Calorie for each percentage of crude protein of the food, offers an acceptable method of estimating the metabolizable energy of foods.

SUMMARY

During each of the years 1957–'58–'59 biscuits and/or muffins were prepared from a dry mixture that when eaten in amounts daily to meet energy needs furnished the nutrients called for in the Canadian Dietary Standard. They were prepared from the same recipe each year and formed the sole diet during 4- (or 5-) day periods for 103 senior university students at Macdonald College. A statistical study using methods of covariance and partial regression was made of voluntary food intake, and of the coefficients of apparent digestibility of dry matter, energy (calories), crude protein, ether extract, and of carbohydrate by difference. The range of voluntary caloric intake varied from as low as 25 to as high as 175% of the computed daily maintenance needs; but neither sex, weight of subject, nor diet intake, relative to computed maintenance requirement significantly affected the coefficients of apparent digestibility for any of the above 5 diet components. Weight of subject and level of diet intake together accounted for (i.e., R^2) only about 7% of the variability in the digestibility coefficients, and of this insignificant total the relative beta values indicated that 90% was traceable to the varying amounts eaten. It was shown that digestible energy, determined by bomb calorimeter combustion of food and of feces, could be converted directly to metabolizable energy by deducting from the digestible calories per 100 gm of diet, one Calorie for each percentage unit of crude protein in the dry matter of the diet.

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Utilization of Methionine by the Adult Rat

I. DISTRIBUTION OF THE ALPHA-CARBON OF DL-METHIONINE-2-C^{14} IN TISSUES, TISSUE FRACTIONS, EXPIRED CARBON DIOXIDE, BLOOD AND EXCRETA[1]

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Present knowledge of the metabolism of methionine in the adult rat has been largely derived from experiments which have demonstrated its need for general tissue synthesis and the formation of special compounds. The labile methyl group of methionine is transferred to various acceptors, resulting in the formation of creatine (du Vigneaud et al., '40), anserine[2] (Schenck et al., '43), N'-methylnicotinamide (Perlzweig et al., '43; Ellinger, '48), adrenaline (Keller et al., '50), and choline (du Vigneaud and co-workers, '40). Du Vigneaud and his collaborators ('44) demonstrated that methionine sulfur, but not the carbon chain of this amino acid, is used for the synthesis of cystine.

Several investigators have reported studies of the distribution in tissues of sulfur from methionine-S^{35} (Levin et al., '56; Gaitonde and Richter, '55; Maass et al., '49) and of the methyl group fed as methionine-methyl-C^{14} (Keller et al., '49; Mackenzie et al., '49).

Although there is fairly extensive knowledge of the metabolism of the methyl and sulfur moieties of methionine, there is little definite information about the fate of its carbon chain. Kisliuk et al. ('56) recently reported the distribution of radiocarbon from 2-, 3-, and 4-C^{14}-DL-methionine in liver glycogen, serine, choline and aspartic acid. Marshall and Friedberg ('51) have reported the occurrence of fumaric acid, labeled in the methine carbons, in the livers of mice injected with DL-methionine-2-C^{14}. The formation of radioactive α-aminobutyric acid from methionine-2-C^{14} in liver homogenates has been observed by Matsuo and Greenberg ('55).

The literature on this subject has been reviewed by Greenberg ('54), Singer and Kearney ('55) and Meister ('57).

The present study was undertaken to investigate the incorporation of the α-carbon of DL-methionine-2-C^{14} into tissues of adult rats. The appearance of radiocarbon at various time intervals in expired carbon dioxide, urine, blood and 23 tissues is described. Subsequent papers will present similar data relating to the methyl carbon of methionine and the identity of radioactive compounds arising from the α-carbon of this amino acid in water-soluble, hexane-soluble and protein fractions of tissues.

EXPERIMENTAL PROCEDURES

DL-Methionine-2-C^{14} containing a specific activity of 0.57 μc/mmole, was used. The radioactive amino acid supplied the only source of methionine in a semi-synthetic diet containing all of the other nutrients required by the adult rat. The ration provided cystine, vitamin B_{12} and choline. The composition of this mixture is shown in table 1. Twelve grams of the diet were mixed thoroughly and made to a volume of 12 ml with distilled water.

The feeding and handling of animals. Male, adult rats of Wistar strain were secured from the Nutrition Laboratory of

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[1] This investigation was supported in part by a research grant (A-1464) from the National Institutes of Health, Public Health Service.

[2] McManus, I. R. 1956 Biological methylation of anserine. Federation Proc., *15:* 312 (abstract).

[3] Purchased from Tracerlab, Inc., Waltham, Massachusetts.

TABLE 1
Composition of diet

Component	Quantity
	%
Amino acids[1]	14.1
Non-nutritive cellulose[2]	3.0
Butterfat	10.0
Dextrin	7.8
Dextrose	46.6
Salt mixture[3]	4.0
Shortening	10.0
Vitamin mixture[4]	4.6

[1] Quantities in milligrams: DL-isoleucine, 200.7; L-leucine, 190.7; L-lysine, 133.0; DL-methionine-2-C^{14}, 54.4; DL-phenylalanine, 80.3; L-threonine, 60.2; DL-tryptophan, 28.1; DL-valine, 216.8; DL-α-alanine, 86.3; L-arginine, 76.4; L-aspartic acid, 48.7; L-cystine, 6.0; L-glutamic acid, 367.3; glycine, 8.0; L-histidine, 52.1; L-tyrosine, 99.4.

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Wesson ('32).

[4] Quantities in milligrams: Thiamine·HCl, 0.04; riboflavin, 0.06; pyridoxine·HCl, 0.04; inositol, 10.0; nicotinic acid, 0.5; Ca pantothenate, 0.1; ascorbic acid, 1.0; choline chloride, 5.0; biotin, 1.0; folic acid, 0.05; *p*-aminobenzoic acid, 10.0; vitamin B_{12}, 0.004; dextrin, 450. The diet also contained 0.9 mg α-tocopherol and 58.5 mg cod liver oil in 12.1 gm.

Iowa State University of Science and Technology. After the rats were fasted for 10 hours, portions of the radioactive diet were administered by stomach tube as a single dose to each of three animals. One animal (rat 5) received 8 ml (8 gm) of the radioactive diet providing 36 mg of radiomethionine. Tissues of rat 5 were examined for radioactivity at the end of 4 hours.

Approximately 50% of the diet had passed from the stomach at the end of 4 hours. The contents of the stomach of this animal, rat 5, were removed. Aliquots were chromatographed two-dimensionally in water-saturated phenol and butanol: propionic acid: water and exposed to medical no-screen x-ray film. All radioactivity was present in the form of methionine. Four milliliters of material obtained from the stomach of the rat was fed by tube to rat 9, which was sacrificed at the end of 24 hours. Rat 8 received 4 ml of the original diet and was also sacrificed 24 hours later.

Collection of blood, carbon dioxide and excreta. Samples of blood and expired carbon dioxide were collected prior to administration of the ration and at specific intervals up to 24 hours afterwards. Urine and feces were collected at these times if the sample had been excreted, and at other intervals when they appeared.

The intermittent, quantitative and simultaneous collection of these samples was made possible by placing the animal in a special metabolism apparatus designed and constructed in our laboratory (Edwards et al., '59a). The rat was placed in this cage immediately after it was fed, with access to water at all times.

The tail of the animal extended through an opening in the cage, permitting samples of blood to be obtained by removal of small segments of the tail at the desired time interval. Blood samples were placed in an ice bath immediately and kept cold until they were analyzed a short time later. Urine and fecal samples were frozen after they were obtained.

Treatment of tissues, blood and expired carbon dioxide. At the time of sacrifice, the animals were anesthetized with ether and exsanguinated by cardiac puncture. About 8 to 9 ml of blood were obtained by this method. All organs and tissues were removed, quickly weighed, and their activities measured with a thin mica end window Geiger-Müller tube (counting efficiency 5%). Portions of the tissues were fixed with Bouin's solution, Carnoy's fluid and 10% formalin for subsequent histological and histochemical studies. The remaining tissues were frozen immediately by placing them on solid CO_2. Tissues and urine samples were kept at $-20°C$ until analyzed.

Expired carbon dioxide was collected in 0.1 N NaOH, contained in 100-ml tubes connected in series in a manifold which led from the metabolism cage. Two collection tubes were used to contain the expired CO_2 for each time interval. The carbonate in the samples was precipitated with barium chloride. The amounts of the samples required to give uniform quantities (16 mg) of barium carbonate were determined. Barium carbonate was plated on filter paper discs in samples of uniform thickness by use of a microprecipitation apparatus and counted with a Geiger-Müller tube and gas flow counter (counting efficiency 30 to 35%).

Globin was precipitated from blood samples with acetone and hydrochloric acid

according to the procedure of Anson and Mirksy ('30). Heme was precipitated from the filtrate by addition of 2 N sodium acetate. All samples were washed until no evidence of radioactivity could be detected in the wash liquids. Samples of heme and globin of uniform thickness were prepared on filter paper discs by use of a microprecipitation apparatus, desiccated over soda lime, weighed, and counted with a gas flow counter.

Aliquots of blood were placed on weighed planchets, desiccated for 24 hours over calcium chloride, reweighed, and counted with a gas flow counter.

One-gram cut sections of tissues were distributed over identical surface areas on aluminum planchets prior to counting. When the tissue weighed less than one gram, the activity of the whole tissue was measured. The radiocarbon in the carcasses of the animals (after removal of organs and muscle) was not determined.

The procedure of Katz and Chaikoff ('54), with modifications, was used for fractionation of tissues into water-soluble, fat-soluble, and protein components. Approximately 1 gm of tissue was minced finely with a razor blade. Four extractions with hot distilled water (5, 1.5, 1.5, and 1.5 ml,), each followed by centrifuging, separated water-soluble materials from the solid tissue residue. The combined supernatant fluids were made to a volume of 10 ml. The residue was extracted 5 times with ethanol, ether and mixtures of the two (5 ml of ethanol; 5 ml of 1:1 ethanol and ether twice; 5 ml of 1:3 ethanol and ether, and 5 ml of ether). The ethanol-ether extracts were combined and the lipids recovered by evaporating gently to dryness on a steam bath. Five milliliters of hexane and 2 ml of distilled water were added to the dried lipids and the mixture was shaken vigorously. The hexane layer (fraction B) was removed. The residue from fraction B contained protein and small amounts of glycogen. The glycogen was extracted with 10% trichloroacetic acid and precipitated with alcohol. The protein residue was washed several times with alcohol, plated uniformly on filter paper discs by use of a microprecipitation apparatus, desiccated over soda lime, weighed and counted with a gas flow counter. Weighed samples were hydrolyzed with 6 N HCl for 24 hours. The hydrolyzate was evaporated gently to dryness on a steam bath, then desiccated over soda lime for 24 hours. Five milliliters of hot distilled water were added and the solutions filtered. The filtrate was evaporated to dryness, cooled, and taken up in 1 milliliter of 10% isopropyl alcohol. The solutions of hydrolyzed protein were frozen immediately and stored at −20°C.

Two-hundred-and-fifty lambda aliquots of all tissue fractions and urine were applied by use of a micropipet to a small circle on Whatman no. 1 filter paper sheets and counted with a Geiger-Müller tube. All measurements were corrected for background. Because the samples were infinitely thin, no correction for self-absorption was made.

RESULTS AND DISCUSSION

Activity in tissues. The specific activities of 23 tissues are shown in table 2. At the end of both 4 and 24 hours, the concentrations of radioactivity arising from the α-carbon of methionine were higher in bone marrow than in other tissues.

TABLE 2

Radiocarbon in tissues of rats fed DL-*methionine-2-C*^{14}

Tissue	Specific activity of tissue[1]		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min./gm	*c/min./gm*	*c/min./gm*
Adrenals	1420	3260	2810
Bone marrow	7560	7163	10550
Brain	180	420	300
Cecum	240	470	360
Duodenum	280	390	150
Hair	40	40	10
Heart	—	318	225
Ileum	90	210	140
Jejunum	100	280	160
Kidney	90	1657	173
Large intestine	120	160	110
Liver	204	405	188
Lungs	390	540	320
Muscle	26	31	21
Pancreas	141	600	258
Prostate	390	1490	470
Seminal vesicles	90	260	130
Skin	20	30	30
Spleen	—	650	380
Stomach	1110	340	230
Submaxillary	130	340	230
Testes	30	90	50
Thymus	310	860	860

[1] Measured with a Geiger-Müller tube.

The specific activities of adrenals, kidney, prostate, thymus, and pancreas also were high at 24 hours. In contrast, concentrations of radioactivity in muscle, hair, and skin remained essentially constant.

Bone marrow and adrenal proteins were more highly labeled than those of other tissues at both 4 and 24 hours (table 3).

Activity in tissue fractions. Table 4 presents data relating to the concentrations of radioactivity in the tissue fractions. With the exception of the stomach, the concentrations of radiocarbon in the water-soluble fractions at 4 hours were highest in bone marrow and pancreas; at 24 hours, in bone marrow.

Other than the alimentary tract, water-soluble fractions of kidney, prostate, seminal vesicles and spleen contained more radiocarbon at 24 hours than at 4 hours. In contrast, concentrations of activity were lower at 24 hours than after 4 hours in adrenals, lungs, muscle, pancreas and skin. The decrease in specific activities of adrenals and pancreas, no doubt, was due to the transfer of radioactive water-soluble compounds from these tissues to other parts of the body.

TABLE 3

Radiocarbon in tissue protein of rats fed DL-*methionine-2-*C^{14}

	Specific activity of protein[1]		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min./mg	*c/min./mg*	*c/min./mg*
Adrenals	53	111	111
Bone marrow	65	621	120
Cecum	6	28	18
Duodenum	45	38	19
Hair	0.04	0.1	0.06
Heart	3	8	5
Ileum	4	32	21
Jejunum	20	56	36
Kidney	6	22	10
Large intestine	6	10	10
Liver	3	11	7
Lungs	5	18	10
Muscle	0.5	2	1
Pancreas	29	—	16
Prostate	13	57	23
Seminal vesicle	2	23	6
Skin	0.3	1	1
Spleen	3	44	22
Stomach	12	25	10
Submaxillary	4	10	4
Testes	6	17	11
Thymus	17	24	24

[1] Measured with a gas flow counter.

A different situation appeared to prevail in prostate, thymus, kidney, seminal vesicles and spleen. The concentration of radiocarbon in the water-soluble fractions of these tissues was lower at 4 hours than at 24 hours. This would suggest that the adrenal glands, pancreas and bone marrow may be sites of priority for acceptance of methionine and/or compounds formed from the α-carbon of methionine.

Whereas different trends in the incorporation of the α-carbon into the various fractions of other tissues can be noted, the activity of the water-soluble and hexane fractions of hepatic tissue remained essentially constant at 4 and 24 hours. This may suggest that the turnover of methionine in the liver occurs at a fairly constant rate, the amino acid being received and dispatched, rather than being retained.

Similarly, the specific activity of the water-soluble fraction of duodenum remained constant at 4 hours as at 24 hours. Chromatograms of the tissue at these times revealed that most of the activity was present in the form of methionine. However, a faintly radioactive spot was present at 4 hours in an area bounded by lysine, arginine and hydroxyproline at R_f 0.49 in water-saturated phenol and 0.25 in butanol: propionic acid: water. In the duodenum and jejunum, after 24 hours, a radioactive compound in the position of α-aminobutyric acid was also observed; however this compound did not react with ninhydrin. These observations pose the question as to whether methionine is changed into other substances prior to absorption into the blood and its uptake by tissues.

Paper chromatograms (Edwards et al., '59b) of the water-soluble fraction of pancreas revealed the presence of several free radioactive amino acids. In contrast, the activity in the water-soluble fraction of liver was present in bound form. Of the various treatments applied, only hydrolysis with 6N HCl made possible the detection of free radioactive methionine and other compounds containing its α-carbon.

The specific activities of the water-soluble and protein fractions of the adrenal glands were high, both at 4 and 24 hours. That the methyl moiety of methionine is used for the synthesis of adrenaline is well documented. Though we have no evidence on this point, it would be of interest to

TABLE 4

Radiocarbon in tissue fractions of rats fed DL-*methionine-2-C*^{14}

Tissues	Water-soluble fraction			Hexane-soluble fraction			Hydrolyzed protein fraction		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min./gm tissue			*c/min./gm tissue*			*c/min./gm tissue*		
Adrenals	5714	2051	2051	1429	0	0	7714	5410	5410
Bone marrow	14286	12498	6667	714	309	800	—[1]	720	—[1]
Cecum	640	1780	1602	0	208	0	398	1402	738
Duodenum	4492	4414	3024	314	475	60	714	452	392
Hair	0	388	855	0	0	0			
Hair and skin							625	630	1276
Skin	561	143	311	30	13	0			
Heart	1468	1818	2000	0	0	0	426	1905	1015
Ileum	1120	1937	2094	49	127	98	272	1414	973
Jejunum	2914	5910	3913	0	122	132	354	1372	754
Kidney	3600	6630	4162	210	387	243	714	3822	3429
Large intestine	708	1250	163	50	109	0	309	829	1175
Liver	4272	4507	3030	272	394	278	733	4565	4176
Lungs	1654	955	642	267	167	0	752	1411	1640
Muscle	580	196	74	68	69	0	144	588	427
Pancreas	12235	4800	2182	329	280	424	61	—[1]	1230
Prostate	2913	4348	2374	0	435	0	456	1786	1395
Seminal vesicles	828	2206	1493	0	0	131	248	1994	1502
Spleen	1633	6000	4000	0	700	0	90	1235	1512
Stomach	22703	1494	1020	473	87	107	1099	2286	972
Submaxillary	2553	1717	1869	0	300	0	153	1000	794
Testes	193	126	519	135	88	27	72	369	372
Thymus	2500	—[2]	4571	0	—[2]	0	1063	—[2]	3629

[1] Not enough sample for analysis.
[2] Sample used for histological examination.

determine whether the α-carbon of methionine is also used in this process.

No radiocarbon could be detected in the water-soluble fraction of hair at 4 hours, though the specific activity of this fraction of skin was 600 c/min./gm.

The hexane-soluble fractions of 17 tissues contained radiocarbon, though less activity occurred in this fraction than in the water-soluble and protein fractions. The activities of this fraction from kidney, liver, spleen, prostate and submaxillary were higher after 24 hours than at 4. Though the concentration of radiocarbon in the lipid fraction of the adrenals was strikingly higher than that in other tissues at 4 hours, no radiocarbon was observed after 24 hours. It would appear that fat-soluble compounds formed in the adrenal are quickly sent to other tissues.

Axelrod and co-workers[4] have reported the incorporation of phenylalanine-C^{14} into a lipid-like substance by isolated rat liver microsomes and particle-free cytoplasm, either separately or combined. However, the incorporation of amino acid into lipid under these conditions appears to differ from protein incorporation in that the former reaction was not inhibited by RNase, or reagents active on lipids. It also appears not to require an energy-generating source.

No activity had been incorporated into hexane-soluble compounds within 4 hours in prostate, spleen and submaxillary gland, though a substantial amount was present at 24 hours in the lipid fractions of these tissues.

The specific activities of all proteins, except those of adrenals and duodenum were higher after 24 hours than after 4 hours. Splenic activity was approximately 14 times higher; that of seminal vesicles was 8 times greater. Similarly, the specific activity of the hydrolyzed protein of pancreas at 24 hours was 20 times that observed at 4 hours. The concentration of radiocarbon in the proteins of skin and hair remained constant.

Table 5 shows a comparison of the total radiocarbon in the water, hexane and protein fractions of all tissues. It is apparent that the total activity in the water-soluble fractions decreased over the 24-hour interval, whereas that in the protein-soluble fraction increased. Total activity in the fat-soluble fraction remained constant in the period from 4 to 24 hours.

Heme and globin. Radiocarbon was detected in heme after 7 minutes. The peak of radioactivity occurred between 4 and 6 hours, after which a level characteristic of the 30-minute interval was reached (fig. 1).

The incorporation of radioactivity into globin, low at early intervals, increased steadily throughout the 24-hour period, the highest value being observed in the last sample (fig. 2). Globin was radioactive as early as 12 minutes after the administration of the radioactive diet.

Expired carbon dioxide, and urine and feces. The pattern of elimination of radiocarbon in carbon dioxide is shown in figure 3. The appearance of $C^{14}O_2$ could be detected as early as 15 minutes after the administration of DL-methionine-2-C^{14}. The metabolism of the α-carbon as $C^{14}O_2$ reached a maximum in 4 to 6 hours after which a gradual decline was observed.

Mackenzie and his co-workers ('50) have studied the rate of oxidation of the methyl group of dietary methionine. They state that, in determinations of this kind, the sharpness with which the oxidation curve changes reflects the extent to which

[4] Axelrod, B., J. L. Haining and T. Fukui 1959 Incorporation of amino acids into lipoidal material by cell-free rat liver preparations. Federation Proc., *18:* 184 (abstract).

TABLE 5

Summary of radioactivity in tissue fractions of rats fed DL-*methionine-2-*C^{14}

Tissue fractions	Total radioactivity			Percentage of absorbed dose		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min.	*c/min.*	*c/min.*	%	%	%
Water-soluble	170,154	88,585	69,464	6.6	3.4	2.9
Hexane-soluble	12,294	10,857	3,525	0.5	0.4	0.1
Hydrolyzed protein	48,899	110,502	120,227	1.9	4.3	5.1

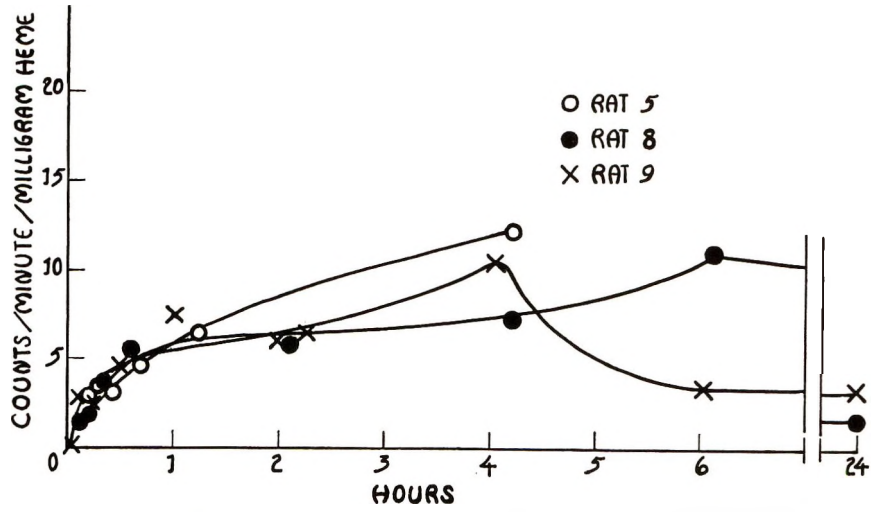

Fig. 1 Specific activity of heme from rats fed DL-methionine-2-C^{14}.

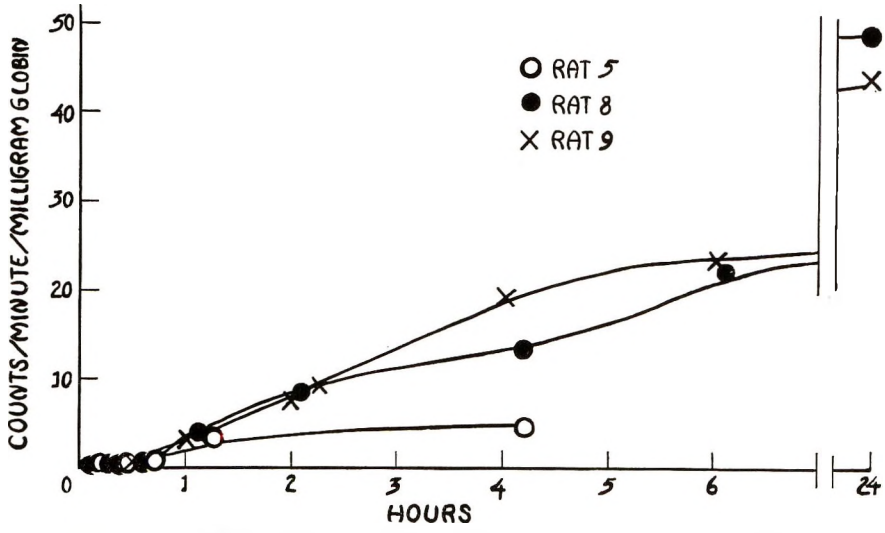

Fig. 2 Specific activity of globin from rats fed DL-methionine-2-C^{14}.

the animal is absorbing the amino acid, destroying or excreting it, and integrating it with endogenous methionine.

In addition to measuring the percentage of the ingested α-carbon of methionine converted to carbon dioxide, the quantity of the isotope excreted in urine and feces in 24 hours was determined (table 6). It was thus possible to compare the 24 hour elimination and retention of exogenous α-carbon labeled methionine.

The first samples of urine were obtained at 6 hours. Only 4 samples were secured from rat 8; three samples were excreted by rat 9. No samples were given during the 4-hour period by rat 5. The peak of activity occurred in urine samples of rat 9 at the 6 hour interval; for rat 8 at 8 hours. After 24 hours, the feces of rats 8 and 9 contained 0.05 and 0.01% of the administered dose, respectively.

We are presently engaged in studies leading to the identification of radioactive compounds in urine, tissue fractions and globin.

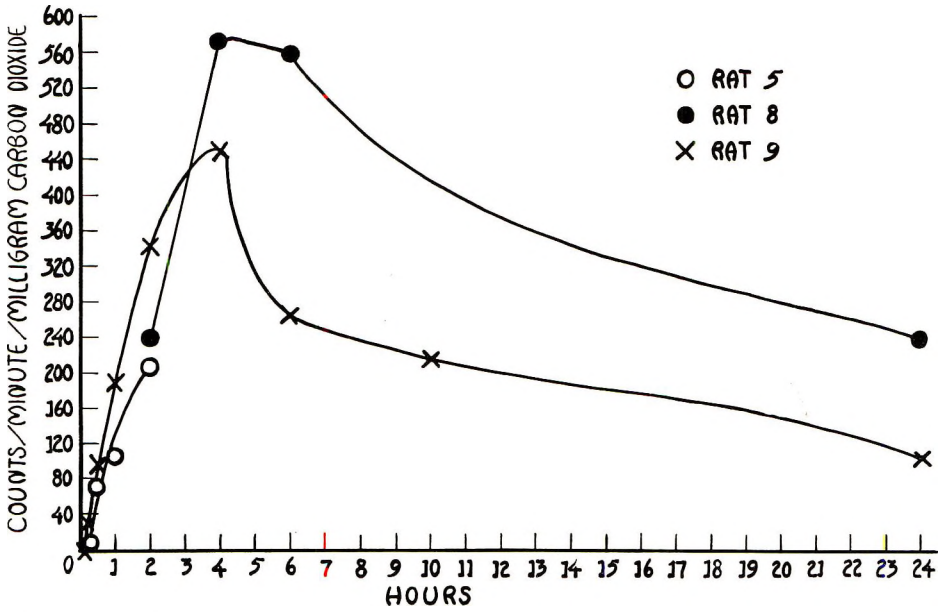

Fig. 3 Specific activity of expired carbon dioxide from rats fed DL-methionine-2-C^{14}.

The data suggest that methionine or compounds containing its α-carbon are taken up preferentially by bone marrow and tissues which secrete hormones. The need of methionine for the elaboration of special compounds thus appears to have priority over general protein synthesis. Further work in our laboratory on rats fed DL-methionine-2-C^{14} and sacrificed after 16 minutes substantiates this interpretation. Of 25 tissues analyzed, the following tissues contained the highest specific activities in decreasing order as listed: bone marrow, pituitary, liver, thyroid, thymus, spleen, kidney, adrenals and pancreas. These data lend support to the hypothesis that bone marrow, certain glandular tissues and liver exert priority in the utilization of methionine. One may speculate as to the role of methionine in bone marrow. Studies are in progress in our laboratory to gain information on this question.

In table 7 is given a summary of the radiocarbon concentrations in tissues, expired carbon dioxide, blood, excreta and the digestive tract. It is of interest that the total activity in tissues was approximately the same (254,501 versus 236,337 c/min.) at 4 and 24 hours. However, it can be observed from table 5 that the total concentration of radiocarbon in the water-soluble fraction decreased strikingly over the 24 hour period, occurring concomitantly with an increase in activity in the protein fraction.

SUMMARY

The appearance of the α-carbon of methionine in 23 tissues and tissue fractions, globin, heme, excreta, and expired carbon dioxide of adult rats at various time intervals has been investigated.

The specific activity of bone marrow was higher than that of other tissues both at 4 and 24 hours. The activity of bone marrow protein, similarly, was higher than that of other tissues.

At 4 hours, the concentrations of radiocarbon in the water-soluble fractions of bone marrow, pancreas and adrenal glands were higher than those of other tissues; after 24 hours, in bone marrow, kidney, spleen and jejunum. The hexane fraction of 17 tissues contained radiocarbon.

The total concentration of radiocarbon in the water-soluble fraction decreased sharply over the 24-hour period, occurring concomitantly with an increase in the carbon fourteen content of tissue proteins.

The α-carbon of methionine appeared in expired carbon dioxide within 15 minutes; in heme, in 7 minutes; and in globin,

TABLE 6

Radiocarbon in expired carbon dioxide and urine of rats fed DL-*methionine-2-C*^{14}

Time interval in minutes	Specific activity			Total activity			Percentage of absorbed dose		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min./mg[1]			*c/min.*			%		
Carbon dioxide									
0–3	0	—	0	0	—	0	0	—	0
3–8	0	—	0	0	—	0	0	—	0
8–15	5.6	—	9.2	5315	—	2196	0.2	—	0.1
15–30	21.6	—	19.9	10044	—	5014	0.4	—	0.2
30–60	29.0	—	38.8	15134	—	21967	0.6	—	0.9
60–120	50.9	47.2	67.8	40613	14564	53430	1.6	0.6	2.3
120–240	40.6	78.3	74.9	49196	59472	87500	1.9	2.3	3.7
240–360	—	96.0	40.5	—	93424	50713	—	3.6	2.1
360–600	—	—	40.1	—	—	30519	—	—	1.3
360–1440	—	41.1	—	—	180961	—	—	7.0	—
600–1440	—	—	16.8	—	—	46905	—	—	2.0
Urine									
0–360	—	—	—	—	5504	—	—	0.2	—
360–498	—	—	—	—	4048	—	—	0.2	—
498–643	—	—	—	—	1560	—	—	0.1	—
643–698	—	—	—	—	1672	—	—	0.1	—
360–780	—	—	—	—	—	2888	—	—	0.1
780–1440	—	—	—	—	—	1040	—	—	0.04

[1] Measured with a Geiger-Müller tube.

TABLE 7

Distribution of radiocarbon in rats fed DL-*methionine-2-C*^{14}

	Total radioactivity[1]			Percentage of absorbed dose		
	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour	Rat 5 4 hour	Rat 8 24 hour	Rat 9 24 hour
	c/min.	*c/min.*	*c/min.*	%	%	%
All tissues (except carcass)	254,501	236,337	204,818	9.8	9.1	8.7
Expired CO_2	120,301	348,421	298,244	4.6	13.4	12.6
Urine	—[2]	12,784	3,928	—	0.5	0.2
Feces	—[2]	1,220	190	—	0.05	0.01
Globin	2,541	14,903	12,209	0.10	0.6	0.5
Heme	605	192	317	0.02	0.01	0.01
Contents of digestive tract	2,606,980	35,444	18,021			
Total dose fed	5,400,737	2,634,869	2,385,461			
Total dose absorbed	2,593,757	2,599,425	2,367,440			

[1] Measured with a Geiger-Müller tube.
[2] No sample.

within 12 minutes. Incorporation of the α-carbon of methionine into globin proceeded slowly, reaching a maximum at 24 hours.

When administered by stomach tube as a component of an adequate semi-synthetic diet, 17 mg of DL-methionine-2-C^{14} was absorbed during the 4-hour interval. Of the absorbed dose, 4.6% was eliminated as $C^{14}O_2$ and 9.8% was present in tissues. At the end of 24 hours, 9% of the α-carbon of methionine had been taken up by tissues, and 13% had been eliminated as $C^{14}O_2$.

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ADDENDUM

In experiments completed after this manuscript was submitted for publication, we have observed that the methyl carbon, and, presumably, the labile methyl group, of methionine is used for the formation of cholesterol. The α-carbon of DL-methionine-2-C^{14} does not appear to be used in this process. Further work on this aspect of the overall problem is now in progress in our laboratory.

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Effects of Purified Linoleic Ester on Cholesterol in the Rat[1]

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The literature contains abundant evidence that certain natural oils, especially those high in polyunsaturated acids, will lower blood cholesterol levels in man and several other species. From these data a number of attempts have been made to deduce the effects of individual fatty acids (Page et al., '57; Anderson et al., '57; Hegsted et al., '57). Considerable lack of agreement is evident, perhaps because of the complexity of composition of the fats or fat products used.

Experiments to test the effects of individual fatty acids on cholesterol levels have been reported in only a few cases; usually commercial fatty acid preparations were used without reference to composition or quality. The purpose of the present study was to observe the effect of a highly purified unsaturated ester, ethyl linolate, on the plasma and tissue cholesterol levels of hypercholesteremic rats fed low- and high-fat diets. A preliminary report was presented previously.[3]

EXPERIMENTAL

Materials and methods. Ethyl linolate was prepared from safflower oil by fractional crystallization of the urea adducts (Parker et al., '55) and subsequent distillation of the converted ester in an all-glass still (130 to 140°C; less than 1 mm Hg pressure). Tests on the distillate (table 1) showed it to be a non-conjugated *cis* product in which oleic acid was the only measurable contaminant; it was believed to be at least 97% *cis* 9, 12-linoleic ester.

Diets. Two basal diets[4] were used, a high-fat (20%) diet and a low-fat (2%) diet, in which the ratio of all other nutrients to calories supplied as fat and carbohydrate was constant. The high-fat diet contained the following (in per cent): purified casein, 18; celluflour[5], 2; salts no. 185 (McCollum and Simmonds, '18), 4.0;

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[3] Quackenbush, F. W., and M. Pawlowski 1959 Linoleic ester effect on hypercholesteremia in rats. Federation Proc., *18:* 542 (abstract).

[4] The authors express their appreciation to the Procter and Gamble Company, Pacific Coast Vegetable Oil Corporation and Ralston Purina Company for generous supplies of hydrogenated coconut oil, safflower oil and soybean oil, respectively.

[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.

TABLE 1
Properties of the ethyl linoleate

Distillation temp. (< 1 mm p.)	127–130°, 20 drops/min. (first 10% discarded)
Refractive index	1.4518 to 1.4520
Iodine value	161–164.6 (Theo. 164.57)
Infrared	No *trans* absorption
Diene conjugation	0.06%
Digitonin ppt.	Nil
Free fatty acids	3% or less
Oleate (gas-phase chrom.)	< 3%
Linolenate (gas-phase chrom.)	Nil
Palmitate (gas-phase chrom.)	Nil
Stearate (gas-phase chrom.)	Nil

TABLE 2

Male rat responses to linolate and to different dietary fats

Group no.	Dietary lipid			Weeks on diet	Body weight	Plasma cholesterol[2]		Liver		Carcass[3]	Total "cholesterol": liver + carcass
	SBO[1]	HCO[1]	EL[1]			Free	Total	Total fat	Total cholesterol	Total "cholesterol"	
	%	%	%		*gm*	*mg/100 ml*		%	*mg*	*mg*	*mg/100 gm body weight*
1	20			10	339	20	139	23	2287	749	896
1	20			24	429	19	121	20	1838	905	639
2		20		10	236	144	600	18	1455	599	870
2L		10	10	5[4]	266	27	164	17	1392	518	718
3		2		10	215	31	268	13	671	654	603
3		2		24	216	82	445	17	1270	658	892
3F[5]		2		24	—	126	666	—	—	—	
3L			2	5[4]	276	13	111	8	259	557	275
3L			2	19[4]	334	20	103	5	133	873	301
4	2			5[4]	287	15	96	11	741	644	453
5	4			5[4]	292	24	72	12	775	565	460
6		2	0.2[6]	5[4]	267	43	333	16	784	587	492

[1] SBO, soybean oil; HCO, hydrogenated coconut oil; EL, ethyl linolate.

[2] Plasma cholesterol values are averages of three to 15 analyses from all rats bled during 9th to 10th weeks or 20th to 24th weeks.

[3] Less intestinal tract, liver, spleen, thymus and adrenals; total sterol expressed as cholesterol.

[4] Groups 2 and 3 were divided at the end of 5 weeks to form groups 2L and 3L, which were fed linolate from the beginning of the 6th week. Groups 4, 5 and 6 received 2% of HCO for the first 5 weeks.

[5] Fasted overnight prior to removal of blood sample.

[6] Each rat received ca. 25 mg of ethyl linolate daily by dropper.

glucose[6], 56; and fat, 20 (table 2). To this diet cholesterol was added at 1% and sodium glycocholate at 0.5% levels. For each kilogram of diet, the following vitamins were included (expressed as mg/kg except where otherwise noted): thiamine·HCl, 24; riboflavin, 12; pyridoxine·HCl, 6; Ca pantothenate, 72; nicotinic acid, 30; *p*-aminobenzoic acid, 12; biotin, 2.4; inositol, 1400; choline chloride, 1750; folic acid, 12; vitamin B_{12}, 0.02; menadione, 60; DL-α-tocopherol, 120; vitamin A powder, 2400 units; crystalline vitamin D_3, 240 units. The tocopherol and vitamin D were added directly to the fat at the time of mixing the diet. All other vitamins were prepared as a premix with the casein. The diets were prepared frequently and refrigerated until used.

For the low-fat (2%) diet, glucose was increased to 78%, and the quantities of all other dietary components were divided by the factor 1.2.

Animals. Weanling rats of an inbred Wistar strain, in individual cages, were fed ad libitum. Twelve males and 12 females were used for each of groups 1, 2 and 3. Eight males were used in each of groups 4, 5 and 6. Group 1 was fed the 20% soybean oil diet throughout the experiment. Group 2, 20% hydrogenated coconut oil (HCO), was divided at the end of the 5th week, and half the animals of each sex (group 2L) were fed 10% of HCO plus 10% of linoleic ester for a subsequent 5-week period. Group 3 (2% HCO) was divided at the end of the 5th week; half of the animals of each sex (group 3L) subsequently were fed 2% of linoleic ester instead of 2% of HCO. Groups 4, 5 and 6 also received 2% of HCO through the 5th week. During the next 5 weeks group 4 received 2% of soybean oil; group 5, 4% of soybean oil; and group 6 continued to receive 2% of HCO but with the addition of 25 mg of linoleic ester per day.

Blood was drawn from the ether-anesthetized rats by cardiac puncture into heparinized tubes, and the plasma was analyzed for free and total cholesterol by the method of Sperry and Webb ('50). Beginning with the 4th week, samples were taken at intervals of one to three weeks. No evidence of any effect on plasma cholesterol levels was observed by bleeding animals as often as once a week.

The statistical significance of differences between means was determined by t tests, and F tests were used when comparing variances, for plasma cholesterol values.

Livers and carcasses of individual rats (usually 6 per group) were analyzed at the 10th week, and in the longer-term experiments, also at the 24th week. The intestinal tract, liver, spleen, thymus and adrenals were removed prior to carcass analysis. Liver lipids were prepared by the method of Thompson et al. ('49), and carcass lipids by the method of Quackenbush and Steenbock ('42). Cholesterol was then determined by the Sperry and Webb method.

RESULTS AND DISCUSSION

Growth and dermatosis. Substantial differences were observed in rate of growth between animals fed soybean oil and HCO (tables 2 and 3). The poor growth of the rats in group 2 was improved substantially by the substitution of linolate for half of the coconut oil during the second 5-week period. Scaliness of paws and tail, beginning at the third week, gave further evidence of EFA deficiency in groups which lacked linoleic acid in the diet. These dermal symptoms persisted throughout the experiment and were more pronounced in males than in females. Rats in group 6 remained essentially free from symptoms.

In general the animals fed high-fat diets made faster gains than those receiving low-fat diets.

Plasma cholesterol. Analysis of plasma from weanling rats in the colony showed average values of 50 mg/100 ml of total cholesterol, about 1/5th of which was in the free form. After feeding the basal diet for 4 weeks with either high or low fat, supplied as HCO, total cholesterol had risen as high as 1,000 mg/100 ml in some animals. In animals which received the soybean oil, it averaged slightly over 200 mg/100 ml.

Substitution of linolate for either part or all of the HCO (groups 2L and 3L) at the end of the 5th week resulted in a prompt lowering of blood cholesterol levels. This is exemplified in the comparison of total plasma cholesterol of the males fed

[6] Cerelose, Corn Products Refining Company, New York.

TABLE 3

Responses of female rats to linolate and to different dietary fats

Group no.	Dietary lipid			Weeks on diet	Av. body weight	Plasma cholesterol[2]		Liver			Carcass[3] Total "cholesterol"	Total "cholesterol": liver + carcass
	SBO[1]	HCO[1]	EL[1]			Free	Total	Av. weight	Total fat	Total cholesterol		
	%	%	%			*mg/100 ml*		*gm*	%	*mg*	*mg*	*mg/100 gm body weight*
1	20			10	246	29	197	16.6	29	1950	427	966
1	20			24	262	46	250	15.9	31	2362	527	1103
2		20		10	170	191	940	10.0	14	621	391	595
2L		10	10	5[4]	210	47	303	15.2	28	1415	375	852
3		2		24	196	82	464	12.3	16	861	749	822
3F[5]		2		24		146	911	—				
3L			2	19[4]	246	22	128	9.1	6	110	469	235

[1] SBO, soybean oil; HCO, hydrogenated coconut oil; EL, ethyl linolate.
[2] Plasma cholesterol values are averages of three to 15 analyses from all rats bled during 9th to 10th weeks or 20th to 24th weeks.
[3] Less intestinal tract, liver, spleen, thymus and adrenals.
[4] Groups 2 and 3 were divided at the end of 5 weeks to form groups 2L and 3L, which were fed linolate from the beginning of the 6th week.
[5] Fasted overnight prior to blood sample.

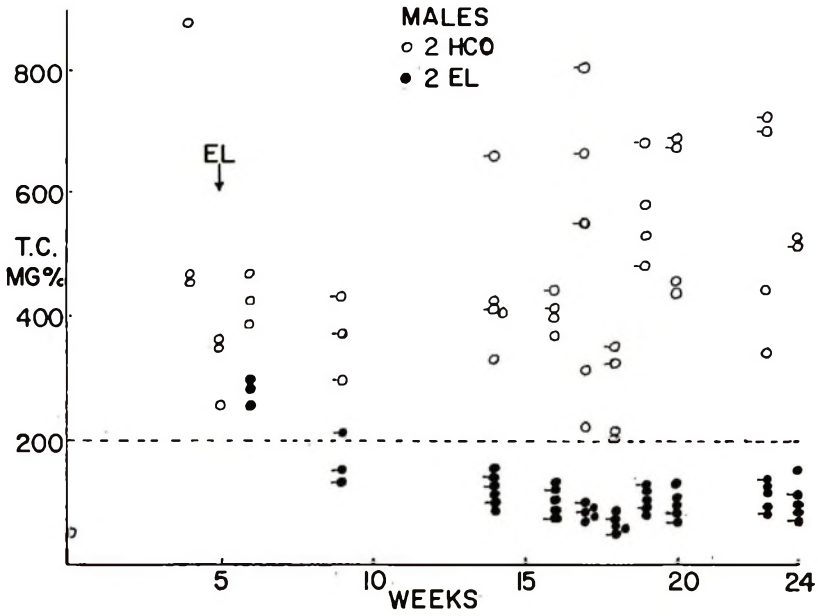

Fig. 1 Total cholesterol in the blood plasma of male rats fed 2% of hydrogenated coconut oil (group 3) vs. 2% of ethyl linolate (group 3L). Values for animals which fasted overnight before blood was drawn are indicated by bars.

diets 3 and 3L (fig. 1). The two groups were separated sharply at the 200 mg/100 ml line. That the response to linolate does not merely reflect a relief of classical EFA-deficiency symptoms is evident from the lack of a similar response of the animals in group 6 (table 2). Substitution of half of the 20 % of HCO with 10% of linolate (groups 2 and 2L, table 2) produced a response similar to that of complete substitution; the average total cholesterol in samples from 10 males in group 2L, bled during the 9th and 10th weeks, was 164. Males which received soybean oil (group 1) showed slightly, though significantly ($P = 0.05$), higher values than those fed 2% of linolate (group 3L) during the 10th and 24th weeks, while those fed 2% of HCO (group 3) were much higher (fig. 1).

It is apparent that the fat level of the diet had no marked effect upon the blood cholesterol level in the rat. This is in agreement with the observations of Nath et al. ('59) who observed no differences between 1 and 25% of fat in the diet.

Female rats showed responses parallel to those of males; however, their levels of plasma cholesterol were significantly higher than those of males. Females which received HCO also showed a significantly larger variance than the corresponding males. Those which received 20% of soybean oil also showed significantly higher plasma cholesterol levels than the corresponding males from the 4th through the 24th week.

Fasting of the animals overnight, prior to the removal of a blood sample, was initiated because of wide variations which were observed between plasma cholesterol levels of animals within a group. These variations were especially marked in groups with high cholesterol levels. Part of the animals in groups 1, 3 and 3L were fasted prior to sampling during the 9th to the 24th weeks. Typical results are shown for males in figure 1. Observations of animals in group 3L showed no evidence of any difference in levels of total plasma cholesterol between those fasted and non-fasted of either sex. Group 1 also showed no evidence of any trend. However, in group 3 the levels were significantly higher in the fasted than in the non-fasted animals of both sexes. On the other hand, variance was not diminished significantly by fasting in any of the groups except those receiving soybean oil. Kohn et al. ('50) have also reported that fasting prior to cholesterol analysis did not affect plasma levels.

On the other hand, Bragdon et al. ('57) have observed a rise in plasma cholesterol level as a result of fasting. Yacowitz et al. ('57) observed a drop in blood volume and a corresponding rise in blood cholesterol during prolonged starvation of roosters.

The rise observed in our EFA-deficient animals (group 3) after overnight fasting is possibly associated with a decreased plasma volume as a reflection of the water imbalances which occur in EFA deficiency. Water requirements of EFA-deficient animals are known to be increased (Basnayake and Sinclair, '54).

Liver and carcass cholesterol. In most of the groups the liver was found to be the animal's main storage depot for the cholesterol (tables 2 and 3). Livers from rats fed the 20% fat diets contained 50 to 80% of the total cholesterol in the animal. A similarly high liver cholesterol was found at 24 weeks in rats of group 3 which received 2% of HCO. This was in sharp contrast with those in group 3L which received 2% of linolate instead of 2% of HCO from the 5th week to the 24th week, where liver cholesterol was only 13 to 19% of the total. Furthermore, the total sterol value, when liver and carcass data were added, showed clearly that both the males and females which received 2% of linolate (group 3L) as the only dietary lipid had much lower cholesterol stores as well as lower blood plasma cholesterol than the animals in any other group. Liver cholesterol levels of males from group 3L at 5 weeks were substantially lower than those of the other groups. Soybean oil at 2 or 4% seemed able to suppress levels in the plasma but not in the liver. A minimal daily level of linolate, to prevent classical EFA-deficiency symptoms, was ineffective for both plasma and liver (group 6).

The results reported in this paper show clearly that linoleic ester is an effective agent in lowering cholesterol levels in the rat. They also indicate that caution should be used in attempting to deduce the effect of individual fatty acids on blood- and liver-cholesterol levels from the data obtained in feeding various natural fats.

Caution is also needed in interpreting results obtained with fatty acid products of unknown purity. Many commercial products sold as such are not worthy of the label they bear. This probably accounts for the failure of some workers (Swell et al., '55; Rona et al., '59)[7] to observe a cholesterol-lowering effect from the dietary addition of products which were purchased as linoleic acid. Peifer et al.[8] had no difficulty in observing the effect in rats fed a tallow-containing diet when a small amount of linolate of known high quality was used, and Kinsell et al. ('58) observed it in humans.

SUMMARY

Linoleic ester of high purity was shown to lower markedly the plasma cholesterol level of hypercholesteremic cholesterol-fed rats. It also lowered liver cholesterol of the rats receiving a low-fat diet (2%) but not those fed a high-fat diet (10% of linolate and 10% of hydrogenated coconut oil).

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The Unique Role of Ascorbic Acid in Peripheral Vascular Physiology as Compared with Rutin and Hesperidin; a Micromanipulative Study[1]

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The need for citrus fruit in the diet for the prevention of scurvy, its hemorrhages into various parts of body tissue, and other probable consequential phenomena, has been recognized for over two-hundred years. Isolation and identification of vitamin C as ascorbic acid (King and Waugh, '32) made specific dietotherapy possible for clinical scurvy, and allowed the deficiency of this single compound to be studied experimentally. It has been shown that lack of ascorbic acid in the diet of guinea pigs is accompanied by widespread histopathological lesions (Wolbach, '37; Dalldorf, '38) and by profound vasomotor disturbances in the capillary bed (Lee and Lee, '47). These latter, in particular, are associated with a greatly increased mortality rate and altered plasma humoral characteristics during experimental hemorrhagic shock in ascorbic acid deficiency (Lee and Holze, '51). Ascorbic acid, therefore, also has a major role in maintenance of vasocompensatory mechanisms.

That this vitamin may not be the only factor in citrus products important in peripheral vascular physiology has been emphasized recently by numerous reports. A group of compounds, the "bioflavonoids," has been described as "vitamins" that increase peripheral vascular resistance to trauma (Rusznayak and Szent-Gyorgyi, '36; Martin, '55). These substances are thought to act, perhaps in association with ascorbic acid (Martin et al., '54; Rhinehart, '55) to influence either the vessel wall or the perivascular connective tissues in some manner and thereby to prevent or reduce hemorrhagic diatheses (Rusznayak and Szent-Gyorgyi, '36; Rapaport and Klein, '42). Enhanced vascular responses to certain vasoconstrictor drugs (Crismon et al., '51; Clark and Geissman, '49; Haley et al., '47), altered blood coagulation (Bourgain et al., '54), and improvement in several clinical states or experimentally produced abnormalities have been ascribed to the action of flavonoids (Arons et al., '54; Biskind and Martin, '54; Boines, '55; Boines and Horoschak, '56; Sokoloff et al., '45; Sokoloff and Redd, '56; Macon, '56). Because of the importance assigned to these materials and the lack of specific descriptions concerning the vascular abnormality induced by their absence in the diet, micromanipulative techniques were used to examine the capillary bed of animals supplied with or known to be deficient in flavonoid substances. This report describes our findings.

METHODS

All rats were supplied with a completely synthetic diet.[2] Those given flavonoids were divided into two groups of equal number (see tables 1–3); one group was fed 10 mg/100 gm of body weight of the substances by gavage, and the second received approximately equal amounts included in the consumed daily ration. Guinea pigs were divided into several groups (tables 4–6). Certain controls received laboratory chow with supplemented greens, and were

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[1] This study was supported by the Nutrition Foundation, Inc., New York, and by the A. H. Robins Company, Richmond, Virginia.

[2] The percentage composition of the supplemented diet was as follows: casein, 18; lard, 8; cornstarch, 49; sucrose, 14; agar, 2; salt, 4; cod liver oil, 1; wheat germ oil, 2; corn oil, 1; vitamin mixture, 1. The vitamin mixture included (in milligrams/100 gm of diet) thiamine·HCl, 0.8; riboflavin, 0.8; pyridoxine·HCl, 0.8; Ca pantothenate, 1.5; nicotinic acid, 1.5; nicotinamide, 1.5; choline chloride, 400; and inositol, 100.

TABLE 1

The appearance of petechiae in rats fed bioflavonoids[1]

Dietary treatment	No. of animals	Spontaneous petechiae	Fresh petechiae after brushing
		no./field	
Normal diet	10	7 (2–13)[2]	2 (0–5)
Hesperidin-supplemented	20	9 (3–15)	2 (0–4)
Rutin-supplemented	20	6 (3–12)	4 (0–6)

[1] Rats received 20 to 100 mg of Dicoumarol 24 hours previous to observation.
[2] Numbers in parentheses indicate range of petechiae.

TABLE 2

The effect of electrical stimulation (VM)[1] *upon clotting tendency in the vascular bed of the rat*

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20–50 μ	20 μ		20–50 μ	20 μ
Normal diet	9	107	70	0	82	28
Hesperidin-supplemented	20	98	87	0	63	46
Rutin-supplemented	20	110	106	0	78	34

[1] VM indicates volts × milliseconds.

TABLE 3

Topical application of epinephrine to vessels of rats fed bioflavonoids[1]

Dietary treatment	No. of animals	Epinephrine threshold	Vasomotor activity
Normal diet	9	1.6×10^{-7}	+
Hesperidin-supplemented	20	1.6×10^{-7}	++
Rutin-supplemented	20	1.6×10^{-7}	+++

[1] No effect was observed from application of hesperidin complex, 1%, hesperidin methyl chalcone, homovanillic acid, dihydroxyphenylacetic acid, calcium flavonoglucoside, or hydroxyphenylacetic acid.

fed additional flavonoids by gavage in half the instances. A second series was given a synthetic guinea pig diet (Reid and Briggs, '53), with direct feeding in the experimental groups of rutin, or hesperidin, (10 mg/100 gm of body weight) with or without added vitamin C (15 mg/100 gm of body weight). Animals fed by stomach tube were individually housed, while the remainder without direct oral supplementation were maintained in cages of two to 6 animals each. Prior to feeding the deficiency diet, a laboratory pellet diet with added fresh greens was supplied for at least 7 to 10 days. Previous studies have indicated that adequate blood- and tissue-vitamin C levels as determined with a standard method (Roe and Kuether, '43) can be maintained using the diet with suitable supplementation by greens or ascorbic acid by mouth (Lee and Holze, '51). During ascorbic acid deficiency, blood levels of this vitamin fall to zero at the end of 14 to 16 days, with tissue levels at approximately 5% of normal. The deficient guinea pigs were therefore not examined until after 16 to 18 days, and it was determined that their blood ascorbic acid levels had reached this stage.

The capillary bed of the mesentery or mesoappendix of rats and the mesentery of guinea pigs were prepared and studied as described previously (Chambers and Zweifach, '44; Lee and Lee, '47). In brief, the animal was anesthetized with pentobarbital sodium and an intestinal loop or the appendix and its mesentery were exteriorized through an abdominal incision. The

preparation was maintained on a warm stage and continuously washed with warmed Ringer-gelatin solution. Studies of the capillary bed were made at magnifications of 100 power. Notes were made with regard to the general character of overall bloodflow, the state of vasomotor tone (partial constriction and occasionally the presence of spontaneous vasomotor activity), and in certain studies the reactivity of the terminal arterioles and precapillary sphincters to topically applied epinephrine (Chambers and Zweifach, '44).

Fragility, bleeding time and intravascular thrombotic phenomena of single microscopic vessels, were studied with several micromanipulative techniques.

1. *Direct trauma.* Methods were used that previously had proved satisfactory to produce petechiae in specific vessels of guinea pigs (Lee and Lee, '47). After exposing the vessel area to be studied, single strokes were applied to the whole region with a fine camel's hair brush while the vessels were under direct observation. The number of strokes necessary to produce red blood cell leakage and petechiae formation, as well as the size of the petechiae and their number per low power field, were noted for arterioles, capillaries and venules.

2. *Microtransection.* Because the flavonoids have been reported to have an influence on blood clotting (Bourgain et al., '54), it was decided to determine whether the reported reduction in petechiae formation on flavonoid therapy could be attributed to an influence on *in vivo* blood coagulation. A microscalpel fixed to the micromanipulator was used to transect certain specific vessels directly under the microscope. With a fast stop-watch, the vessels were observed immediately after cutting until bleeding stopped and the severed ends of the vessel could be seen to be completely plugged. This procedure, used previously elsewhere (Zucker, '47), was found to give satisfactory end points and very reproducible results in our studies.

3. *Intravascular experimental thrombosis.* With suitable apparatus, electrical stimulation of the vascular wall in the capillary bed will produce intravascular thrombus formation as well as hemorrhage (Fulton et al., '53). Microelectrodes, either hand pulled from silver wire to an external diameter of not more than 15 to 20 μ, or made with exceedingly fine-guage silver wire, were connected to an electric stimulator and the electrode mounted in the manipulator arm. With practice, it was possible to apply the electrode tip directly against the side of any vessel selected in the capillary bed without lacerating the tissue or indenting the wall. Single spike as well as repetitive stimulation could then be applied to the site on the vessel selected with various millivolt-second stimuli while continuous observations were made on blood flow velocity, and the time necessary for "white cell" thrombus formation and blood flow stoppage. With care in the construction of the electrodes and experience in observation, these procedures also made it possible to study and determine several stages in intravascular thrombosis that were unrelated to our present purposes.

RESULTS

Observations on rats

Dicoumarol treated rats. Fifty animals fed the synthetic diet, with 40 of them receiving hesperidin or rutin for 4 weeks, were given dicoumarol by stomach tube (average dose 50 mg/100 gm of body weight) and 24 hours subsequently the vessels in the mesoappendix and mesentery were examined. The results are shown in table 1. The numbers of initial and "spontaneous" petechiae per low power field were closely comparable in all three groups. A single stroke with a camel's hair brush to areas under observation produced approximately the same number of fresh petechiae (table 1). These could be seen to appear and to reach approximately the same size (20 to 50 μ in diameter), primarily along the smaller collecting venules. This region of the capillary bed has been described previously as the major site of petechiae formation (Lee and Lee, '47; Fulton et al., '53).

Microthrombosis. Table 2 lists the mean volt millisecond values (VM) found necessary to produce a "white thrombus" and stoppage of blood flow. The ranges in each instance were approximately 50% of the VM shown on either side of the average

level; for example, for arterioles less than 20 μ in diameter, the VM necessary for thrombosis ranged from 36 to 118. Analysis of the standard error of means for arterioles of this size (where the means were most widely separated) indicated no significant difference ($P = 0.2$). Animals receiving hesperidin or rutin, therefore, showed no change in the tendency to form either arteriolar or venular microthrombi. Thrombosis in the capillaries could not be produced; electrical stimulation of these vessels at VM values far below those needed to cause thrombosis invariably produced a complete closure of precapillary sphincter and drainage of all capillary blood into the collecting venular system.

Responsiveness to epinephrine. The response to epinephrine of vessels in animals receiving either flavonoid was not altered (table 3). Of the animals fed rutin, however, 60% showed a moderate but definite increase in the observed spontaneous vasomotor activity of the precapillary sphincters, such that capillary blood flow in these animals was more intermittent.

Direct application of flavonoids to vessels. When the mesenteric or mesoappendiceal capillary of normal rats was bathed with 1% aqueous "solutions" of hesperidin complex, hesperindin methylchalcone, homovanillic acid, dihydroxyphenylacetic acid, calcium flavonegluco-side and hydroxyphenylacetic acid (5 animals with each substance), no influence was found on the responsiveness to topical epinephrine, the spontaneous vasomotor activity, or the overall nature of peripheral vascular blood flow.

Observations on guinea pigs

Bleeding time. Duration of bleeding in various vessels completely transected while being observed was measured until closure by the "platelet-white cell plug" and by partial vasoconstriction. The observations in normal animals, vitamin C-deficient, and hesperidin- and rutin-supplemented animals (both supplemented and vitamin C-deficient) are shown in table 4. The range in bleeding time for each type of vessel was extreme. Analysis of mean values for larger arterioles (20 to 50 μ in diameter) where the differences were greatest, indicate that vitamin C deficiency, or flavonoid feeding had no significant effect ($P = 0.1$).

Microthrombosis. Table 5 illustrates mean VM necessary to produce white cell thrombosis in the larger arterioles, smaller arterioles, and venules in the 6 guinea pig diet groups. It is apparent that no difference was found that could be associated either with vitamin C deficiency or with feeding with either flavonoid. As with rats, thrombosis of capillaries could not be pro-

TABLE 4

Bleeding time of guinea pigs (in seconds) after direct microtransection[1]

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20–50 μ	20 μ		20–50 μ	20 μ
Normal diet	18	170	209	21	210	160
Normal diet + rutin	20	182	210	22	219	178
Normal diet + hesperidin	20	190	198	20	206	180
Vitamin C-deficient	24	206	220	23	218	174
Vitamin C-deficient + hesperidin	22	184	198	19	204	168
Vitamin C-deficient + rutin	20	197	188	22	216	157

[1] Data in tables 4, 5 and 6 are from studies on those guinea pigs given the "semi-synthetic" basal diet (Reid and Briggs, '53). Findings in approximately equal numbers of control animals fed laboratory chow and greens, laboratory chow alone, or supplemented with rutin or hesperidin are comparable.

TABLE 5

Clotting tendency in guinea pigs (volts × milliseconds)

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20–50 μ	20 μ		20–50 μ	20 μ
Normal diet	20	55	48	0	60	35
Normal diet + rutin	30	62	60	0	54	44
Normal diet + hesperidin	28	58	66	0	72	36
Vitamin C-deficient	32	48	56	0	76	26
Vitamin C-deficient + hesperidin	24	54	62	0	54	41
Vitamin C-deficient + rutin	24	42	51	0	41	52

TABLE 6

Effect of direct trauma to capillary bed of guinea pigs (petechiae/low power field)

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20–50 μ	20 μ		20–50 μ	20 μ
Normal diet	42	0	0	0–2	0–2	0–2
Normal diet + rutin	29	0	0	0–4	0–3	0–2
Normal diet + hesperidin	40	0	0	0–3	0–2	0–4
Vitamin C-deficient	34	0	1–8	2–5	3–18	5–12
Vitamin C-deficient + hesperidin	28	0	1–9	2–6	4–16	7–11
Vitamin C-deficient + rutin	28	0	0–8	2–4	4–20	6–11

duced because of precapillary sphincter closure at VM levels far below those producing microthrombus formation.

Direct trauma to the capillary bed. Repeated single brush-strokes to the vessels under observation produced petechiae in occasional capillaries and various-sized venules of normal animals (table 6). Similar trauma was followed by numerous petechiae in all vitamin C-deficient guinea pigs, regardless of flavonoid feeding. Induced hemorrhages were far more frequent in smaller venules.

DISCUSSION

A review of the summarized data presented in tables 1 through 3 indicates that the flavonoid substances studied have no influence on spontaneous petechiae formation in the dicoumerol-fed rats, on microthrombus formation following electrical stimulation of the specific vessels directly with microelectrodes, or on responsiveness to topical epinephrine. The enhancement of spontaneous vasomotor activity by rutin was the only phenomenon observed in rats that could possibly be attributed to supplemental feeding of a flavonoid. The reported protection by flavonoid substances against hemorrhages produced in rats by various means could not be confirmed by the techniques described with direct study of the vascular structures thought to be involved. In the guinea pig, rutin or hesperidin had no observed influence on the normal vascular bed, and failed to reduce both "spontaneous" and induced petechiae, and failed also to influence to any degree the symptoms of vitamin C deficiency.

In sharp contrast with the absence of findings on rats and guinea pigs deficient in rutin and hesperidin, were those on guinea pigs deficient in vitamin C. Here the arterioles and capillaries appeared engorged, spontaneous ruptures were numerous, and direct mechanical trauma produced widespread venular hemorrhages. Feeding either rutin or hesperidin (or both) failed to affect these manifestations; supplements of ascorbic acid, however, prevented them.

This failure to detect any physiological action of rutin or hesperidin on the peripheral microscopic vessels is paralleled by a division of opinion, generally, with regard both to the physiological importance and to the therapeutic value of flavonoids. In repeated experiments, the original workers could not completely duplicate their earlier findings (Szent-Gyorgyi, '38) and attempts to confirm reported benefits of treatment have not been successful (Franz et al., '56; Tebrock et al., '56). The present studies do not support the concept that there is an additional intravascular thrombotic defect in vitamin C deficiency or that flavonoids influence blood coagulation. *In vivo* thrombus formation occurred as quickly and at generally comparable VM stimuli in the same vessel classes of all animals.

It was interesting to find that rats and guinea pigs fed synthetic diets failed to display any manifestation that could be explained or relieved by added flavonoids. This observation reaffirms the current concept that although flavonoids may exert certain physiological influences on the organism, they are not essential nutrients.

SUMMARY AND CONCLUSIONS

Using several previously established micromanipulative techniques, the peripheral vascular system has been studied in rats fed (1) a synthetic diet with and without rutin or hesperidin, and in guinea pigs fed (2) laboratory chow with added greens, (3) laboratory chow with greens and added rutin or hesperidin, (4) a synthetic diet with added vitamin C, with and without rutin or hesperidin, and (5) a synthetic diet devoid of ascorbic acid, with and without rutin or hesperidin. Increased susceptibility to "spontaneous" and induced hemorrhages in minute vessels proved to be unique in its association with ascorbic acid deficiency, regardless of whether the animals were fed supplements of either flavonoid. Neither gross signs of dietary deficiency nor any peripheral vascular phenomena were found that could be explained by a lack of rutin or hesperidin in the diet, or corrected by their supplementation.

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Nutritional Value of Mustard and Rape Seed Meals as Protein Source for Rats

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The feeding of livestock is gaining great impetus in the area of the State of Montana and the Province of Alberta. Although this area has an abundance of carbohydrate feed it is deficient in protein sources. In the search for potential local protein sources, it was observed that two possible crops stand out, namely, rape seed and mustard seed. Rape seed has been known for years to have some toxic factor present. An excellent review of this data has been made by Bell ('55). Mustard protein, on the other hand, has been almost neglected as a potential protein source due to the unpalatability of the meal. Feed trials have been made on preparations of doubtful purity with the results varying from satisfactory to quite unsatisfactory (Bunger, '43; Edin, '41; Jarl, '46; Nehring et al., '51; O'Neil, '48; Tretsven et al., '46). This investigation was initiated to see whether the mustard flavor could be removed from the mustard meal and to study the quality of mustard protein as compared with rape and soybean meal.

EXPERIMENTAL

The compound responsible for the "horse-radish" flavor in mustard meal is commonly called sinigrin. This is a glucoside which on hydrolysis yields potassium acid sulfate, glucose, and allyl isothiocyanate. It is the allyl isothiocyanate which is responsible for the flavor. It was observed by Goering ('59)[1] that the allyl isothiocyanate could be removed competely by an enzyme treatment.

Both solvent-extracted and expeller-treated rape seed meals were treated in a manner similar to that used on mustard using both fresh Oriental mustard seed (*Brassica juncea*) and fresh Golden rape seed (*Brassica napus*) as the sources of sinigrinase to decompose the glucosides present.

Amino acid analyses were made on both Oriental mustard seed and Golden rape seed, processed in the above manner, using the method described by Moore and Stein ('51).

Feeding trials were conducted using weanling rats of the Holtzman strain weighing between 40 and 45 gm. They were housed in cages with screen floors in a room maintained at 22°C. Four males and 4 females were used for each feed treatment.

Semipurified diets were used in which the sole protein source was the vegetable protein being tested. The various lots of protein were analyzed for nitrogen according to a modified Kjeldahl procedure (A.O.A.C., '55) and the protein calculated on the basis of N × 6.25. All diets were made up to 20% protein and to 10% fat by the addition of corn oil. In addition, all diets contained 2% of vitamin mix, 4% of salt-mineral mix and the balance of the ration was corn starch. The vitamin mix was the standard vitamin diet fortification mixture[2] and the salt-mineral mix was Salt Mixture U.S.P. 14.

DISCUSSION

When it was observed that the undesirable flavor could be removed from mustard meal, it was considered essential to run an amino assay on this material. At the same time, an assay was run on rape seed meal in order to compare these two protein

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[1] Process of obtaining proteinaceous feed material from mustard seed, rape seed and similar seeds. Belgian Patent 578,452, November 6, 1959.

[2] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Amino acid assay on enzymatically-treated mustard and rape seed meal

Amino acid[1]	Oriental mustard seed meal[2]	Golden rape seed meal[2]
Alanine	6.1	1.6
Arginine	2.1	11.4
Aspartic acid	9.7	6.8
Glutamic acid	22.8	35.5
Glycine	3.9	17.2
Histidine	1.9	5.3
Isoleucine	2.9	7.0
Leucine	3.5	4.0
Lysine	5.8	1.3
Methionine	5.7	1.3
Phenylalanine	3.3	3.1
Proline	15.6	10.0
Serine	4.5	1.4
Threonine	4.2	2.4
Tryptophan	2.6	1.8
Tyrosine	1.8	0.6
Valine	2.2	1.3

[1] Cystine, cysteine and hydroxyproline were not determined.

[2] Grams of amino acid per 100 gm of original protein on dry basis.

sources. These results are tabulated in table 1.

The high content of lysine, methionine, and tryptophan in mustard meal indicated it to be a very unusual protein. Bell ('55) reported on the composition of rape seed meal as received in private communications from three independent investigators, namely, Roche, Agrew and Wetter. The limited data reported do not agree and furthermore are different from those reported herein. This would suggest that either different varieties of rape seed have a substantial difference in composition or that environment plays a factor in amino acid content. This should be more thoroughly investigated.[3]

In order to determine whether any toxic factors were present in enzyme-treated mustard meal, a feeding experiment was set up. Since soybean meal has a trypsin inhibitor which has been crystallized by Kunitz ('47), it was decided to heat-treat one lot of mustard meal to see whether such an inhibitor was present. The heat treatment consisted of heating for one-half hour in an autoclave at 15 p.s.i. This trial consisted of feeding rats for 40 days and then continuing for an additional 35 days, the only change then being to feed unheated mustard meal to those rats which had been eating heated meal. These results are reported in table 2.

It was apparent that the heat treatment was detrimental to weight gains probably either because of the destruction of lysine or to the conversion of the protein to a more insoluble form. The response to unheated meal was quite significant indicating that the heat treatment of mustard meal was undesirable.

To study the possibility of detrimental factors being present which might influence reproduction, pregnancy tests were run comparing the females raised with mustard meal with those raised with soybean meal. Two of the 4 females receiving soybean meal died during birth of young. The other two produced a total of 14 young. The 4 females fed mustard meal produced 27 young with no deaths.

Although it is recognized that this is very limited data on which to draw any conclusions, it would appear that mustard seed meal was not detrimental for reproduction. Because enzyme treatment was effective in eliminating the irritating properties of mustard meal, it was decided to try this treatment on Golden rape seed and feed this material to rats. An experiment was run for 40 days using various blends of rape seed and soybean meal. This data is reported in table 3.

Although the gains were somewhat less than with soybean meal, it was observed that the feed efficiency was good when not more than 25% of the protein was from rape seed meal.

To check the effect of the enzyme treatment compared with untreated rape seed meal a feeding test was run for 44 days. This data is presented in table 4.

From this data it was apparent that enzyme treatment definitely improved the solvent - extracted rape seed meal. The treatment with fresh mustard seed (table 5) is essentially the same as the one involving rape seed (table 4). It will be noted that the results are approximately the same although these experiments were conducted several months apart. The use of raw mustard seed appeared to have some advantage

[3] Collaborative work with Dr. Wolff of the Northern Utilization Research Laboratory, Peoria, Illinois, indicates that the methionine content may vary within a species.

TABLE 2

Comparison of heated and unheated enzyme-treated mustard meal as protein source for rats

Sex	Protein source	First 40 days		Additional 35 days	
		Average gain	Feed/ 100 gm gains	Average gain	Feed/ 100 gm gain
		gm		*gm*	
M	Soybean	204	202	104	430
F	Control	124	330	47	735
M	Unheated	108	300	86	533
F	Mustard	82	384	40	983
M	Heated[1]	36	998	122	338
F	Mustard	36	973	64	581

[1] Rats fed heated mustard were fed unheated mustard for final 35 days.

Analysis of variance for data covering first 40 days in table 2 based on weight gain

Source	D.F.	Mean square	F value at 1% level
Sex	1	7,632	8.28
Treatment	2	33,025	6.01
Treatment × Sex	2	3,434	6.01
Remainder	18	238	

TABLE 3

Effect of increasing concentrations of enzyme-treated rape seed meal in rape seed-soybean combinations as protein sources for rats

Sex	Protein[1] source	Average gain	Feed/100 gm gain[2] Average of males and females
		gm	
M	control	235	407
F		135	
M	12.5%	222	411
F		119	
M	25%	187	404
F		106	
M	50%	146	436
F		77	
M	100%	75	610
F		44	

[1] Base was soybean meal with indicated percentage substitution of soybean by enzyme-treated solvent-extracted rape seed meal.

[2] This experiment was run with rats averaging 73 gm–beginning weight, resulting in lower efficiencies.

over rape seed as an enzyme source for the removal of toxic factors.

In order to determine whether toxic factors are involved or if the poor results observed were due to amino acid inbalance, blends of mustard and rape seed meal were made because these two protein sources have a distinctly different composition.

From the calculations made using the requirements for rats as expressed by Rose ('37), it would appear that mustard meal is adequate in all amino acids but valine. Soybean meal appears to be slightly deficient in methionine whereas rape seed is deficient in lysine, methionine and valine. A blend of 25% mustard-75% rape seed appears to be deficient in lysine,

TABLE 4

Effect of enzyme treatment on solvent-extracted rape seed meal as protein source for rats

Sex	Treatment	Average gain	Feed/100 gm gain
		gm	
M	none	—[1]	—
F		—	—
M	0.1% fresh ground rape seed	37	575
F		34	632
M	0.1% fresh ground mustard seed	69	388
F		52	491

[1]Six rats died in one week. The other two were sacrificed for observation.

TABLE 5

Effect of combinations of solvent-extracted enzyme-treated rape seed and mustard meal as protein sources for rats

Sex	Protein source	Average gain	Feed/100 gm gain
		gm	
M	100% SBM	240	280
F		140	358
M	100% MSM	200	286
F		121	397
M	75% MSM	163	301
F	25% RSM	118	367
M	50% MSM	113	335
F	50% RSM	93	394
M	25% MSM	72	399
F	75% RSM	50	533
M	100% RSM	33	680
F		24	870

Analysis of variance for data in table 5

Source	D.F.	Mean square		F value at 1% level
		Weight gain	Feed efficiency	
Sex	1	23,586	203,450	7.39
Treatment	5	28,975	326,202	3.58
Treatment × Sex	5	2,221	19,664	3.58
Remainder	36	121	18,490	

methionine and valine whereas the 75% mustard-25% rape blend is deficient only in valine.

The data from the feeding trials with these blends are reported in table 5.

From these observations it was apparent that factors other than amino acid content were responsible for the poor results with rape seed meal even when some of the toxic factors were removd by enzyme treatment. Although mustard meal was very close to soybean meal in promoting rate of gain and feed efficiency, with increasing content of rape seed both rate of gain and efficiency dropped off.

It would appear that a deficiency in valine might be the reason for the slightly lower biological value of mustard meal as compared with soybean meal. If this is true, then blending mustard meal with a cereal protein high in this acid or mixing with soybean meal would give good results. In view of the fact that solvent-extracted rape seed meal showed some improvement

TABLE 6

Effect of treatment on rape seed and mustard meal as protein source for rats

Treatment[1]	Sex	Average gain	Feed/100 gm gain
		gm	
Soybean control	M	188	257
	F	132	332
Expeller rape seed meal control	M	42	627
	F	35	699
½ SBM + ½ PMSM	M	189	248
	F	130	329
½ SBM + ½ PERSM	M	125	325
	F	97	396
½ SBM + ½ PSERSM	M	148	296
	F	99	412
PERSM with water from enzyme treatment discarded	M	109	366
	F	118	336
SBM + Water from enzyme treated ERSM	M	145	345
	F	123	360
SBM + Water from enzyme treated SERSM	M	121	377
	F	119	374
SBM + Water from ERSM without enzyme treatment	M	159	298
	F	126	362
SBM + Water from SERSM without enzyme treatment	M	155	314
	F	108	422
SBM + Water from enzyme treated ERSM after treating with growing yeast	M	143	314
	F	110	367

[1] SBM, soybean meal; ERSM, expeller rape seed meal; SERSM, solvent-extracted rape seed meal; PERSM, processed expeller rape seed meal; PMSM, processed mustard seed meal and PSERSM, processed solvent extracted rape seed meal.

and since most of the rape seed processed in this area is the expeller type, it was decided to see whether enzyme treatment could improve this type of meal. An experiment was set up to test both of these factors with one group of rats. The treatments for each group and the results are expressed in table 6.

From this data the following conclusions were reached:

1. Mustard meal in a 50% combination with soybean meal showed improved results over the soybean meal alone. This would be expected on the basis of the amino acid analysis as long as other toxic factors were not present. It is possible that a 75% mustard-25% soybean meal ration would show even more satisfactory results or that combinations of mustard meal and other cereal proteins would give good results.

2. Expeller rape seed meal was a very unsatisfactory protein source as the rats fed this meal made no appreciable gain and appeared starved. Unfortunately, due to lack of facilities a solvent-treated rape seed control was not run. However, from the basis of previous data it would appear that solvent-treated meal is superior to expeller meal as the gains observed were about double using the solvent-treated type.

3. On comparing a mixture of 50% soybean and 50% processed expeller rape with 50% soybean and 50% processed solvent-extracted rape, a 15% increase in growth and 10% increase in feed efficiency was observed in favor of the solvent treated material. Both of these samples had the water extract added back, a procedure which in the light of these experiments would seem undesirable.

4. The processed expeller rape seed meal which did not have the process water added back to it was considerably better than the other meal. However, an interesting observation was that the males gained less than the females. The female weights were only about 10% below the weight of the control samples. This would infer that some hormone disturbance was caused by this factor in the process water. If this type of meal was blended with soybean meal satisfactory results might be obtained.

5. The process water removed from expeller rape seed meal was added back to soybean meal to determine whether any factors were present which would be detrimental to the feed. It was observed that the rate of gain for the males was reduced by 35.6% and the feed efficiency reduced 47.3%. For the females these figures are 10 and 12.7% respectively. A straight water extract without the usual process treatment was not quite as effective in removing these factors. Here the reduction in gain was 15.4 and 4.5%, respectively, for males and females. Likewise, there was less reduction in feed efficiency as evidenced by the figures of 16.0 and 9.1%, respectively, for males and females. This same difference in the effect of rape seed meal on males and females was observed by Bell ('55) when working with mice. This indicated that an appreciable amount of toxic material was removed by enzyme treatment which was not removed by a simple water extraction.

6. Autopsies were made on these rats. The soybean meal control and the mustard-soybean blend appeared normal. All rats having rape seed or water extract from rape seed showed mottled thymus. No other abnormal conditions could be observed. The thyroids appeared normal which is in contradiction to results reported in the literature (Bell, '55). Experiments are now under way to check the possibility of producing a satisfactory expeller rape seed meal by enzyme treatment.

SUMMARY

Mustard seed meal has been successfully treated to remove the sharp flavor, producing a very bland material. This meal appeared to be a satisfactory protein source for rats as no toxic factors were in evidence even when this material was used as a sole protein source for two generations. This protein is high in lysine, methionine, threonine and tryptophan. Being to some degree deficient in valine it was most satisfactory when blended with proteins containing adequate amounts of this amino acid. A 50% blend of mustard and soybean meal gave equal gains and better feed efficiency than straight soybean meal. Rape seed meal is quite inferior to mustard meal as a protein source. Enzyme treatment improved rape seed meal but the toxic factors appeared to concentrate in the liquid from the enzyme treatment. By discarding this fraction it might be possible to produce a satisfactory protein especially when blended with other protein sources. Experiments are now under way to test this thesis.

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Manganese Metabolism in College Women[1]

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The essentiality of manganese in trace amounts has been demonstrated for the rat, rabbit, mouse and chick. Because of its established role in enzymatic reactions, it is to be expected that this element also is required by higher animals including man. Extremely few studies have been undertaken, however, to determine the manganese requirement of human subjects. Everson and Daniels ('34), as a result of balance studies on 7 children ranging in age from 3 to 5 years, suggested that 0.2 to 0.3 mg of manganese per kilogram of body weight was required for normal development. Basu and Malackar ('40), based on a study of three adult male subjects, estimated that a mean daily intake of 4.6 mg was required for manganese equilibrium. No balance studies employing adult women as subjects have been noted in the literature.

Underwood ('53) estimated that adults receiving typical Australian diets had intakes of 6 to 8 mg of manganese daily and Monier-Williams ('49) calculated the manganese intake of adults using a typical winter diet in England as 7 mg. The average daily intake of two adults was found by Kent and McCance ('41) to be 2.2 to 2.7 mg when 40 to 50% of their calories were derived from white flour and 8.5 to 8.8 mg when the same percentage of calories was derived from 92% extraction flour.

Plant tissues have been found to be much more variable in manganese content than animal tissues. Richards ('30) and Hodges and Peterson ('31) reported that not only did the manganese content vary from plant to plant, but differed markedly in various tissues even within the same plant. On the other hand, animal tissues were relatively constant in manganese content. Furthermore, Gilbert ('57) noted that soil from different locations in the same field may vary as much as several hundred parts per million in this element. Hopkins and Eisen ('59) showed that the manganese content of fresh vegetables from different geographic areas varied widely depending on the locality of production, season of year and possibly other factors. Carrots, for example, ranged from 0.06 to 0.83 mg per 100 gm; celery from 0.03 to 0.98 mg.

The present investigation was undertaken for the purpose of determining the manganese intake and retention of young adult women receiving a carefully controlled diet. A balance study in which a diet providing two levels of protein intake was fed presented the opportunity of simultaneously investigating manganese metabolism in these young women.

PROCEDURE

The study extended for 41 days and consisted of eight 5-day periods and one post-experimental day. The subjects were 9 healthy college women, ranging in age from 18 to 21 years and in weight from 45.2 to 74.2 kg. Four of the subjects received a low-protein diet (37 gm of protein per day) during the first 4 periods and 5 a high-protein diet (76 gm of protein per day). At the beginning of the 5th period the subjects using the low-protein diet were transferred to the higher level, and those receiving the high-protein diet to the lower level.

Five menus, planned for the two levels of protein intake, were repeated for each of the eight 5-day balance periods. A typical day's menu is given in table 1. To meet the needs of those subjects whose energy requirement exceeded the amount provided in the basal diet, certain foods

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TABLE 1

Typical day's menus

Food	Diet	
	Low protein	High protein
	gm	*gm*
Breakfast		
Orange juice	100	100
Cream of Wheat, regular	125	100
Raisins	10	10
Cream, light	60	55
Canadian bacon (raw weight)	—	40
Bread, white	25	25
Butter	6	6
Jelly	10	—
Lunch		
Cheese, cheddar	15	15
Tomato soup	200	—
Beef patty (raw weight)	—	80
Salad		
Cabbage	30	30
Orange	50	50
Dates	30	—
Peanuts	—	15
Marshmallow	8	8
Lettuce, head	10	10
Mayonnaise	14	14
Strawberries, frozen	75	50
Baked meringue		
Egg white	—	16
Sugar	—	24
Bread, white	25	25
Butter	12	6
Dinner		
Ham (cooked weight)	50	100
Sweet potato, candied	100	100
Asparagus, frozen	80	80
Butter	6	6
Lettuce, head	40	40
French dressing	11	11
Jello	100	100
Banana	25	30
Whipped cream	16	20
Bread, white	25	25
Butter	6	6

were allowed ad libitum. These included sugar, candy mints, candied orange peel and especially prepared cookies. Tea, coffee and sodium chloride also were unrestricted. A record was kept for each subject of the amounts consumed of these ad libitum foods. Thus, the basal diet plus the ad libitum foods gave a mean daily caloric intake of 2200 Cal. (range, 2125 to 2300 Cal.). Essentially no change in weight occurred in any subject throughout the study since these young women showed no more than the usual fluctuations in weight from period to period. Distilled water was used in the preparation of the foods and for drinking purposes.

To adjust the level of phosphorus intake with the low-protein diet to that of the high-protein diet, at each meal the subjects receiving the low protein diet were given 1.07 gm of sodium glycerophosphate (equivalent to 325 mg of phosphorus per day). Vitamin D (400 I.U.) was included in the noon meal of all subjects.

Food composites for analysis were prepared for each experimental period by weighing an amount equivalent to one fifth of each serving of food at the time the foods were weighed for the subjects' meals. A torsion balance was used for all weighings.

Total fecal and urine collections were made by the subjects throughout the study. Acid digests of the food composites and of the 5-day fecal composites for each subject were prepared (Leichsenring et al., '58) and an aliquot was retained for analysis. The 24-hour urine collections for each subject were measured and one fifth of the total volume was retained and acidified with concentrated HCl, using 10% of the volume of the aliquot. At the end of each period, the acidified urine composite for each subject was mixed well and an aliquot was retained for analysis.

Approximately 25-gm samples of the acid digests of the foods and 10-gm samples of those of the feces were weighed and dry-ashed at 550°C in tared silica crucibles in quadruplicate and in triplicate, respectively. One-hundred-milliliter samples of the urine composites were dry-ashed in triplicate in new procelain evaporating dishes. The ash was acidified with HCl and made up to 100 ml.

To obtain a color which could be read accurately in a Bausch and Lomb Spectronic 20 colorimeter, it was necessary to concentrate the food and urine ash solutions. During re-evaporation on a steam bath two ml of concentrated HNO_3 were added to drive off the chlorides (Sandell, '50). The ash solution was then transferred to 15-ml centrifuge tubes in which the color was developed using a modification of the periodate method of the Association of Official Agricultural Chemists ('55) and Milton ('55). Sulfuric acid was not used in the oxidation of the manganese to permanganate since it was noted that color development was less satisfactory

when this acid was added during the oxidative phase.

RESULTS AND DISCUSSION

The mean daily manganese content of the basal diet is given in table 2. In spite of great care in the selection of the foods and in the preparation of the food composites, considerable variation in the manganese content occurred from period to period. In view of the variability of plant products in this element this is perhaps not unexpected. To insure maximum uniformity in the composition of the diets, certain frozen fruits and vegetables were secured in one lot at the beginning of the study.[2] These included peas, corn, asparagus, strawberries and orange juice. Other fruits and vegetables (potatoes, lettuce, celery, cabbage, carrots, green peppers, apples, onions, oranges and bananas) were purchased in the local market in such quantities as storage facilities would permit.

In general, there was fair agreement between the values for the high- and low-protein diets. In those instances in which differences were appreciable, presumably they occurred in the sampling of the foods for the food composite. For example, a sample wedge taken from a head of lettuce may contain a certain proportion of leaves and core at one time and a somewhat different proportion at another time. Because of the variations in manganese content in plant tissues, one would expect differences in intake even in a controlled diet. The mean value for the 8 low-protein food composites was 3.23 mg of manganese and for the 8 high-protein, 3.08 mg (table 2). Since this difference is not significant, it is evident that the protein level was not a determining factor in the manganese content of the diets and, in consequence, in the discussion which follows, the mean values of the high- and low-protein diets will be used.

TABLE 2

Mean daily manganese content of the basal diet

Period	Low protein	High protein	Mean
	mg	*mg*	*mg*
1	2.70	3.29	3.00
2	2.63	2.83	2.73
3	2.71	2.54	2.63
4	2.21	2.33	2.27
5	2.94	3.04	2.99
6	4.29	2.57	3.43
7	4.68	3.62	4.15
8	3.65	4.40	4.02
Mean	3.23	3.08	3.16

The results of the analyses of the ad libitum foods were: tea per cup, 0.29 mg; coffee per cup, 0.05 mg; ranger cookies, 0.23 mg per 20 grams; ice box cookies, 0.10 mg per 20 grams. These results show that the tea and coffee contained appreciable amounts of manganese, as has been reported by Coleman and Gilbert ('39) and Monier-Williams ('49). Consequently, these beverages, especially tea, contributed significant amounts of this element to the intake of those subjects who used them.

During the 40 days of observation, the mean daily manganese intake of the 9 subjects was 3.70 mg (range, 3.26 to 4.22 mg) a value which is on the low side of the estimated intakes reported (table 3). Although the number of servings of fruits and vegetables in the diet was generous, the fact that only white bread was included may account for the relatively low manganese content. Cotzias ('58) has stated that, as a rule, low values are probably more representative of the actual manganese content of food than some of the higher values reported in the literature.

According to Greenberg and Campbell ('40) manganese is poorly absorbed and poorly stored in the body. For the subjects in the present study total manganese excretion (fecal plus urinary) amounted to a mean of 2.17 mg or 59% of the intake, of which 91% was in the feces. Thus, there was a mean retention of 1.54 mg or 41% of the intake. For the individual subjects mean retentions ranged from 1.30 to 1.78 mg per day or 37 to 46% of intake. These values suggest that the young women were still growing at a rate sufficient to require a greater amount of manganese than the mature adult. It is also possible that the observed retentions may have been due to the repletion of body reserves of this element or that they represented losses through the skin, hair and nails which were not determined.

Since these subjects varied considerably in body weight, in order to determine

[2] The authors wish to express their appreciation to the Birdseye Division of General Foods Corporation for their contribution of these products.

TABLE 3

Mean daily manganese intake, excretion and retention of nine young women for eight 5-day periods

Diet	Subject (weight)	Period	Intake	Excretion		Retention
				Fecal	Urinary	
			mg	*mg*	*mg*	*mg*
1[1]	A	1	2.91	1.08	0.12	1.71
	(52.4 kg)	2	2.89	1.71	0.19	0.99
		3	2.89	1.71	0.14	1.04
		4	2.67	2.20	0.14	0.33
2[2]		5	3.24	1.55	0.19	1.50
		6	3.03	1.45	0.17	1.41
		7	3.92	2.04	0.20	1.68
		8	4.52	2.20	0.27	2.05
		Mean	3.26	1.74	0.18	1.34
1	B	1	3.13	0.73	0.16	2.24
	(54.9 kg)	2	3.11	2.63	0.00	0.48
		3	3.11	1.55	0.23	1.33
		4	2.99	1.96	0.16	0.87
2		5	3.54	2.62	0.45	0.47
		6	3.20	1.68	0.18	1.34
		7	4.11	2.44	0.30	1.37
		8	4.93	2.37	0.18	2.38
		Mean	3.51	2.00	0.21	1.30
1	D	1	3.30	1.38	0.00	1.92
	(54.5 kg)	2	3.40	1.61	0.27	1.52
		3	3.32	1.65	0.20	1.47
		4	2.91	2.45	0.24	0.22
2		5	3.65	1.61	0.19	1.85
		6	3.33	2.26	0.25	0.82
		7	4.21	2.05	0.22	1.94
		8	5.02	2.80	0.24	1.98
		Mean	3.64	1.98	0.20	1.46
1	E	1	3.37	1.68	0.20	1.49
	(45.2 kg)	2	3.51	1.60	0.00	1.91
		3	3.51	1.47	0.22	1.82
		4	3.14	1.20	0.26	1.68
2		5	3.77	3.84	0.12	−0.19
		6	3.62	1.89	0.22	1.51
		7	4.45	2.61	0.43	1.41
		8	5.00	2.70	0.18	2.12
		Mean	3.80	2.12	0.20	1.46
2	C	1	3.42	1.05	0.24	2.13
	(51.4 kg)	2	3.18	2.41	0.15	0.62
		3	2.75	1.52	0.16	1.07
		4	2.88	1.86	0.27	0.75
1		5	3.19	1.39	0.00	1.80
		6	4.82	2.55	0.17	2.10
		7	5.00	2.12	0.13	2.75
		8	4.09	1.50	0.27	2.32
		Mean	3.67	1.80	0.17	1.70
2	F	1	4.09	2.01	0.27	1.81
	(50.7 kg)	2	3.94	1.44	0.20	2.30
		3	3.30	3.14	0.25	−0.09
		4	3.49	2.02	0.34	1.13
1		5	3.67	1.98	0.14	1.55
		6	5.28	2.84	0.20	2.24
		7	5.57	2.29	0.22	3.06
		8	4.48	2.15	0.11	2.22
		Mean	4.22	2.23	0.22	1.78

TABLE 3 (*Continued*)

Mean daily manganese intake, excretion and retention of nine young women for eight 5-day periods

Diet	Subject (weight)	Period	Intake	Excretion		Retention
				Fecal	Urinary	
			mg	*mg*	*mg*	*mg*
2	G	1	3.51	1.36	0.00	2.15
	(57.4 kg)	2	3.16	1.52	0.19	1.45
		3	2.80	1.16	0.16	1.48
		4	2.73	1.85	0.32	0.56
1		5	3.24	2.13	0.15	0.96
		6	4.79	2.33	0.21	2.25
		7	5.10	2.55	0.24	2.31
		8	3.99	2.27	0.00	1.72
		Mean	3.66	1.90	0.16	1.61
2	H	1	3.78	1.69	0.20	1.89
	(62.4 kg)	2	3.51	1.47	0.19	1.85
		3	3.13	2.22	0.20	0.71
		4	2.98	2.03	0.27	0.68
		5	3.63	2.20	0.00	1.43
		6	5.28	2.53	0.17	2.58
		7	5.15	2.51	0.23	2.41
		8	4.28	3.50	0.22	0.56
		Mean	3.96	2.27	0.19	1.51
2	I	1	3.51	1.76	0.20	1.55
	(74.2 kg)	2	3.25	0.97	0.38	1.90
		3	2.83	1.39	0.32	1.12
		4	2.46	1.70	0.36	0.40
1		5	3.21	2.09	0.29	0.83
		6	4.70	2.06	0.33	2.31
		7	5.20	1.52	0.00	3.68
		8	3.86	2.17	0.00	1.69
		Mean	3.62	1.71	0.24	1.68
Mean of all			3.70	1.97	0.20	1.54

[1] Low-protein diet.
[2] High-protein diet.

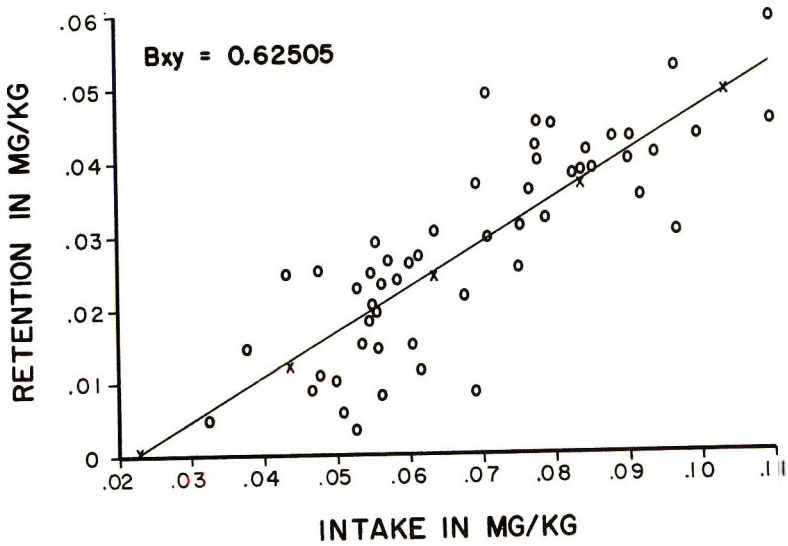

Fig. 1 The regression of manganese retention on manganese intake. One value for one individual, intake 0.065 mg per kg and retention −0.0017 mg per kg, was omitted from the figure, but the values were included in the calculation of the regression coefficient.

whether there was evidence that the amount of manganese retained was associated with the body weights of the subjects, intakes and retentions, expressed as milligrams per kilogram of body weight per day, were computed. These calculations showed that the mean intake for these subjects was 0.068 mg per kilogram with a range from 0.049 to 0.084 mg. The mean retention was 0.028 mg per kg; the range from 0.023 to 0.035 mg. Manganese retention based on the data for the 5-day periods was significantly related to intake as shown by a highly significant coefficient of correlation ($R = 0.801$; $P = < 0.01$). In these calculations the data for periods 1 and 5 were omitted since they were considered adjustment periods. At the intake levels used in this study, all balances were positive except for two subjects during one 5-day period each. These two negative balances appeared to be the result of incomplete separation of the carmine-marked fecal specimens between two consecutive periods.

The regression coefficient of manganese retention on manganese intake also was calculated (figure 1). The observed linear regression suggests that a somewhat higher manganese intake might have resulted in even greater retentions. Further investigations are planned to test the effect of higher manganese intake levels on the manganese retention of young women and to determine, if possible, the intake level at which no further increases in retention are noted.

SUMMARY

Manganese metabolism was studied in 9 healthy college women ranging in age from 18 to 21 years. A basal diet supplied subjects with adequate amounts of all essential nutrients. To provide additional food energy for those subjects whose requirements exceeded the amount included in the basal diet, certain foods were allowed ad libitum, and a record kept of their consumption.

The study consisted of eight 5-day periods, with one post-experimental day, making a total of 41 days.

Composites of the basal diet and of the fecal and urine specimens for each 5-day period were analyzed for manganese. The ad libitum foods also were sampled and analyzed. The results of the analyses showed that the basal diet provided a mean daily intake of 3.16 mg of manganese with some variation from period to period. The manganese intake for the 9 subjects, (basal diet plus ad libitum foods) was 3.70 mg per day.

The results of the analyses of the urine and fecal specimens gave a mean daily manganese excretion for all subjects of 2.17 mg, of which 91% was in the feces. The mean daily retention of the subjects was 1.54 mg or 41% of the intake.

Manganese intakes and retentions also were computed for the subjects on the basis of body weight. The mean daily intake was 0.068 mg per kg (range, 0.049 to 0.084 mg) and the mean retention, 0.028 mg per kg (range, 0.023 to 0.035 mg). For these subjects manganese retention was significantly related to intake, based on the data for the 5-day periods. The regression coefficient also was computed on these data. A linear relationship between intake and retention was observed. It is possible that intake levels higher than those used in this study would have resulted in greater retentions.

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The Influence of Dietary Factors upon the Composition of Mineralized Tissues and upon the Susceptibility of Enamel to Erosion in vivo

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During the last 10 years evidence has been accumulating at a fast rate that the mineral composition of the diet plays an important role in the formation of mineralized tissues, and in the susceptibility of enamel to erosion and to formation of caries. Variations in the dietary ratios of calcium to phosphorus have been found to affect the calcium, phosphorus and carbonate content of bone, dentin and enamel of both rats and cotton rats (Armstrong, '52; Bethke et al., '32; Dymsza et al., '59; Likins et al., '58; Sobel and Hanok, '58; Steel, '60). A decrease in the incidence and severity of dental caries has been observed in experimental animals receiving cariogenic diets supplemented by certain phosphate salts (Barnard and Johansen, '58; McClure and Muller, '59; Nizel and Harris, '60; Van Reen and Glassford, '59; Wynn et al., '56, '57).

Erosion of enamel *in vivo* has been produced by acidic fluids at a pH of 3.5 or lower (McCay and Will, '49), by lactate at pH 4.5 and by citrate at both pH 6.3 (Zipkin and McClure, '49) and pH 5.0 to 6.0 (Hills and Sullivan, '58). Enamels with a higher carbonate content have been found to dissolve at a faster rate in acidic solution *in vitro* (Sobel, '55). With the exception of the fluoride ion and sucrose, the role of dietary constituents on susceptibility of dental enamel to erosion *in vivo* has received little attention.

Fruit juice and acidified beverages have been credited for lingual erosion of teeth in humans (Holloway et al., '58). The extent to which the results from erosion studies with experimental animals can be applied to erosion problems in man is uncertain at the present time.

The purpose of the studies reported here was to determine the interrelationship between certain dietary constituents and the resistance of enamel *in vivo* to the erosive action of commonly consumed, acidic fluids.

EXPERIMENTAL

In a preliminary experiment, series 1, littermate, weaning, male rats of the Sherman strain were divided into two groups and fed either the high-calcium or high-phosphorous diet which Sobel and Hanok ('48) designed for their study of caries by adding either calcium carbonate or disodium phosphate to a basal diet. The two groups were subdivided equally and given either distilled water or the water replaced three times weekly by 25 ml per rat of a cola beverage. There were 4 rats fed each of the dietary combinations hereafter designated as High-Ca-W, High-P-W, High-Ca-C and High-P-C (W for water and C for cola). They were caged in pairs and fed ad libitum. The body weight, food consumption and fluid consumption were recorded weekly.

At the end of a 40-day experimental period the rats were chloroformed. The jaws were boiled for 15 minutes in distilled water and then cleaned. The molars were scored for erosion by the method of Re-

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starski et al. ('45). The same two molars from the lower jaw of each rat were dissolved, as pairs, in 5 ml of 2 N HCl at room temperature for 24 hours. After bringing the filtered solution to 50 ml with distilled water the calcium content was determined in 20-ml aliquots by the oxalate method (Kolthoff and Sandell, '52) and the phosphorus content in 0.8-ml aliquots by the colorimetric method (Fiske and Subbarow, '25).

A more refined experiment, series 2, was then undertaken in which a highly purified diet and a larger number of animals were used. Littermate, weanling rats of the Wistar strain were distributed according to weight between two diets and given one of three fluid combinations. The basal diet, which was low in phosphorus, consisted, in per cent, of white, technical dextrin, 79.1; vitamin-free casein, 9.0; cystine, 0.3; tryptophan, 0.1; threonine, 0.15; liver extract (concentrate, 1:20), 0.5; cod liver oil, 1.0; hydrogenated vegetable fat,[3] 4.0; cellulose powder, 1.7; salt mixture (Feaster, '53), 4.0; choline chloride, 0.1; and vitamin mixture (Frost et al., '52), 0.05. For the high-phosphate diet 1.25 gm of the dextrin was replaced by an equal quantity of monosodium phosphate. By analysis the two diets contained a percentage of phosphorus of 0.09 and 0.25 and a Ca:P ratio of 4.9 and 1.8 by weight and 3.8 and 1.4 by moles. The high-phosphorus diet contained calcium, phosphorus and Ca:P levels within the normal range (Gaunt and Irving, '40; Wynn et al., '56); the low-phosphorus diet contained an adequate level of calcium but an abnormal ratio of Ca:P. The level of phosphorus in the latter diet was lower than the 0.124% used by Sobel and Hanok ('48) or the 0.25% by Wynn et al. ('56) but was higher than the 0.018% used by Zipkin et al. ('59).

The three fluids used were distilled water ad libitum; distilled water replaced three times weekly by 20 ml of a commercially-canned, orange juice, unsweetened, pH 3.7 to 3.8; or distilled water replaced by 20 ml of the orange juice previously neutralized to pH 6.0 by the addition of solid, C.P., sodium carbonate. The fluids were dispensed from glass drinking bottles. Food consumption was equalized within litters. There were 6 dietary combinations: High-P-W, High-P-OJ, High-P-NOJ, Low-P-W, Low-P-OJ, and Low-P-NOJ (W for water, OJ for orange juice, NOJ for neutralized orange juice).

In this series 2 there were two groups of littermate rats: group A contained 8 rats, fed each of the 6 dietary combinations; group B, composed of litters with much higher initial weights, contained 4 rats each receiving the High-P-W, High-P-OJ, Low-P-W and Low-P-OJ combinations.

At the end of an 8-week experimental period the rats were chloroformed and the head and right tibia autoclaved at 5 pounds pressure for 15 minutes. The jaws were cleaned and the lower molars given scores for the extent of erosion as in series 1. The jaws and tibia were immersed in neutral, 80%, ethyl alcohol for 24 hours, drained, immersed in diethyl ether for another 24-hour period and then oven-dried for one hour at 95 to 100°C. The 6 lower molars of each rat were pooled, ground to pass through a 100-mesh screen and separated into dentin and enamel fractions (Brekhus and Armstrong, '35). The whole tibias were ground to the same fineness.

Four analyses were made on each powder sample, in duplicate for dentin and tibia, on samples ranging from 3 to 12 mg. Glass distilled water was used throughout. The carbonate content of each sample was determined manometrically as carbon dioxide (Deakin and Burt, '44) first at 25°, later at 30°, after shaking for one hour. It was found convenient to weigh the powdered sample, previously dried at 100° for 30 minutes, into a small glass cup which was slipped into the side arm of the Warburg flask. The solubility factor for CO_2 in 2 N HCl was used in calculating the flask constant.

The contents of the Warburg flask were filtered into a 25 ml-volumetric flask and, after repeated washings, brought to volume with water. A 1-ml aliquot was removed for the determination of phosphorus as in series 1.

The remaining 24 ml were washed into a 50-ml beaker, concentrated to about 10 ml on a hot plate, and adjusted to pH 6.0 by the successive addition of 40%, 20%

[3] Fluffo, Procter and Gamble.

and very dilute NaOH. The phosphate ions were removed by allowing this solution to flow through a Dowex-1-X4 (Cl^-, 100 to 200 mesh) column, 9 by 250 mm, under gravity. It was found necessary to concentrate the effluent, readjust to pH 6.0, and re-run it through the column in order to remove all of the interfering phosphate ions. About 30 ml of distilled water were used to wash the solutions through the column.

The second effluent and combined washings were concentrated on a hot plate to about 15 ml and adjusted to pH 12.5 with NaOH. The calcium and magnesium contents were determined by the Hildebrand modification (Hildebrand and Reilley, '57) of the Lott and Cheng ('57) method.

The accuracy of the methods was checked by testing samples of appropriate, C.P. salts by the same procedures of analysis. The carbonate content of 38 trials of $CaCO_3$ was 92.5% of theory and checked with the 92.6% found by Deakin and Burt ('44) without using the solubility factor. The recovery of magnesium and of calcium from the Dowex-1 column was found to be 98.9 and 98.7% respectively.

Aliquots of 4 bone and dentin samples were ashed at 500°C, dissolved in 2 N HCl and analyzed for calcium, magnesium and phosphorus as before. No measurable differences were found between these results and those for the corresponding, nonashed samples.

RESULTS

In series 1 the consumption of a cola beverage caused an erosion of the enamel which was readily seen under low power magnification. The extent of erosion, as measured by the Restarski scale, in the 6 molars was somewhat greater for the diet supplemented with the phosphate salt than that with calcium salt and showed, therefore, a dependence both upon the kind of fluid and the mineral composition of the diet.

The percentage levels of calcium and of phosphorus in the two teeth analyzed were very similar (table 1) and difficult to evaluate since they represented both dentin and enamel which varied in their proportion to each other according to the extent of the erosion. The molars from the High-P-C dietary combination had a higher level of phosphorus and consequently a lower Ca:P ratio. The growth response was much lower for the phosphate supplementation than for the calcium supplementation.

The Low-P diets in series 2 were theoretically at a ricketic level for phosphorus and in a few instances produced malformed tibia. The addition of a phosphate salt to this basal diet to give a High-P diet had a beneficial effect upon growth and the weight of mineralized tissues (table 2). The average gain in weight per week, length of tibia, weight of tibia, and total weight of the lower molars when using the High-P diets were statistically higher than the corresponding values with the Low-P diets for the rats in group A (P for W-groups < 0.1, for OJ < 0.2, for NOJ < 0.05; P for W < 0.01, for OJ and NOJ < 0.05; P for W, OJ and NOJ < 0.001; P for W, OJ and NOJ < 0.05). The heavier weight of the 6 molars was an accumulation of the heavier weights for each of the individual teeth. In group B only the weight of the tibia was significantly higher (P for W < 0.1; P for OJ < 0.001) due, probably, to a much higher starting weight of the rats.

The orange juice was an additional source of mineral elements and by analysis

TABLE 1

Effect of diet upon growth and composition of teeth during in vivo erosion (series 1)

Diets[1]	High-Ca-C	High-P-C	High-Ca-W	High-P-W
Weight gain, gm/40 days	54 ± 8[2]	22 ± 5	61 ± 5	33 ± 3
Erosion score	1.0	1.5	0.2	0.2
Calcium in molars, %	27.8 ± 0.5	28.0 ± 0.3	28.2 ± 0.4	27.1 ± 0.8
Phosphorus in molars, %	14.0 ± 0.2	17.0 ± 1.0	14.8 ± 0.4	14.8 ± 0.2
Ca:P, moles	1.54	1.28	1.46	1.42

[1] Symbols used in diet descriptions indicate the following: Ca, calcium; P, phosphorus; C, cola; W, water; OJ, orange juice; NOJ, neutralized orange juice.

[2] Mean ± mean deviation.

TABLE 2

Effect of diet upon the growth and the weight of mineralized tissues (series 2)

Diets[1]	Group	Low-P-W	Low-P-OJ	Low-P-NOJ	High-P-W	High-P-OJ	High-P-NOJ
Weight gain, gm/week	A	16 ± 1[2]	17 ± 1	18 ± 1	20 ± 1	22 ± 1	20 ± 1
	B	24 ± 2	23 ± 3	—	28 ± 2	25 ± 3	—
Length of tibia, mm	A	30 ± 0.7	31 ± 0.7	31 ± 0.8	33 ± 0.6	33 ± 0.6	33 ± 0.3
	B	36 ± 3	36 ± 1	—	37 ± 1	37 ± 1	—
Weight of tibia, mg	A	106 ± 10	121 ± 11	122 ± 12	223 ± 13	213 ± 7	215 ± 10
	B	273 ± 15	270 ± 15	—	334 ± 20	338 ± 8	—
Weight of 6 molars, mg	A	68.0 ± 1.5	68.9 ± 1.6	69.9 ± 1.1	73.5 ± 1.5	75.0 ± 1.6	74.8 ± 1.4
	B	79.4 ± 2.8	77.9 ± 2.3	—	80.8 ± 1.3	77.9 ± 2.9	—
Erosion score	A	0.5 ± 0.1	1.0 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	3.2 ± 0.6	0.4 ± 0.1
	B	0.2 ± 0.2	2.4 ± 0.5	—	0.3 ± 0.2	5.8 ± 0.1	—

[1] See footnote 1, table 1, for description of diet symbols.
[2] Mean ± mean deviation.

increased the phosphorus, calcium and magnesium intake by one to two per cent, respectively. The beneficial effect upon the rats seemed to be minimal since the only differences observed between the water and orange juice combinations of diet were with the Low-P diets but these differences were statistically significant only for the length of the tibia (P for W versus OJ and for W versus NOJ < 0.2).

Erosion of enamel with variable exposure of dentin was observed whenever orange juice was provided as the fluid but not when water or neutralized orange juice was provided. The results confirmed those of Restarski et al. ('45) that a pH 3.5, or lower, was required for *in vivo* erosion.

A difference in the degree of erosion was found for the two types of diet, a higher degree occurring on the High-P-OJ combination for both groups A and B (for group A the P for Low-P-OJ versus High-P-OJ < 0.01; P for Low-P-OJ versus Low-P-W < 0.05; P for High-P-OJ versus High-P-W < 0.01; and for group B all P values < 0.001). These results demonstrated what had been indicated in series 1, namely, that the enamel was eroded to a greater degree by an acidic beverage when the diet was enriched with a phosphate salt with a resultant decrease in the Ca:P ratio.

The supplementation of the basal diet with a phosphate salt affected the chemical composition of both the tibia and the dentin (table 3). For the tibia the percentages of calcium, of phosphorous and of carbonate were higher for each of the High-P diets than those of the Low-P diets with the same kind of fluid intake (calcium-P for W < 0.05, for OJ < 0.01 and for NOJ < 0.1; phosphorus-P for W < 0.2, for OJ < 0.01 and for NOJ < 0.1; carbonate-P for W < 0.01, for OJ < 0.2 and for NOJ < 0.01). The Ca:P ratios were generally the same for all of the dietary combinations but the $Ca:CO_3$ and $PO_4:2CO_3$ ratios were higher on the Low-P diets with the exception of the Low-P-OJ dietary combinations. There seemed to be no consistent differences between the values found for the W and OJ groups. The nutritional effect of the orange juice, at the levels provided in this study, again seemed to be negligible.

TABLE 3

Effect of diet upon the composition of mineralized tissues during in vivo erosion (series 2)

Diets[1]		Low-P-W	Low-P-OJ	Low-P-NOJ	High-P-W	High-P-OJ	High-P-NOJ
Ca, %	Enamel	24.2 ± 1.4[2]	23.5 ± 0.7	27.8 ± 1.3	24.0 ± 1.9	24.7 ± 1.2	26.7 ± 0.7
	Dentin	19.1 ± 0.3	18.8 ± 0.5	19.4 ± 0.5	19.7 ± 0.6	19.5 ± 0.6	19.6 ± 0.4
	Tibia	16.3 ± 0.4	16.2 ± 0.6	17.4 ± 1.0	18.1 ± 0.6	19.3 ± 0.8	20.2 ± 0.8
Mg, %	Enamel	1.22 ± 0.15	1.09 ± 0.22	0.87 ± 0.10	1.04 ± 0.26	1.15 ± 0.24	1.29 ± 0.11
	Dentin	1.21 ± 0.08	0.86 ± 0.07	1.16 ± 0.12	1.06 ± 0.11	0.80 ± 0.07	1.25 ± 0.13
	Tibia	1.10 ± 0.13	1.03 ± 0.07	1.05 ± 0.11	1.22 ± 0.10	1.12 ± 0.09	1.01 ± 0.10
P, %	Enamel	14.5 ± 0.3	14.8 ± 0.2	15.0 ± 0.2	14.4 ± 0.5	14.6 ± 0.8	15.2 ± 0.2
	Dentin	12.2 ± 0.2	11.9 ± 0.2	11.4 ± 0.3	12.5 ± 0.2	11.9 ± 0.3	12.1 ± 0.4
	Tibia	10.0 ± 0.4	9.4 ± 0.4	9.8 ± 0.5	10.9 ± 0.3	11.5 ± 0.1	11.0 ± 0.4
CO_3, %	Enamel	2.86 ± 0.20	2.65 ± 0.11	2.74 ± 0.08	2.84 ± 0.12	2.19 ± 0.10	2.49 ± 0.08
	Dentin	2.95 ± 0.07	2.88 ± 0.16	3.41 ± 0.13	2.76 ± 0.08	2.78 ± 0.09	2.99 ± 0.09
	Tibia	3.17 ± 0.21	3.26 ± 0.16	3.10 ± 0.18	3.91 ± 0.07	3.50 ± 0.06	4.09 ± 0.17
Ca:P	Enamel	1.29[3]	1.23	1.44	1.29	1.31	1.33
	Dentin	1.21	1.22	1.32	1.22	1.27	1.26
	Tibia	1.26	1.34	1.38	1.29	1.30	1.29
$Ca:CO_3$	Enamel	12.69	13.30	15.22	12.67	16.92	16.09
	Dentin	9.71	9.79	8.53	10.24	10.52	9.83
	Tibia	7.71	7.38	8.43	6.94	8.27	7.41
$PO_4:2CO_3$	Enamel	4.91	5.41	5.30	4.91	6.45	5.91
	Dentin	4.00	4.00	3.24	4.38	4.14	3.92
	Tibia	3.05	2.79	3.06	2.70	3.18	2.60

[1] See footnote 1, table 1, for description of diet symbols.

[2] Mean ± mean deviation.

[3] Molar ratios.

The composition of the dentin varied with the diet but in a manner distinct from that of bone. For all of the High-P diets the percentage of calcium was higher, but not statistically so, than that for the Low-P diets; the percentage of carbonate was lower, in contra-distinction to that of the tibia, and in some cases statistically so (P for W < 0.1; for OJ < 0.6 and for NOJ < 0.05). No consistent differences in composition seemed to exist between the W and OJ groups, yet the highest values for the ratios of Ca:P and the lowest for those of $Ca:CO_3$ and $PO_4:2CO_3$ were found in the NOJ groups. The composition of the tibia, closely related to that of the serum and of the diet, would contra-indicate a dietary role as the direct cause for these differences in the dentin.

When water was the sole fluid the chemical composition of the enamel was the same whether the diet was high or low in phosphorus. In the orange juice groups the carbonate content of the eroded enamel was lower and significantly so for the High-P-OJ diet (P for High-P-W versus High-P-OJ < 0.01; for Low-P-W versus Low-P-OJ < 0.4; for High-P-OJ versus Low-P-OJ < 0.01). An inverse relationship existed between the erosion score and the mean carbonate content for the High-P and Low-P diets as well as for the individual enamel samples in the two orange juice groups. The Ca:P ratios of these enamels were similar to those of the water groups but the $Ca:CO_3$ and $PO_4:2CO_3$ ratios were consistently higher.

The composition of the enamel from the NOJ groups gave evidence of chemical changes even though typical erosion had not been observed. The carbonate content was lower than that of the W groups (P for High-P-W versus High-P-NOJ < 0.1) but not as low as that of the OJ groups (P for High-P-OJ versus High-P-NOJ < 0.05) and was lowest for the High-P-diet. The levels of calcium were higher than those for either the W (P for Low-P-W versus Low-P-NOJ < 0.1; for High-P-W versus High-P-NOJ < 0.2) or the OJ groups (P for Low-P-OJ versus Low-P-NOJ < 0.1; for High-P-OJ versus High-P-NOJ < 0.2). The levels of phosphorus showed a similar trend but with no significant differences. The net results were higher ratios of Ca:P, $Ca:CO_3$ and $PO_4:2CO_3$ for the NOJ enamels than were found in the W groups.

The values found in these studies for calcium were lower and those for magnesium higher than those generally reported in the literature. There seems to be little value in trying to make any direct comparisons since so many variables exist such as age and breed of animal, composition of the diets, preparation of the dental samples and methods of chemical analyses.

DISCUSSION

The data in these studies further confirmed the fact that an increase of dietary phosphorus above a low level affects the mineral composition of bone and dentin. Since this subject has been covered in a number of papers it will be discussed only as it relates to the major contributions of these studies, namely, the role of dietary phosphorus in the susceptibility of enamel to erosion *in vivo* and the changes in the chemical composition of teeth which occur as the result of erosion.

The enamel from the High-P-W and the Low-P-W groups, in which no erosion was observed, showed no difference in the composition with respect to calcium, phosphorus, magnesium or carbonate when a purified diet containing either 0.09 or 0.25% of phosphorus was provided. Yet, when littermates had their drinking water replaced three times a week by canned orange juice the enamel was eroded and to a higher degree with the diet containing the higher concentration of phosphorus, and a lower ratio of Ca:P. Similar results were obtained in a preliminary experiment in which the erosion of enamel by a cola fluid *in vivo* was more extensive when the natural diet was supplemented with a phosphate salt than with a calcium salt.

In these studies the ratio of Ca:P in the diet seemed to be the factor determining the extent to which erosion occurred when teeth were exposed *in vivo* to an acidic fluid. With two ratios of Ca:P and two types of diet the erosion was more severe when the Ca:P was decreased. Studies recently completed but unpublished further confirm these results since an increase of dietary calcium, with a resultant increase of Ca:P, was found to decrease the erosive action of orange juice. Dalderup

('59) concluded from his studies and those reported in the literature that the Ca:P ratio was an important factor in the development of caries, but Wasserman[4] using essentially the same studies felt the ratio to be a factor only at very low levels of dietary phosphorus.

The carbonate content of the eroded enamel layer was lower than that for non-eroded enamel and varied inversely with the extent of the observed erosion. The decrease in carbonate content would seem to have been the result of, rather than the causative agent for, the erosion; for before eruption the carbonate content of the enamels should have been the same, as shown in the later stages by the water groups. In addition, the dentin layer below the eroded enamel had a lower content of carbonate for the teeth in which a greater extent of erosion had been observed.

The process of erosion *in vivo* appeared to be unlike that *in vitro* since Sobel ('55) found that enamel with the higher carbonate content had the faster rate of solution in acids *in vitro*. The process of erosion *in vivo* would seem to be associated with a metabolic action which could not be detected by the method used in these studies.

The ratios of $Ca:CO_3$ and of $PO_4:2CO_3$ in the enamels increased with erosion but the ratios of Ca:P remained fairly constant. The apatite structure of these enamels corresponded more closely to that proposed by Hendricks (Armstrong, '52) in which carbonate ions were described as distributed randomly in the calcium phosphate lattice. Calcium and phosphorus must have been lost during erosion but, apparently, in a fixed, molar ratio.

The enamels exposed to neutralized orange juice did not appear to have been eroded. The weights of the separated enamel fractions were similar to those of the groups receiving water. Yet the carbonate content and the ratios of both $Ca:CO_3$ and $PO_4:2CO_3$ of these enamels corresponded more closely to those from the orange juice groups than to those from the water groups. Erosion *in vitro* has been reported at a pH of 5.0 to 6.0 for citrate ions (Hills and Sullivan, '58). Citrate solutions of pH 4.0 have been classed as moderately strong decalcifying agents (Little et al., '54).

Although the presence of an acid is required for the production of both erosion and caries, the metabolic processes seem to have little in common. Dietary increase of phosphate has been shown repeatedly to reduce the incidence of caries in experimental animals and also in children (McClure and Muller, '59). However, it now becomes evident that, at least in rats, this same dietary practice has the adverse effect of making the enamel more susceptible to the erosive action of acidic fluids. In view of the results of the data reported here caution should be exercised in the supplementation of human diets with phosphates for the control of caries.

SUMMARY

In a preliminary experiment the Sobel-Hanok diet supplemented with either a calcium or phosphate salt was fed in conjunction with water or water-cola to Sherman strain rats for 40 days. Erosion of the enamel layer occurred with the cola fluid and to a greater degree when using the phosphate supplemented diet. The levels of calcium and of phosphorus in the whole teeth were similar for all the diet-fluid combinations.

In a second experiment a highly purified diet containing either 0.09 or 0.25% of phosphorus was fed in conjunction with water, water and orange juice, or water and neutralized orange juice to Wistar-strain rats for 8 weeks. Irrespective of the fluid the following measurements were found at a higher level for the higher phosphate-containing diet: length and weight of tibia; weight of 6 lower molars; percentages of calcium, phosphorus and carbonate in the tibia; and percentage of calcium in the dentin. The level of carbonate in the dentin was lower for the higher phosphate diet. The composition of the enamel showed no differences for the two levels of dietary phosphate when water was the sole fluid.

When canned orange juice, pH 3.7 to 3.8, was provided as the fluid, erosion of

[4] Wasserman, R. H. 1960 Calcium and phosphorus interactions in nutrition and physiology. Symposium on the interaction of mineral elements in nutrition and metabolism. American Institute of Nutrition.

the enamel layer occurred and to a greater extent on the higher phosphate diet. The carbonate content of the eroded enamel showed an inverse relationship with the extent of erosion but the Ca:P ratio remained unchanged. When neutralized orange juice was provided no erosion was observed but chemical changes occurred which were similar to, but not as marked, as the changes which occurred with untreated orange juice.

The level of carbonate in the eroded enamel was the result, and not the primary cause, of erosion. The Ca:P ratio, rather than the level of phosphorus, in the diet seemed to determine the degree of availability of carbonate ions for loss by erosion.

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Studies on Growth, Copper Metabolism and Iron Metabolism of Rats Fed High Levels of Zinc[1,2]

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High levels of dietary zinc (0.5 to 1.0%) fed to rats cause a depression in growth, the development of anemia, a decrease in heart and liver cytochrome oxidase activities, and a decrease in the copper content of the liver (Sutton and Nelson, '37; Smith and Larson, '46; Gray and Ellis, '50; Van Reen, '53; Duncan et al., '53; Grant-Frost and Underwood, '58). Smith and Larson ('46) found that a supplement of a liver extract partially alleviated the depression in growth. The nature of the active factor(s) in liver extract has not been determined. Duncan et al. ('53) reported some beneficial effect on the subnormal growth by adding folic acid to the high-zinc diets of rats. Supplements of copper to diets containing 0.75 to 1.0% of zinc partially overcome the zinc-induced anemia (Smith and Larson, '46; Duncan et al., '53; Grant-Frost and Underwood, '58). Additional copper also prevents the depression in cytochrome oxidase activity (Van Reen, '53; Duncan et al., '53). Thus, there is ample evidence to support the hypothesis that zinc interferes with copper metabolism in some way that has, as yet, not been determined.

In view of the lack of information concerning certain aspects of the zinc toxicity problem in rats, the present study was conducted to investigate the nature of the active factor(s) in liver, capable of alleviating subnormal growth in zinc-fed rats, to examine the nature of the interference of zinc with copper metabolism, and to determine the possibility of iron involvement in the zinc-induced syndrome.

MATERIALS AND METHODS

Male and female rats (Wistar and Sprague-Dawley strains) from 4 to 6 weeks old were used for the various phases of this study. The animals were maintained on experiment for 5 weeks, housed in individual wire-bottom cages, and given free access to feed and water. Rats in each experiment were randomized into replications according to initial body weights. The test treatments were assigned at random to individual cages within a replication.

The basal diet, the control, used in this study consisted of the following (in per cent): casein 19;[3] corn starch 63;[4] vegetable fat 10;[5] mineral mix 4;[6] vitamin mix 2;[7] cellulose 2;[8] and oleum percomorphum.[9] Materials tested were added to the basal diet at the expense of equal amounts of starch. Since previous workers have fed

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[3] Vitamin-Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[4] Corn Products Sales Co., Baltimore.

[5] Crisco, Procter and Gamble Company, Cincinnati.

[6] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland.

[7] Each 100 gm of the vitamin mix contained (in milligrams) biotin 1; folic acid 5; 0.1% vitamin B_{12} (with manitol) 0.1; thiamine·HCl 25; pyridoxine·HCl 25; 2-methyl-naphthoquinone 50; riboflavin 50; nicotinic acid 50; Ca pantothenate 150; and (in grams) *p*-aminobenzoic acid 5; inositol 5; choline 7.5; DL-methionine 30; and corn starch 52. Folic acid was furnished by American Cyanamid Company, Pearl River, New York; all other vitamins were contributed by Merck and Company, Rahway, New Jersey.

[8] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[9] Each 1000 gm of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

zinc in the forms of carbonate, chloride or oxide, the first experiment was designed to compare the effects of these three zinc compounds on growth, hemoglobin formation, liver copper and iron and heart cytochrome oxidase activity. In all other experiments zinc was fed as the carbonate. Supplements of copper and iron were in the forms of sulfate.

Oxyhemoglobin determinations, by the method of Shenk et al. ('34), were made on blood samples taken from the tails of the rats, at the termination of each experiment.

At the end of an experiment, rats from several replications were sacrificed, and the hearts and the livers were removed. The hearts were homogenized immediately in a Potter-Elvehjem homogenizer with 5 ml of cold 0.03 M phosphate buffer (pH 7.4). Cytochrome oxidase activities of appropriate dilutions of the crude homogenates were assayed spectrophotometrically at 550 mμ with a Beckman DU spectrophotometer (Cooperstein and Lazarow, '51). A stock solution of cytochrome C was prepared by dissolving cytochrome C[10] in cold 0.03 M phosphate buffer (pH 7.4) so that there were 12 mg of cytochrome C per ml. This solution was reduced with sodium hydrosulfite. A standard assay mixture consisted of 40 μ moles of phosphate buffer (0.1 M) at pH 7.4, 0.021 μ moles of reduced cytochrome C, 0.02 to 0.04 ml of diluted enzyme (volume depended on activity), and sufficient water to bring the total volume to 1 ml. The enzyme was added to the assay mixture, and the decrease in optical density between 15 and 75 seconds was recorded. Enzyme activity was expressed as the optical density change per minute per milligram of protein rather than as the first-order velocity constant (Cooperstein and Lazarow, '51) because repeated tests showed that under the conditions of these assays the oxidation rates remained linear for at least three minutes. This is in agreement with the findings of others (Fritz and Beevers, '55; Miller and Evans, '56). Protein contents of the diluted homogenates were determined by the method of Lowry et al. ('51).

The copper and the iron content of the livers was determined by the methods of Parks et al. ('43) and Kitzes et al. ('44), respectively, as modified by Matrone et al. ('47). The zinc content of the livers was determined by a method outlined by Sandell ('59).

A radioisotope experiment with Cu^{64} was conducted to determine if zinc affected the absorption or the utilization and the excretion of copper in the rat. Four control rats and 4 zinc-fed rats were taken from one of the growth trials after it was completed and placed in individual metabolism cages with access to water and the respective experimental rations. Each rat received 100 μc of radiocopper[11] by stomach tube. Forty-eight hours after the administration of the radiocopper, the rats were sacrificed; the hearts, spleens, livers, kidneys, and gastrointestinal tracts were removed and the radioactivity of each was determined by means of a scintillation counter. Feces and urine samples from each rat were also collected, and the radioactivities of these were measured. Using the same procedures an experiment was conducted with Fe^{59} to observe the effect of zinc on absorption, utilization, and excretion of iron. Each rat received 5 μc of radioiron[11] by stomach tube. The radioactivities of the hearts, spleens, livers, kidneys, right and left femurs, gastrointestinal tracts, feces and urine samples were also estimated by means of a scintillation counter.

In the investigation of the nature of the active factor(s) in liver extract,[12] an ash of the liver was obtained by the wet-ashing method. Solvent fractionation of the liver extract was carried out with either methanol or acetone. The liver extract, in 50-gm amounts, was extracted for approximately 24 to 36 hours with either solvent. A Soxhlet extractor was used for this extraction step. When acetone was used as the solvent, a precipitate settled out in the boiling flask as the acetone cooled. This precipitate, designated as "compound X" in this study, was collected on filter paper by means of a suction filter, allowed to dry at room temperature, and saved for use in an assay. The acetone fraction was concentrated to approxi-

[10] Sigma Chemical Company, St. Louis, Missouri.

[11] Oak Ridge Nuclear Laboratories, Oak Ridge, Tennessee.

[12] Liver Fraction L, Nutritional Biochemical Corporation, Cleveland.

mately 100 ml at reduced pressure. The methanol fraction, obtained when methanol was used as the extracting solvent, was also concentrated to approximately 100 ml at reduced pressure.

A randomized block design was used in the animal experiments and all data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$).

RESULTS AND DISCUSSION

Experiment 1. As shown in table 1, the addition of zinc to the diet of rats caused marked decreases in growth, liver copper, and liver iron. Although the hemoglobin levels of the zinc-fed rats were lower than the controls, the effect of zinc on hemoglobin was not as great as has been reported in the literature. Zinc in the diet was associated with a slight decrease in heart cytochrome oxidase activity and a large increase in the concentration of zinc in the liver. In general, the results from feeding the three zinc compounds were similar.

Experiment 2. Since the work of Smith and Larson ('46) with liver extract was carried out prior to the discovery of vitamin B_{12}, it was reasoned that this compound could be a part of the active factor(s) in liver. This vitamin, in combination with folic acid and ascorbic acid, was added to the zinc diets in a second experiment. The results shown in table 2 indicated that the rats receiving additional vitamins gained more and had a higher concentration of iron in the livers than the zinc-fed rats. However, the difference was not statistically significant. The added vitamins apparently had no effect on the hemoglobin levels, the copper contents of the livers or the cytochrome oxidase activities of the hearts of the zinc-fed rats.

Experiment 3. In this experiment the effectiveness of liver extract for alleviating subnormal growth was compared with that of the ash of liver extract since it has not been established whether or not the active principle was organic or inorganic. The addition of 10% of liver extract to the zinc diet caused a highly significant increase ($P \leq 0.01$) in growth, hemoglobin level, and liver iron (table 3). There was also a significant increase ($P \leq 0.05$) in the liver

TABLE 1

Effect on the rat of high dietary zinc from various sources[1,2]

Source	Zinc in diet	Weight gain at 5 weeks	Hemoglobin	Liver constituents[3]			Heart cytochrome oxidase[3,4]
				Cu	Fe	Zn	
	%	gm	*gm/100 ml blood*	*μg/gm dry weight*			*ΔOD/min/mg protein*
None	—	145 ± 8[5]	13.58 ± 0.46[5]	15.69 ± 0.57[5]	425.72 ± 43.91[5]	37.21 ± 3.26[6]	10.90 ± 1.42[7]
$ZnCO_3$	0.75	121 ± 9[7]	10.03 ± 0.51[7]	6.47 ± 0.69	230.52 ± 29.39	378.39 ± 40.62	6.74 ± 0.73
	1.00	95 ± 9[8]	11.11 ± 0.75[8]	6.16 ± 0.51	240.56 ± 11.91	497.70 ± 40.57	5.91 ± 0.46
$ZnCl_2$	0.75	108 ± 12[8]	11.92 ± 0.10[3]	5.30 ± 0.52	232.54 ± 56.25	398.73 ± 63.92	7.79 ± 1.86
	1.00	82 ± 6[9]	10.97 ± 0.68[10]	3.96 ± 0.07	161.88 ± 10.59	452.25 ± 48.86	6.74 ± 1.13
ZnO	0.75	108 ± 9[7]	12.40 ± 0.37[3]	5.42 ± 0.90	173.26 ± 42.84	367.01 ± 47.98	7.63 ± 2.74
	1.00	76 ± 7[9]	10.39 ± 1.18[3]	4.11 ± 0.23	321.08 ± 91.95	526.66 ± 55.84	5.46 ± 1.74

[1] Wistar rats averaging 58 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 4 animals unless otherwise indicated.
[4] Optical density change at 550 mμ.
[5] Mean of 16 animals.
[6] Mean of 15 animals.
[7] Mean of 8 animals.
[8] Mean of 6 animals.
[9] Mean of 7 animals.
[10] Mean of three animals.

TABLE 2

Effects of extra vitamins on zinc-fed rats[1,2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents			Heart cytochrome oxidase[6,7]
			Cu[4]	Fe[4]	Zn[5]	
	gm	*gm/100 ml blood*	*μg/gm dry weight*			*ΔOD/min/mg protein*
Control	145 ± 8	13.58 ± 0.46	15.69 ± 0.57	425.72 ± 43.91	37.21 ± 3.26	10.90 ± 1.42
Control + extra vitamins[8]	146 ± 7	13.15 ± 0.28	16.61 ± 0.70	476.25 ± 75.62	27.80 ± 3.73	11.42 ± 1.28
Control + 0.75% Zn	114 ± 6	10.82 ± 0.49[9]	5.95 ± 0.56	201.89 ± 26.37	332.31 ± 29.18	7.19 ± 1.32
Control + 0.75% Zn + extra vitamins	125 ± 8	11.36 ± 0.35	6.44 ± 0.59	284.58 ± 38.75	325.28 ± 32.32	5.48 ± 0.47
Control + 1.0% Zn	85 ± 6[10]	10.82 ± 0.62	5.14 ± 0.47	280.82 ± 45.54	512.18 ± 32.41	5.69 ± 0.84
Control + 1.0% Zn + extra vitamins	102 ± 5	10.98 ± 0.28[11]	5.02 ± 0.29	300.68 ± 36.16	453.94 ± 46.48	5.79 ± 0.74

[1] Wistar rats averaging 60 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 16 animals unless otherwise indicated.
[4] Mean of 16 animals in the control treatment. Mean of 8 animals in all other treatments.
[5] Mean of 15 animals in the control treatment. Mean of 8 animals in all other treatments.
[6] Optical density change at 550 mμ.
[7] Each figure is the mean of 8 animals.
[8] Included 10 ppm vitamin B_{12} + 500 ppm folic acid + 0.1% ascorbic acid.
[9] Mean of 12 animals.
[10] Mean of 13 animals.
[11] Mean of 11 animals.

TABLE 3

Response of zinc-fed rats[1] *to supplements of liver extract and liver extract ash*[2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents[4]			Heart cytochrome oxidase[5,6]
			Cu	Fe	Zn	
	gm	*gm/100 ml blood*	*μg/gm dry weight*			*ΔOD/min/mg protein*
Control	196 ± 7	13.28 ± 0.17	12.91 ± 0.44	332.87 ± 37.00	36.13 ± 3.27	6.25 ± 0.49
Control + 0.75% Zn	128 ± 10[7]	7.36 ± 0.36[7]	4.93 ± 0.48	118.46 ± 7.22	334.36 ± 23.47	4.43 ± 0.42
Control + 0.75% Zn + 10% liver extract	163 ± 9	11.14 ± 0.32	6.52 ± 0.41	209.32 ± 12.40	294.86 ± 17.59	3.77 ± 0.29
Control + 0.75% Zn + liver extract ash[8]	87 ± 6[9]	8.64 ± 0.25[9]	5.45 ± 0.42[10]	110.84 ± 5.56[10]	402.93 ± 35.75[10]	4.16 ± 0.71[11]

[1] Sprague-Dawley rats averaging 58 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 16 animals unless otherwise indicated.
[4] Each figure is the mean of 13 animals unless otherwise indicated.
[5] Optical density change at 550 mμ.
[6] Each figure is the mean of 9 animals unless otherwise indicated.
[7] Mean of 14 animals.
[8] Equivalent to 10% liver extract.
[9] Mean of 12 animals.
[10] Mean of 10 animals.
[11] Mean of 6 animals.

copper and a further reduction in cytochrome oxidase activity.

The ash of the liver extract caused an additional reduction in growth when added to the zinc diet (table 3). There was a significant effect from the ash on hemoglobin level, but no significant effect on either liver copper, or liver iron or on the heart cytochrome oxidase activity. The failure of the ash to significantly increase the concentration of copper and iron in the livers was unexpected in view of the increases obtained with equivalent amounts of unashed liver extract which contained 77 μg and 681 μg per gm of dry weight of copper and iron, respectively. Presumably, the oxides of these metals, the form probably prevailing in the ash, were poorly utilized. This possibility seems reasonable in view of the fact that iron oxide has been reported to be poorly utilized by the chick (Elvehjem and Hart, '29), the rat (Mitchell and Schmidt, '26), and the pig (Hart et al., '30).

The results of this experiment suggest that the factor(s) in liver extract capable of alleviating subnormal growth is primarily organic in nature.

Experiment 4. In the observations previously shown, there was a marked decrease in the iron content of the livers of the rats fed zinc as compared with that of the controls. Thus, there is an indication that zinc was also affecting iron metabolism in some manner. Two possibilities are that (1) zinc has a direct effect on iron metabolism, and (2) the adverse effect on iron metabolism is a result of the effect of zinc toxicity on copper metabolism. An experiment was designed to test these two hypotheses by studying the effects of adding copper and iron supplements to the zinc diet. As shown by the data in table 4, a level of 0.02% of copper only partially restored the hemoglobin level of zinc-fed rats. A level of 0.04% of iron effected the same magnitude of increase. When both minerals were added to the diet of zinc-fed rats, the hemoglobin levels equaled those of the control rats. The data also show that 0.02% of copper was sufficient to restore the liver copper to a level above that of the controls, whereas 0.04% of iron did not completely overcome the depression in

TABLE 4

Response of zinc-fed rats[1] to supplements of copper and iron[2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents[4]			Heart cytochrome oxidase[5,6]
			Cu	Fe	Zn	
	gm	*gm/100 ml blood*	*μg/gm dry weight*			*ΔOD/min/mg protein*
Control	194 ± 9	13.08 ± 0.18	12.89 ± 0.58	344.54 ± 47.36	38.73 ± 3.89	6.60 ± 0.52
Control + 0.75% Zn	110 ± 9[4]	6.97 ± 0.38[4]	4.49 ± 0.54	121.76 ± 8.18	341.11 ± 29.97	4.36 ± 0.63
Control + 0.75% Zn + 0.02% Cu	119 ± 9	10.31 ± 0.37	18.35 ± 0.90	108.00 ± 9.00	348.68 ± 31.03	8.10 ± 0.87
Control + 0.75% Zn + 0.04% Fe	116 ± 8	10.31 ± 0.39	4.84 ± 0.33	197.82 ± 16.98	350.04 ± 21.64	4.86 ± 0.40
Control + 0.75% Zn + 0.02% Cu + 0.04% Fe	97 ± 10	12.98 ± 0.38	20.29 ± 2.50	179.00 ± 7.75	408.62 ± 24.61	7.38 ± 0.73

[1] Sprague-Dawley rats averaging 53 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 12 animals unless otherwise indicated.
[4] Each figure is the mean of 10 animals.
[5] Optical density change at 550 mμ.
[6] Each figure is the mean of 6 animals.

liver iron. Supplemental copper did not increase the concentration of iron in the liver, but actually was associated with a further reduction. Iron had no effect on copper accumulation in the liver. These results would tend to support the hypothesis that zinc is directly interfering with iron metabolism. Grant-Frost and Underwood ('58) have suggested that a deficiency of some dietary essential, in addition to copper, affecting hematopoiesis is occurring as a consequence of the high zinc intakes. The results of this experiment indicate that the additional dietary essential involved may be iron.

The effect of added copper on the cytochrome oxidase activity in the zinc-fed rat is similar to that previously reported by Duncan et al. ('53) and Van Reen ('53). Iron would not be expected to have any effect on cytochrome oxidase activity in view of the results of several investigators (Cohen and Elvehjem, '34; Schultze, '39; Gubler et al., '57) who have shown that cytochrome oxidase activity is not influenced by iron deficiency but is markedly reduced in copper deficiency.

Experiment 5. Since supplemental vitamins were not as effective in preventing subnormal growth as the liver extract, it is possible that the adverse effect of zinc on growth is being mediated through the inactivation of some other essential factor(s). Potter and Dubois ('43) showed that zinc was one of the compounds which was capable of inhibiting succinic dehydrogenase by its ability to react with the -SH groups of the enzyme. Barron and Kalnitsky ('47) showed that a linkage between zinc and the -SH groups of succinoxidase resulted in an inactivation of the enzyme. Kunkel ('51) confirmed the inhibition of succinoxidase by zinc and also found that zinc inhibited the oxidation of α-ketoglutarate and citrate. Thus, it may be reasoned that the inactivation of metabolically important sulfhydryl groups by zinc is involved and that the sulfhydryl compounds in liver extract might be responsible, in part, for the alleviating effect observed.

With this possibility in mind, an experiment was designed testing two sources of protein, casein and egg albumen,[13] in combination with 0.02% of copper, 0.03% of iron, and various levels of vitamin B_{12}, folic acid and ascorbic acid. Two additional test diets were included. One contained 1% of cysteine; the other contained 5% of liver extract. The results of this experiment (table 5) indicate that there was apparently no difference between the treatments across the casein diets and the corresponding treatments across the egg albumen diets. The addition of 1% of cysteine was also without effect. The failure of egg albumen and cysteine to alleviate the subnormal growth of zinc-fed rats would suggest that the sulfhydryl groups *per se* present in the liver extract were not the active factor. The results on growth suggest that the amounts of copper and the iron supplements used in this experiment may have approached toxic levels. It is also apparent that the addition of the extra vitamins, in combination with the copper and the iron supplements, was not beneficial in alleviating the subnormal growth. However, a level of 5% of liver extract seems to be sufficient to alleviate the subnormal growth of the zinc-fed rats since the mean weight gain of the rats receiving this treatment was not significantly different from the mean weight gain of the control animals.

The improvement in the hemoglobin levels of the zinc-fed rats supplemented with the other ingredients was probably due to the presence of copper and iron.

The data indicated that increasing levels of vitamins added to the diets containing casein were associated with slight increases in the levels of copper in the livers of the rats. However, when the level of the vitamin supplement was increased, there appeared to be a corresponding decrease in the level of iron in the liver.

The rats fed all supplemented diets, except those containing zinc only, had cytochrome oxidase activities equal to or greater than the controls. The ability of 5% of supplemental liver extract to overcome the depression in cytochrome oxidase activity was unexpected in view of the results of experiment 3 (table 3) which showed that 10% of liver extract had no beneficial effect on the enzyme activity. The reason for this difference is not apparent.

[13] Nutritional Biochemicals Corporation, Cleveland.

TABLE 5

Response of zinc-fed rats[1] fed various supplements[2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents[4]			Heart cytochrome oxidase[5,6]
			Cu	Fe	Zn	
	gm	*gm/100 ml blood*		*μg/gm dry weight*		*ΔOD/min/mg protein*
Basal, casein	220 ± 5	13.14 ± 0.53	11.47 ± 0.41	339.54 ± 43.44	26.14 ± 1.42	14.05 ± 1.46
Basal, casein + 0.75% Zn	150 ± 7	6.73 ± 0.34	3.64 ± 0.22	137.76 ± 14.77	364.74 ± 32.43	8.05 ± 0.81
Basal, casein + 0.75% Zn + 0.02% Cu + 0.03% Fe + 10× vitamins[7]	120 ± 8	12.42 ± 0.31	15.42 ± 0.99	303.33 ± 52.35	464.04 ± 35.24	23.20 ± 2.44
Basal, casein + 0.75% Zn + 0.02% Cu + 0.03% Fe + 100× vitamins[8]	130 ± 8	12.00 ± 0.35	18.72 ± 2.13	244.95 ± 26.79	434.14 ± 46.82	22.66 ± 4.02
Basal, casein + 0.75% Zn + 0.02% Cu + 0.03% Fe + 500× vitamins[9]	140 ± 8[10]	11.84 ± 0.43[10]	18.69 ± 1.25	208.98 ± 15.28	419.70 ± 16.30	16.40 ± 1.59
Basal, casein + 0.75% Zn + 0.02% Cu + 0.03% Fe + 500× vitamins + 1% cysteine	122 ± 11	11.82 ± 0.36	16.08 ± 0.42	289.88 ± 10.62	359.55 ± 37.28	15.99 ± 2.73
Basal, casein + 0.75% Zn + 5% liver	198 ± 6[10]	10.35 ± 0.40[10]	6.80 ± 0.53	146.39 ± 11.43	299.27 ± 21.07	12.44 ± 1.12
Basal, egg albumen	210 ± 7	12.96 ± 0.20	12.38 ± 1.30	361.55 ± 16.37	31.44 ± 1.43	14.48 ± 3.69
Basal, egg albumen + 0.75% Zn	135 ± 6	6.24 ± 0.45	3.70 ± 0.82	143.60 ± 32.12	338.05 ± 16.85	10.43 ± 1.48
Basal, egg albumen + 0.75% Zn + 0.02% Cu + 0.03% Fe + 500× vitamins	157 ± 9	12.42 ± 0.27	14.52 ± 1.49	142.58 ± 17.97	273.20 ± 34.83	19.02 ± 4.78

[1] Sprague-Dawley rats averaging 80 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 8 animals unless otherwise indicated.
[4] Each figure is the mean of 4 animals.
[5] Optical density change at 550 mμ.
[6] Each figure is the mean of three animals.
[7] Included 0.2 ppm vitamin B_{12} + 10 ppm folic acid + 0.1% ascorbic acid.
[8] Included 2 ppm vitamin B_{12} + 100 ppm folic acid + 0.1% ascorbic acid.
[9] Included 10 ppm vitamin B_{12} + 500 ppm folic acid + 0.1% ascorbic acid.
[10] Mean of 7 animals.

Rat assay experiment. In a further attempt to isolate the active factor(s) in liver extract, the materials obtained from the solvent fractionation procedure were incorporated into a rat assay experiment. The data, table 6, indicate that the active factor(s) in liver cannot be extracted with acetone since the mean weight gain of the rats consuming the zinc diet supplemented with the acetone fraction was not significantly different from that of the rats consuming the zinc diet. Also, the mean weight gain of the rats consuming the zinc diet containing 5% of acetone-extracted liver was not significantly different from that of the rats fed the zinc diet supplemented with 5% of liver. The mean weight gain of the rats fed both of these diets was significantly different ($P \leq 0.01$) than that of the rats fed only zinc. The data also suggest that the active principle is soluble in methanol since the rats consuming the zinc diet supplemented with the methanol fraction had a mean weight gain significantly greater ($P \leq 0.01$) than that of the rats consuming only zinc.

Radiocopper experiment. In the experiment with radiocopper, approximately 98 and 96% of the activity recovered was present in the gastrointestinal tracts and the feces of the control and the zinc-fed rats, respectively. The difference between these two values was not significant. Thus, it appears that zinc had little effect on the absorption of copper. Table 7 shows the effect of zinc on the distribution of the absorbed radiocopper in some tissues and urine. There was a significant increase ($P \leq 0.05$) in the amount of activity in the urine of the zinc-fed rats as compared with the activity of the urine of the controls. There was approximately a 20% decrease in the activities of the livers of the zinc-fed rats which was a significant change ($P \leq 0.05$) when compared with the controls. The addition of zinc to the diet caused no significant changes in the uptake of radiocopper by the heart, kidney and spleen. Thus, it appears that high intakes of zinc interfere with the utilization of copper and cause it to be excreted via the urine.

Radioiron experiment. In the experiment with radioiron, approximately 97 and 96% of the recovered activity was present in the gastrointestinal tracts and the feces of the control and the zinc rats, respectively. These two values were not signifi-

TABLE 6

Response of zinc-fed rats to test materials obtained by extraction procedures[1]

Diet treatment	Mean weight gain at 5 weeks
	gm
Control	225 ± 5
Control + 0.75% Zn	106 ± 11
Control + 0.75% Zn + 5% liver extract	170 ± 8
Control + 0.75% Zn + 5% acetone-extracted liver	168 ± 7
Control + 0.75% Zn + acetone fraction (≏ 5% liver)	121 ± 10
Control + 0.75% Zn + 0.1% "compound X"	111 ± 5
Control + 0.75% Zn + methanol fraction (≏ 5% liver)	151 ± 6

[1] Each figure is the mean and the standard error of the mean of 10 rats.

TABLE 7

Effect of zinc on the distribution of absorbed radioactive Cu^{64}

Tissue or urine	Distribution of radioactivity[1]	
	Control rats	Zinc-fed rats
	%	%
Heart	1.6 ± 0.2	1.1 ± 0.2
Spleen	1.4 ± 0.2	2.2 ± 0.4
Kidneys	17.8 ± 1.7	13.4 ± 1.8
Liver	57.5 ± 1.6	37.8 ± 7.2
Urine	21.7 ± 0.7	45.5 ± 7.2

[1] Each figure represents the mean and the standard error of the mean calculated from 4 rats.

TABLE 8

Effect of zinc on the distribution of absorbed radioactive Fe^{59}

Tissue or urine	Distribution of radioactivity	
	Control rats[1]	Zinc-fed rats[2]
	%	%
Heart	3.8 ± 0.1	2.1 ± 0.5
Spleen	14.6 ± 0.6	10.3 ± 1.7
Kidneys	5.4 ± 0.4	3.8 ± 0.3
Liver	65.0 ± 0.9	76.8 ± 2.8
Femurs	10.0 ± 0.7	6.1 ± 0.7
Urine	1.2 ± 0.3	0.9 ± 0.1

[1] Each figure represents the mean and the standard error of the mean calculated from three rats. One rat died while the radioactive iron was being administered.

[2] Each figure represents the mean and the standard error of the mean calculated from 4 rats.

cantly different from each other. This would suggest that zinc had little effect on the absorption of iron. The summary of the results of this experiment (table 8) reveals that zinc caused significant decreases ($P \leq 0.05$) in the uptake of radioiron by the heart and the femurs. There was a significant increase ($P \leq 0.05$) in the radioactivities of the livers of the zinc-fed rats. While these results give some indication that zinc may be interfering with the utilization of iron, it is evident that a complete picture has not been attained. There is the possibility that 48 hours was not enough time for equilibrium conditions to become established among the various iron compartments of the body. Unfortunately, radioactivity data on the blood was not taken.

SUMMARY

Young rats were fed purified diets containing high levels of zinc (0.75 and 1.0%) in the presence and absence of various combinations of copper, iron, vitamin and liver extract supplements. Under these conditions effects of zinc toxicity on growth, hemoglobin formation, liver copper and iron levels, and heart cytochrome oxidase activity were observed in several experiments.

The factor(s) of liver extract that alleviates the subnormal growth of zinc-fed rats appears to reside primarily in the organic portion of the extract. There is an indication that certain vitamins, such as vitamin B_{12} and folic acid, may be involved. The data suggest that the active principle can be extracted from the liver with methanol.

In addition to supporting the accepted hypothesis that zinc interferes with copper metabolism, results of this study indicate that zinc directly interferes with iron metabolism.

Supplements of iron had no apparent effect on the heart cytochrome oxidase activities of rats on a high zinc diet indicating that copper is the important factor connected with restoration of the enzyme activity.

Results of an isotope experiment suggest that zinc interferes with copper metabolism by decreasing the utilization and increasing the excretion of copper in the rat, but apparently has little effect on the absorption of copper.

Results of another isotope experiment indicate that zinc does not interfere with the absorption of iron, but interferes, in some manner, with the utilization of iron.

Throughout this entire study, the addition of zinc to the diet resulted in a marked increase in liver zinc. None of the dietary components tested counteracted this tremendous increase in the accumulation of zinc in the liver.

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Salt Mixtures for Purified-Type Diets

III. AN IMPROVED SALT MIXTURE FOR CHICKS[1]

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The bulletin, Nutrient Requirements for Poultry (National Research Council, '54), has been very useful to poultry nutritionists in formulating experimental diets. More recent studies on the mineral requirements of the chick have shown the need for some revision and addition to the standards set forth in this publication. The National Research Council (NRC) requirements have been found to be too low for potassium (Burns et al., '53; Leach and Norris, '58) and for sodium (Burns et al., '53). Conversely, there have been studies which indicate that the NRC requirement for magnesium is too high (Scott et al., '56; Gardiner et al., '59). The adequacy of certain salt mixtures has been criticized by Briggs ('56).

Recent research has focused attention on some of the trace minerals. The requirement for zinc has been found to be much higher than earlier work indicated (O'Dell et al., '58; Morrison and Sarett, '58; Moeller and Scott, '58; Pensack et al., '58; Young et al., '58; Roberson and Schaible, '58). No clear-cut demonstration of the essentiality of molybdenum has been made for the chick. An apparent molybdenum requirement was created by feeding sodium tungstate to chicks (Higgins et al., '56). Selenium has been shown to prevent exudative diathesis in vitamin E-deficient chicks (Schwarz et al., '57); however, evidence for the essentiality of selenium in the presence of vitamin E is lacking.

Other important considerations in supplying the needed inorganic elements in an experimental diet involve the effects of the salts upon the stability of essential nutrients in the diet. It was found that in experimental diets some of the commonly used salt mixtures accelerated oxidative rancidity (Fox and Mickelsen, '59a), the Maillard-type of browning reaction (Fox and Mickelsen, '59b), and destruction of thiamine (Waibel et al., '54).

This paper is a report of work done to develop an improved salt mixture for the chick. The new Salts N is inert with respect to the Maillard browning reaction in purified diets and the effect in accelerating oxidative rancidity is very small compared with that of many other salt mixtures. All essential inorganic elements are present in amounts adequate for the chick's requirement; however, excesses have been eliminated.

EXPERIMENTAL PROCEDURE

Preparation of salt mixtures. The formula for Salts A (Briggs et al., '52), which has been used in this and other laboratories, is given in table 1. The composition of the new salt mixture, Salts N, is also given in table 1 and the amounts of individual elements supplied by each salt mixture are presented in table 2. For reasons stated in the introduction, moylbdenum and selenium have been set aside as "optional trace minerals" to be used with Salts N at the discretion of each investigator. To facilitate varying the individual constituents, both salt mixtures have been routinely mixed in two parts (see footnote 2 of table 4), but the ingredients can be satisfactorily combined in one mixture. The salt mixtures were prepared from chemicals of at least C.P., U.S.P., or reagent grades. All salts were obtained in finely powdered form; anhydrous magnesium sulfate and anhydrous copper sul-

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[1] Part of these data were presented at the meeting of the American Institute of Nutrition, 1959, Atlantic City, New Jersey.

fate were chosen for this reason. Originally Salts A contained hydrated magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$); when this was replaced by the anhydrous salt, glucose was used to compensate for the water of hydration.

TABLE 1

Composition of Salts A and Salts N

	Salts A[1]	Salts N[1]
	gm	*gm*
$CaHPO_4$	—	2840
$CaCO_3$	1500	1000
$Ca_3(PO_4)_2$	1400	—
K_2HPO_4	900	—
Na_2HPO_4	730	700
NaCl	880	400
KCl	—	700
$MgSO_4$	244	300
Fe citrate	40	20
$MnSO_4 \cdot H_2O$	42	25
KI	4	—
KIO_3	—	1
$ZnCO_3$	2	13
$CuSO_4$	—	1
$CuSO_4 \cdot 5H_2O$	2	—
Glucose	256	—
Total	6000	6000
Optional trace minerals[2]		*mg*
$Na_2MoO_4 \cdot 2H_2O$	—	500
Na_2SeO_3	—	21.9

[1] This quantity of each salt mixture is the amount needed to make 100 kg of diet when the salt mixture is fed at 6% of diet. Salts N is commercially available.

[2] In this paper Salts N refers to the above mixture *exclusive* of the "optional trace minerals." The optional trace minerals are mixed in 100 gm of glucose and then can be mixed with other salts or added to the diet as a separate premix.

Composition of diets. The composition of the three diets fed to chicks in these experiments are presented in table 3. Diet C47D was free of vitamin E and contained Torula yeast to exaggerate the vitamin E deficiency. This diet was used to test the stability of selenium in Salts N, since selenium can prevent the exudative diathesis that is characteristic of vitamin E deficiency in the chick. Diets C50 and C53 were both complete diets. The former is a typical casein-gelatin diet; whereas, the latter is a soy protein diet, a modification of that designed by Machlin and Gordon ('58) to support very rapid growth in the chick. Both of these diets were fed with 4 and 16% levels of fat. Both diet C50 and diet C53 were fed with 6 and 9% levels of Salts A and of Salts N. Supplements of copper, iron, molybdenum and selenium were tested with each of the complete diets and different levels of magnesium were also fed with each diet.

The diet for each group of chicks was prepared in 2-kg quantities. No more than 1 kg of this was kept in the animal room at one time; the remainder was refrigerated. Change in the dietary level of any constituent was compensated by equal alteration of the carbohydrate content so that the concentration of all other dietary constitutents remained constant.

TABLE 2

Elements supplied by chick Salts A and chick Salts N and the NRC requirements

	Salts A % of diet[1]	Salts N % of diet[1]	Requirement % of diet[2]
Ca	1.14	1.24	1.0
P	0.6	0.8	0.6
K	0.4	0.37	0.2 (0.3)
Na	0.58	0.384	0.2 (0.3)
Cl	0.52	0.58	0.3
Mg	0.0493	0.06	0.05 (0.025)
Fe	0.0067	0.00334	0.0020
Mn	0.0136	0.00813	0.0055
I	0.0030	0.00060	0.0001
Zn	0.00112	0.00728	— (0.0055)
Cu	0.00051	0.00040	0.0002
Optional trace minerals		*mg/kg of diet*[1]	
Mo	—	2.	
Se	—	0.1	

[1] When salt mixture fed at 6% of diet.

[2] Requirements different from those of NRC are given in parentheses. For source of original data, see introduction and discussion sections.

TABLE 3

Composition of diets[1]

	Diet C50	Diet C53	Diet C47D
	%	%	%
Vitamin-free casein	20	—	—
Gelatin	8	—	—
Soybean protein[2]	—	35	30
Torula yeast	—	—	30
L-Cystine	—	—	0.3
DL-Methionine	0.3	0.6	0.2
Glycine	—	1	—
Salts	6	6	6
Corn oil	4	16	—
Vitamin E-free lard[3]	—	—	4
Choline chloride	0.2	0.2	0.1
Glucose	61.5	41.2	29.4

[1] Diet C50 contained the following vitamins (mg/kg of diet): thiamine·HCl, 8; riboflavin, 8; Ca pantothenate, 20; nicotinic acid, 100; pyridoxine·HCl, 8; *d*-biotin, 0.3; folacin, 3; vitamin B_{12}, 0.02; vitamin A acetate, 6; vitamin D_3, 0.02; α-tocopherol, 25; α-tocopheryl acetate, 25; 2-methyl-1,4-naphthoquinone, 1. Diet C53 contained the same levels of vitamins except that vitamin B_{12} was increased to 0.1 mg. Diet C47D was vitamin E-free, otherwise it contained the same quantities of all vitamins as diet C50 except that vitamin A was supplied in stabilized form by 0.1% of Nopcay "20" (20,000 U.S.P. units/kg of diet; purchased from Nopco Chemical Company, Harrison, New Jersey).

[2] Drackett C-1 Assay Protein (now designated ADM assay protein) was purchased from Archer-Daniels-Midland Company, Cincinnati, Ohio.

[3] Stripped lard was purchased from Distillation Products Industries, Rochester, New York.

Stability of the diets. The effect of salt mixtures upon the development of oxidative rancidity was evaluated by the detection of a rancid odor in simplified diet mixes stored at 37°C. This was similar to the procedure used previously (Fox and Mickelsen, '59a). To study the effect of browning, mixtures composed of 6% Salts N, 10% glycine, and 84% glucose[2] were stored for 4 to 6 weeks in closed vials at 37°C, and in both closed and open vials at 25°C in an animal room. The content of iodine in Salts N was determined by a standard chemical titrimetric procedure (Willard and Furman, '40). Evaluation of the stability of selenium in Salts N was based on its prevention of exudative diathesis in vitamin E deficient chicks.

Experimental animals. Day-old female New Hampshire chicks were distributed into groups of 8 chicks each. They received the experimental diets ad libitum for 4 weeks; a few experiments were continued for 7 weeks. All chicks were weighed at weekly intervals. All experiments were repeated several times as indicated in the tables. The chicks receiving the vitamin E-deficient diet were plucked of abdominal down and feathers at 10 days. They were checked each morning thereafter for the appearance of exudative diathesis, the severity of which was scored from 1+ to 4+. The location of exudates (abdomen, wings, or neck) and the presence and severity of hemorrhaging were also noted.

RESULTS

Chick Salts A served as the point of departure in the development of a new salt mixture and numerous combinations of minerals were tested. Many mixtures that had negligible effects on diet stability and that appeared to be well-balanced and adequate did not support good growth of chicks. In table 2 are given the amounts of individual elements supplied by Salts A and N as well as what appear to be the best requirement figures for each element. When Salts N is fed at 6% of the diet, it supplies elements only in slight to moderate excess of the chick's requirement. When 1 gm of salt mixture was mixed with 20 ml of distilled water, the solution of the soluble salts was pH 7.58 for Salts A and pH 7.52 for Salts N. The ratio of acid to base-forming elements was 0.92 for Salts A and 1.34 for Salts N. The calcium to phosphorus ratio was 1.90 for Salts A and 1.55 for Salts N.

[2] Cerelose, Corn Products Refining Company, New York.

Salts and stability of dietary constituents. The effects of Salts A and of Salts N in accelerating oxidative rancidity of simplified diet mixes are shown in table 4. Each salt mixture was tested at contentrations of 4, 6 and 8% of the diet mix; equivalent amounts of part 1 and part 2 of each salt mixture was tested at each level (see footnote 2). In general, an increase in the concentration of salts resulted in a decrease in time for the diet mix to smell rancid. Part 2, which contained the trace minerals of each salt mixture, was chiefly responsible for the effect of the whole salt mixture in accelerating rancidity. Salts N-1 had no effect on rancidity; however, Salts A-1 definitely hastened the process of rancidity. With every possible comparison, rancidity developed much more slowly with Salts N than with Salts A (see "stability factor" in table 4). For example, at the 6% level of salts, there was a 9-fold delay when Salts N was compared with Salts A.

The Maillard-type of browning, which can result in extensive dietary loss of amino acids, as well as glucose, was not affected by the presence of Salts N. This was true in the glucose-glycine mixtures

TABLE 4

Development of rancidity in diet mixes[1] containing Salts A and Salts N stored at 37°C (Average of two experiments)

Level of salts	Chick Salts A		Chick Salts N		Stability factor[4]
	Part of salts[2]	Time[3]	Part of salts[2]	Time[3]	
%		*days*		*days*	
0	—	67	—	67	—
3.33	A-1	24	N-1	67	2
0.67	A-2	2	N-2	28	13
4.00	A	3	N	36	11
5.00	A-1	18	N-1	68	3
1.00	A-2	2	N-2	28	13
6.00	A	3	N	32	9
6.66	A-1	12	N-1	64	4
1.34	A-2	2	N-2	26	12
8.00	A	3	N	26	8

[1] Diet mixes contained casein, 20%; cottonseed oil, 4%; sucrose, 68 to 76%; salts, zero to 8%.

[2] Part 2 (i.e., A-2 or N-2) of each salt mixture was composed of the trace minerals plus sufficient $CaHPO_4$ (Salts N-2) or the NaCl and enough $CaCO_3$ (Salts A-2 to make part 2 equal to 1/6th of the total salt mixture. Part 1 was composed of the remainder of each salt mixture. Salts A or Salts N, then, refers to the complete salt mixture (part 1 plus part 2).

[3] Time for development of a rancid odor.

[4] The stability factor is a measure of the slower development of rancidity with Salts N as compared with Salts A; it is calculated from the day rancidity was observed:

$$\frac{\text{Salts N} - \text{Salts A}}{\text{Salts A}}.$$

TABLE 5

Exudative diathesis in vitamin E-deficient chicks fed chick Salts A and chick Salts N in diet C47D and the effect of sodium selenite mixed in Salts N-2

Salts	4-Week weight	No. of exp.	Exudative diathesis		Encephalomalacia	
			Incidence	Mean time[1] ± S.E.	Incidence	Mortality
	gm		%	*days*	%	%
N	291	5	68	17.3 ± 0.7	8	25
N + Se[2]	375	5	0	—	20	18
A	276	3	75	15.8 ± 0.6	8	21

[1] Time for appearance of exudates.

[2] The 0.219 mg of Na_2SeO_3 (0.1 mg Se/kg diet) was mixed in 1 gm of glucose and then mixed in Salts N-2 (see experimental procedure). The same salt mixture contaniing selenium was fed over a 6-month period.

as well as in the numerous types of diets in which Salts N has been used.

The iodine of potassium iodate was found to be stable when mixed in Salts N; there was no loss after a year's storage.

The stability of selenium present in the form of sodium selenite, in Salts N was tested by its ability to prevent exudative diathesis in vitamin E-deficient chicks (table 5). About two-thirds of the chicks receiving Salts N without selenium developed exudates; whereas, chicks receiving selenium were completely protected against this symptom of vitamin E deficiency. The same selenium-containing salt mixture was used during the 6-month course of these experiments and the selenium was still completely effective at the end of this time. Chicks receiving Salts A in the absence of selenium also developed exudative diathesis. There was a suggestion from these experiments that in the absence of selenium exudative diathesis was favored by Salts A as compared with Salts N. There was some encephalomalacia in all groups.

Adequacy of Salts N for growth and development. Salts N was compared with Salts A in two common types of experimental diets for the chick (table 6). In diet C 50, the protein was supplied by casein and gelatin, in diet C53, by purified soybean protein. Growth of the chicks was equally good with Salts N or with Salts A in each of the two complete diets and its modifications (4 and 16% levels of fat). Elevation of the salts content to 9% did not have any adverse effect. All chicks were healthy and appeared normal upon gross inspection at autopsy. Chicks fed diets containing Salts N for 7-week periods continued to grow and develop normally. Purified diets containing Salts N have supported normal growth and development of guinea pigs, mice, and rats.[3]

When either diet C50 or diet C53 was prepared with Salts N, further supplementation with copper sulfate, ferric citrate, molybdic anhydride and sodium selenite did not result in any improvement in growth (table 7). Sodium molybdate, which is much more soluble in water than is molybdic anhydride, has since been fed to chicks and it also had no effect on growth.

Various levels of magnesium were fed in diet C50 and diet C53 (table 8). With 0.02% of magnesium in diet C50, growth was very poor and almost half of the chicks died. With the same amount of magnesium added to diet C53, mortality was less than with diet C50 and the survivors grew normally. Most of the mortality in chicks fed either diet occurred during the first two weeks of the experiment. Results of the three experiments with diet C50 indicate that 0.03% of magnesium is probably slightly below the requirement, but that 0.04% of magnesium is adequate with this diet.

[3] Unpublished data. Salts N was incorporated at a level of 6% of the diet fed all animals. In addition the guinea pigs received 25 gm of potassium acetate and 5 gm of magnesium oxide per kilogram of diet.

TABLE 6

Growth of chicks fed chick Salts A and chick Salts N in casein and soybean protein diets

Diet	Level of salts	Chick Salts A			Chick Salts N		
		No. of exp.[1]	Mean 4-week weight ± S.E.	Feed efficiency[2]	No. of exp.[1]	Mean 4-week weight ± S.E.	Feed efficiency[2]
	%		*gm*			*gm*	
C50 (4% fat)	6	6	379 ± 5.3	0.58	6	365 ± 7.8	0.64
	9	3	369 ± 15.7	0.63	3	371 ± 6.2	0.61
C50 (16% fat)[3]	6	3	388 ± 11.0	0.65	3	363 ± 19.9	0.64
C53 (4% fat)[3]	6	5	349 ± 13.3	0.59	5	359 ± 8.5	0.60
C53 (16% fat)	6	5	387 ± 8.8	0.69	5	403 ± 8.2	0.70
	9	2	361 ± 20.4	0.67	3	390 ± 11.3	0.70

[1] Eight chicks per group in each of the indicated number of experiments. Mortality was negligible.
[2] Grams of weight gain/gram of diet consumed.
[3] In diet C50, corn oil was incorporated at the expense of dietary glucose to give a total fat content of 16%. In diet C53, the fat content was reduced to 4% and the deficit made up with glucose.

TABLE 7

Effects of supplementary copper, iron, molybdenum, and selenium upon growth of chicks fed chick Salts N in casein and soybean protein diets

Diet	Supplement	No. of exp.[1]	Mean 4-week weight ± S.E.
	mg/kg of diet		*gm*
C50	None	4	372 ± 10.1
	$CuSO_4$, 10; ferric citrate, 200	3	358 ± 11.6
	MoO_3, 3; Na_2SeO_3, 1.1	2	384 ± 15.2
C53	None	4	409 ± 10.3
	$CuSO_4$, 10; ferric citrate, 200	4	408 ± 9.4
	MoO_3, 3; Na_2SeO_3, 1.1	3	412 ± 9.3

[1] Eight chicks/group in each of the indicated number of experiments; mortality was negligible.

TABLE 8

Magnesium requirement of chicks fed chick Salts N in casein and soybean protein diets

Diet	Level of magnesium	No. of exp.[1]	Mortality	4-week weight ± S.E.
	%		%	*gm*
C50	0.02	6	46	284 ± 15.6
	0.03	3	8	355 ± 13.9
	0.04	3	0	374 ± 11.3
	0.06	6	2	377 ± 9.7
C53	0.02	3	29	381 ± 16.9
	0.06	4	3	393 ± 11.0

[1] Eight chicks/group in each of the indicated number of experiments.

DISCUSSION

Chick Salts N offers the following advantages. When used in the diet at a level of 6% it provides each essential inorganic element in only slight to moderate excess of the chick's requirement. It produces minimal destruction of dietary constituents by oxidative rancidity and browning. Both of these conditions guard against inadvertently having an undesirable balance of nutrients in a diet or unknowingly masking a sparing effect by an excess of an inorganic nutrient upon a borderline level of some other dietary essential. The good growth that was observed when either Salts A or Salts N was fed at a level of 9% of the diet indicates that each is a well-balanced salt mixture for the chick. The fact that chicks grew as well with Salts A as with Salts N is undoubtedly due to the high nutritional value of these diets that were used and to the precautions in storing and handling these diets to safeguard against loss of nutrients. Under less ideal conditions, Salts N would be expected to be far superior.

The design of a salt mixture to minimize an effect upon oxidative rancidity in the diet was particularly successful. For example, the mean time required for rancidity to be detectable in the diet mix stored at 37°C and containing 6% of Salts N, was 32 days. Thus, one would expect stability of diets for even longer periods of time at room temperature, as well as very low losses under the usual laboratory procedures for mixing, handling, and refrigerated storage of diets. The success in reducing the pro-oxidative character from the high level of Salts A to the low level in Salts N is probably due primarily to the reduction of trace elements (especially copper and manganese), although other components of the salt mixture are thought to be involved also. Elimination of an accelerating effect upon browning is unquestionably due to omission of the dipotassium phosphate from the salt mixture (Fox and Mickelsen, '59b).

It seemed best to leave molybdenum and selenium as optional trace minerals at this time. Current "purified" diets used for chick experiments contain significant amounts of molybdenum. More work is needed to establish clearly the requirement for molybdenum in the chick. Similarly,

more work is needed to clarify the metabolic role of selenium and its relationship to vitamin E and other antioxidants.

An investigation of magnesium requirements seemed warranted because of the report by Scott et al. ('56) that the chick's dietary requirement for magnesium was only 0.01%, one-fifth of the amount recommended by the NRC. Their level of 0.01% was clearly inadequate in the present studies. Of interest is the poorer response to 0.02% of magnesium that was observed with the casein-gelatin diet as compared with the soybean protein diet. The small contamination of magnesium in the soybean protein, 40 mg/kg of diet (based on the manufacturer's analyses), would not be expected to account for this difference.

SUMMARY

A salt mixture for chicks was designed (1) to provide inorganic elements in only moderate excess of the requirement and (2) to minimize destruction of dietary constituents.

The final mixture, Salts N, supported excellent growth of female New Hampshire chicks from one day to 4 weeks of age when the salt mixture was present at a level of 6% in adequate casein-gelatin or soybean protein diets. Each type of diet was fed with 4 and 16% levels of fat; a 9% level of Salts N was also fed in each type of diet. Growth was not improved with either type of complete diet by supplements of moylbdenum or selenium; these elements are recommended as optional trace elements for the "moderately purified" experimental diets in current use. The requirement for magnesium was less than that recommended by the National Research Council.

In a simplified diet mix, the time required for rancidity to occur was 10 times longer with Salts N than with Salts A, previously used in this and other laboratories. Salts N did not accelerate the Maillard-type of browning in glucose-glycine mixtures or in complete diets containing varying quantities of free amino acids. The iodine in potassium iodate and the selenium in sodium selenite were found to be stable in Salts N.

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The Influence of Sleep, Work, Diuresis, Heat, Acute Starvation, Thiamine Intake and Bed Rest on Human Riboflavin Excretion[1]

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Riboflavin is excreted in urine, feces and sweat, but the amount found in the urine is the only useful index of its metabolic fate. The influence of various nutritional and physiological factors on the urinary excretion of riboflavin has been reviewed by Bro-Rasmussen ('58). The present studies were conducted to assess the effects on urinary riboflavin excretion of several environmental factors which up to now have been investigated insufficiently, and to elucidate some of the physiologic mechanisms involved in the metabolism of riboflavin as reflected in its urinary excretion.[4]

METHODS

Riboflavin was determined fluorometrically by a modification of the procedure of Conner and Straub ('41). The blank values were determined by irradiation or by alkaline digestion as described by Swaminathan ('42). Excretion of thiamine was determined as described by Mickelsen et al. ('45). The measurement of renal plasma flow during moderate exercise has been described by Chapman et al. ('48). Specimens of urine were collected in dark-brown bottles, preserved with 5 ml of glacial acetic acid and 3 ml of toluene, and stored in darkness at 3°C until analyzed.

EXPERIMENTS AND RESULTS

A. Normal rate of urinary excretion of riboflavin. The rate of urinary excretion of riboflavin was determined for 12 normal men receiving uncontrolled diets. From 4 to 8 urine samples were collected when the subject was awake and one for the time he was asleep. To save space, the observations for only a few subjects are shown. The data for the subjects omitted from table 1 are similar to those for subject WOC.

A highly significant decrease in the hourly rate of riboflavin excretion occurred during sleep ($P < 0.01$). The rate of excretion during the waking hours when the subjects were receiving uncontrolled diets was surprisingly constant for each individual on any particular day. For most subjects, the highest hourly rate of excretion was no more than three times the lowest rate. For some subjects, especially those who showed some low rates of excretion, the ratio between the two extreme values was as much as 8.5 (LS, HG, SK). In one subject (AH) this ratio was 12 and was probably due to the ingestion of liver at the meal just preceding the high rate of riboflavin excretion. In contrast with the small intra-individual variation, the interindividual values varied by as much as 40-fold. From published figures relating dietary intake to urinary excretion (Keys et al., '50) it would appear that only two subjects (FL and SK) were ingesting much less than the recommended allowance of

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[4] Merck and Company, Inc. provided a generous supply of pure vitamins. Most of the food materials used in some of the dietary studies were supplied by the Subsistence Branch, Office of the Quartermaster General, U. S. Army.

TABLE 1

Urinary excretion of riboflavin by normal men fed uncontrolled diets (μg/hour)

Subject		Age	Daytime periods[1]							Average		24-hour total
			1	2	3	4	5	6	7[2]	Day 1–7	Night 8	
WOC	1	28	40.6	30.5	27.2	38.6	37.3	91.8	—	58.1	35.8	1270
	2		34.3	29.8	85.5	53.2	44.9	44.7	—	47.2	22.5	928
OM	1	35	55.4	71.2	61.4	86.7	65.7	51.9	63.3	64.4	45.5	1381
	2		58.5	52.0	71.2	66.6	41.4	48.2	48.7	54.7	41.4	1129
AH	1	41	21.2	27.4	16.3	36.8	184.2	199.0	—	91.3	81.5	2148
	2		30.2	23.5	36.6	32.2	17.0	26.1	—	27.4	15.0	545
LS	1	26	58.2	60.0	35.1	102.4	50.9	12.1	—	44.3	13.8	831
	2		108.1	76.6	67.3	50.7	77.8	53.5	—	68.7	29.3	1316
HG	1	20	14.6	12.6	11.2	11.2	5.7	19.8	—	13.6	10.0	298
	2		37.2	44.6	26.7	49.1	32.7	37.8	—	37.4	33.3	865
RP	1	23	86.5	39.2	98.9	77.4	78.3	36.4	—	66.3	39.5	1324
	2		42.9	71.3	43.9	58.6	83.4	51.0	—	60.2	56.0	1397
SK	1	35	33.4	35.3	38.9	19.9	6.9	15.8	(16.0)[2]	22.1	10.2	464
	2		33.3	19.2	18.0	—	—	—	(23.8)	21.4	13.3	441

[1] Periods 1–7 were of 2–4 hours duration while the men were awake.
[2] Samples indicated in parentheses were collected on the following day in order to complete the 24-hour test period.

1.8 mg of riboflavin per day (Food and Nutrition Board, '58).

The decreased rate of excretion of riboflavin during sleep can be accounted for partly by the tapering off of absorption of riboflavin from the intestine and partly by decreased renal clearance. Since the ingestion of food is interrupted longer during sleep than at any other part of the 24-hour cycle, the amount of riboflavin taken into the circulation by gastrointestinal absorption is curtailed. Under normal conditions of eating and sleeping, less blood passes through the kidneys during sleep; consequently, the amount of riboflavin filtered into the urine decreases.

B. Effect of work. Experiment 1. The effect of work performed on a motor-driven treadmill was determined in 7 normal men (age 20 to 46 years). Control urine samples were collected during a two-hour period while the men rested. The subjects then walked for 45 minutes on a motor-driven treadmill in an air-conditioned room. The walking ranged from 3 to 5 miles per hour with the angle of the treadmill from 5 to 7%. The work was tiring but not exhausting. Sweating was negligible. After walking for this period, the men rested for another hour. Urine was also collected after the work-stress, and after the hour's rest (table 2). Seven subjects collected samples of urine on days when they did not walk on the treadmill; apart from the work, the procedure followed was exactly the same as that on the exercise day.

For the work-days, the average urinary excretion of riboflavin during the first, second and third rest periods was 158, 138

TABLE 2

Hourly rates of urinary riboflavin excretion during rest as a percentage of the excretion during work

Subject	Rest		Work	Rest
	Work days			
RT	127	82	100	97
JA	322	190	100	183
JA	263	301	100	148
JA	258	145	100	185
LG	120	120	100	217
LG	149	145	100	158
CM	125	127	100	144
BS	59	49	100	112
OM	113	132	100	177
OM	89	141	100	92
AA	108	87	100	137
Group mean	158	138	100	150

Subject	Rest			
	Control days			
OM	168	161	100	119
OM	111	59	100	100
EB	176	133	100	107
CP	106	50	100	56
RY	122	118	100	107
ZT	104	100	100	124
RK	80	49	100	38
MS	111	56	100	61
Group mean	122	91	100	89

TABLE 3

Effect of constant injection of epinephrine on renal clearance of p-aminohippurate and rate of urinary excretion of riboflavin in a fasting woman

Period	Rate of injection of epinephrine	Renal plasma clearance	Excretion of riboflavin
	μg/hour	*ml/minute*	*μg/hour*
1	0	316	39.0
2	0	385	52.0
3	50	287	39.3
4	50	306	37.7
5	100	266	28.1
6	300	234	23.8

and 150% of the work values, respectively. These values are statistically greater than those for the work stress ($P < 0.05$). A similar decrease did not occur on control days indicating that the reduction during work was not due to a diurnal variation.

From a theoretical standpoint, one might expect a reduction in excretion of riboflavin during strenuous work associated with a reduction in renal plasma flow. This has been shown by Chapman et al. ('48) to occur during similar work on a treadmill. To assess the effect of reduced renal plasma flow on urinary excretion of riboflavin, 6 observations were made on a fasting female subject whose renal clearance was reduced by constant injection of epinephrine (table 3). The product-moment correlation between renal plasma clearance of *p*-aminohippurate and urinary riboflavin in this subject was 0.967, which is statistically significant ($P < 0.01$). In other words, as the renal clearance of *p*-aminohippurate decreased, the excretion of riboflavin was reduced proportionately.

Experiment 2. Nine normal young men were maintained on a constant intake of 2 mg of riboflavin per day. During the control period of a number of months these men became accustomed to a work schedule of walking on a treadmill at a rate requiring 3300 Cal. per day for maintenance of body weight. Urinary excretion of riboflavin during this control period averaged 285 μg per day (fig. 1).

The walking schedule was increased suddenly so that 5500 to 6000 Cal. per day were required for maintenance of body weight. In spite of a constant intake of riboflavin, the severe physical work reduced the mean urinary excretion of riboflavin to 137 μg per day by the third day ($P < 0.01$).

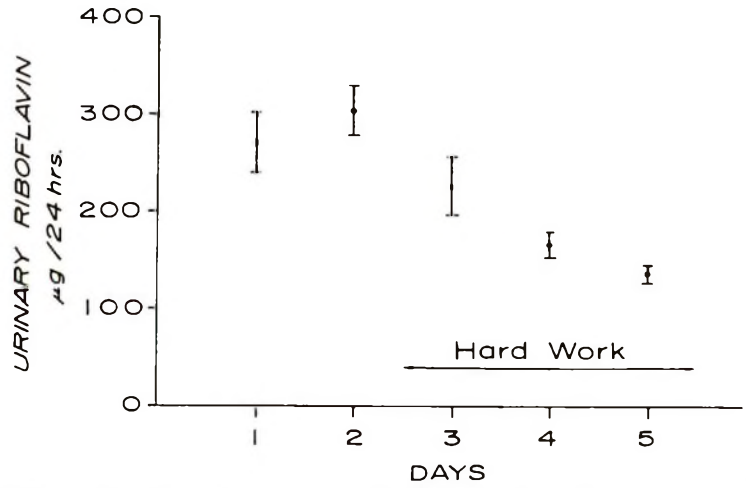

Fig. 1 Effect of hard work on excretion of riboflavin (exp. 2); mean data ± standard error.

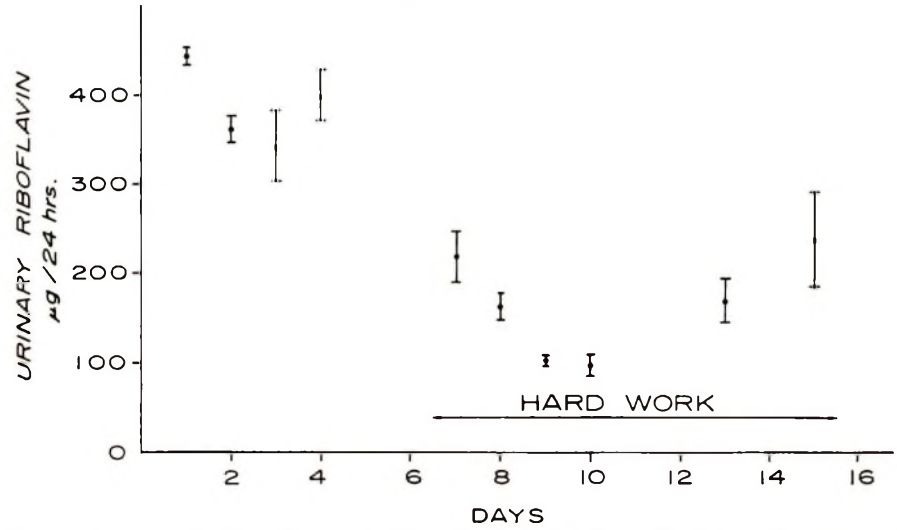

Fig. 2 Effect of hard work on excretion of riboflavin (exp. 3); mean data ± standard error.

Experiment 3. A month after the above experiment 5 of the men were subjected to a repetition of the above work-stress except that the test was continued for 9 days. The 24-hour urinary excretion of riboflavin averaged 386 µg per day in the control period, decreased to 97 µg on the 4th day of hard work ($P < 0.01$), and then increased gradually to 237 µg by the 9th day (fig. 2). The latter value still differed significantly from the control value ($P < 0.05$).

All three experiments produced a reduction in the rate of urinary riboflavin excretion during work. In the acute experiment, which lasted only 45 minutes (exp. 1), the decrease can be attributed largely to a reduced renal circulation. However, the decreased excretion when hard work was performed for three or 9 days must be explained in a different way. It cannot be attributed to a change in renal blood flow, since these men were relatively inactive during the major portion of the day. The riboflavin excretion during the latter period should have overcome in part the reduced excretion of riboflavin associated with reduced renal blood flow during the period of hard work. Delachaux and Ott ('43) observed decreased urinary excretion of riboflavin during several hours of mountain climbing and during three weeks of physical training. Our results are consistent with their findings. Friedmann et al. ('49) also observed that urinary riboflavin tended to decrease during physical activity.

There is no explanation for the reduced riboflavin excretion during work. *A priori* reasoning suggests that physical exercise is associated with the formation of new muscle tissue. Axelrod et al. ('41) have shown that human muscle contains about 3 µg of riboflavin per gm of fresh tissue. From these data one can calculate that it would require no more than the synthesis of 30 to 50 gm of new muscle tissue daily to account for the observed decrease in riboflavin excretion during prolonged hard physical work.

C. Effect of diuresis. Three experiments were conducted to determine whether or not riboflavin could be "washed" out of the body:

Experiment 1. Eight normal men received a basic diet supplying 0.64 mg of riboflavin daily; one-half received a supplement of 1.0 mg of riboflavin daily (Keys et al., '45). On certain days each subject measured his total intake of fluid. On the following day, the intake of fluid was doubled. Tweny-four hour urine samples were analyzed for riboflavin (table 4). For both the subjects on the high and those on the low intakes of riboflavin there was no change in urinary excretion values for the vitamin in spite of an almost three-fold increase in urinary volume.

Experiment 2. Nine normal young men were fed an approximately constant daily intake of 1.76 mg of riboflavin for two months. On the last two days, the intake of fluids was measured. On the following two days the intake of fluids was doubled and the intake of riboflavin was increased inadvertently to an average of 1.92 mg. The 24-hour urinary excretion of riboflavin averaged 643 μg during the control period and 751 μg during the periods of forced fluids; the urinary volumes average 1175 and 2786 ml, respectively. The slight increase in riboflavin excretion during the period of diuresis ($P < 0.05$) becomes insignificant when the excretion is calculated as percentage of intake (36.6 and 39.1% for the control and diuretic periods, respectively). The data for this experiment have been omitted, since they are similar to those obtained in experiment 1.

Experiment 3. Along with studies of renal plasma clearance (Chapman et al., '48) the effect of severe diuresis on the excretion of riboflavin was tested. Five tests were conducted on 4 normal young men. Urine was collected during a control period of several hours in the morning. The subjects then rested in bed while drinking large quantities of water. Urine was collected during the following two-to-three-hour period of diuresis, and analyzed for riboflavin (table 5). In 4 of the 5 tests the diuresis, which resulted in an almost 9-fold increase in urinary volume, was associated with a reduced rate of riboflavin excretion; the reduction, however, was not statistically significant.

The above experiments indicate that water-diuresis has no effect on the urinary excretion of riboflavin by normal subjects. This holds true at both high and low intakes of this nutrient.

TABLE 4

Effect of diuresis on urinary excretion of riboflavin (experiment 1). These men were maintained on a constant intake of riboflavin

Subject	Riboflavin intake	Control period			Period of Diuresis		
		Date	Riboflavin excretion	24-hour urine vol.	Date	Riboflavin excretion	24-hour urine vol.
	mg/day		*μg*	*ml*		*μg*	*ml*
G	0.64	6/29	101	890	6/30	206	3600
		8/30	162	885	8/31	248	2300
WI	0.64	6/29	155	580	6/30	114	1165
		8/30	204	730	8/31	112	1500
WA	0.64	6/29	207	1340	6/30	186	3810
		8/30	140	1235	8/31	180	3180
T	0.64	6/29	188	1485	6/30	122	3150
Average			165	1021		169	2672
S	1.64	6/29	422	1175	6/30	367	4370
		8/30	282	925	8/31	351	3260
JA	1.64	8/30	526	755	8/31	527	1870
N	1.64	8/30	490	1085	8/31	425	2600
JO	1.64	8/30	488	1320	8/31	643	4210
Average			442	1052		482	3262

TABLE 5

Effect of severe diuresis on the urinary excretion of riboflavin

Subject	Control period			Period of Diuresis		
	Duration	Volume	Riboflavin	Duration	Volume	Riboflavin
	hours	*ml/hour*	*μg/hour*	*hours*	*ml/hour*	*μg/hour*
RH 1	3.8	53	32.6	1.9	500	20.7
RH 2	4.2	96	31.8	2.6	830	26.2
BS	4.0	56	55.0	3.0	359	39.6
ET	3.8	74	68.1	2.1	492	38.5
SN	3.5	76	63.1	2.1	876	78.6
Mean values		71	50.1		611	40.7

D. Effect of heat-stress. Twelve normal men not acclimated to high temperatures were kept for 7 weeks in quarters maintained at approximately 26°C and 50% relative humidity. They were allowed outdoors daily. The outdoor temperature during the experiment was near freezing or below. They were trained to walk on a motor-driven treadmill for 4 to 6 hours daily (Taylor et al., '43). For 6 days the temperature in the working chamber was maintained at 49°C and 30% relative humidity. The men spent 10 hours each day under these conditions continuing the work output of the control period. The remainder of the day was spent in living quarters kept at 32 to 5°C and 30% relative humidity. During the heat-stress, the men were not permitted out-of-doors. They drank adequate amounts of water at this time, and the diet contained sufficient sodium chloride, and provided 2.1 mg of riboflavin per day throughout the control and test periods. None of the men showed signs of severe distress as a result of the exposure to the high temperature.

During the control period, the 24-hour urinary excretion of riboflavin averaged 443 μg (fig. 3). Riboflavin excretion increased gradually during the heat-stress and reached a maximum of 692 μg on the 6th day of stress ($P < 0.01$). This increased riboflavin excretion occurred in spite of a constant intake. Riboflavin excretion returned to control values slowly. Some subjects showed elevated excretions 8 days after the termination of the heat-stress.

The increased excretion of riboflavin observed during heat-stress in man is consistent with previous reports on animals. Mitchell et al. ('50) reported that swine showed increased excretion of riboflavin during heat-stress. Similar results were reported by Worden and Waterhouse ('55) for the dog. This could be interpreted as showing a decreased requirement of riboflavin at high temperatures.

E. Effect of acute starvation. Experiment 1. Three young men were deprived of all food for three days (water ad libitum) while they worked at a rate which would have required 5800 Cal. per day for caloric equilibrium. These men were conditioned physically before the experiment to eliminate any training effect. During the 7-day control period the men ingested 2.1 mg of riboflavin daily and excreted an average of 586 μg in the urine (fig. 4). During acute starvation, the average urinary excretion rose to 1.76, 1.87 and 1.44 mg, on the first, second and third days, respectively. The average weight lost during the three days of starvation was 4.99 kg per man. Although the body-weight of each man decreased at a fairly uniform rate during this period, his urinary excretion of riboflavin increased very markedly on the first day or so and then returned in one subject, almost to the pre-starvation level.

Experiment 2. In a second experiment three men received nothing but water ad libitum for 7 days, during which they led

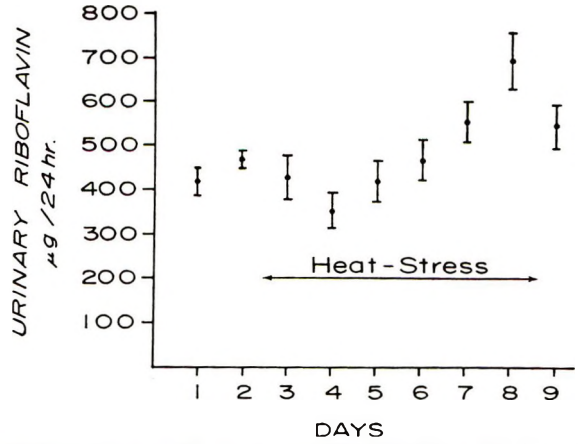

Fig. 3 Effect of heat-stress (49°, 30% relative humidity, 10 hours/day) on excretion of riboflavin, mean data ± standard error.

a very sedentary life. At the end of the control period the men were excreting an average of 32 μg of riboflavin per day (fig. 5). The rate of excretion of riboflavin increased slowly during starvation and reached an average maximum of 167 μg on the 7th day. When the subjects again were placed on the control regimen, the average rate of excretion dropped to 48 μg by the third day of the recovery period.

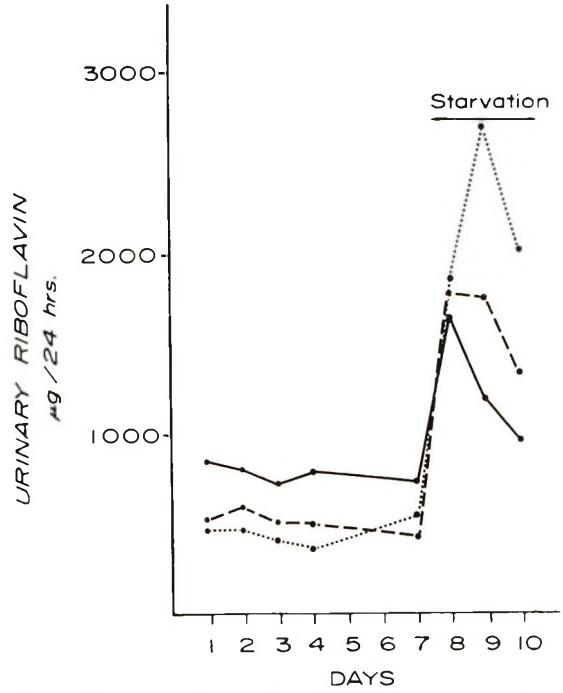

Fig. 4 Effect of a combination of hard work and acute starvation on excretion of riboflavin (exp. 1).

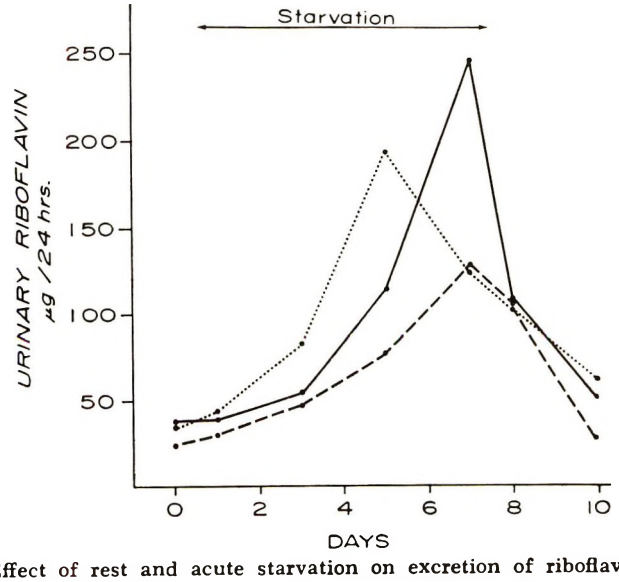

Fig. 5 Effect of rest and acute starvation on excretion of riboflavin (exp. 2).

TABLE 6

Relation between increased excretion of riboflavin and loss of protein during bed rest

Subject	Period	Average increase in riboflavin excretion[1]	Average loss of protein	Δ riboflavin excretion / Daily protein loss
	days	*μg/day*	*gm/day*	
RM	21	544	5.6	97.1
	28	154	8.8	17.5
DM	21	646	20.1	32.1
	28	434	13.6	31.9
AW	21	190	8.5	22.4
ES	21	681	11.6	58.7
	7	348	21.0	16.6
	21	1355	11.1	122.1
LB	21	237	5.3	44.7
GW	21	285	15.5	18.4
Average		487	12.1	40.2

[1] The excretion of riboflavin during the pre-bed-test period was subtracted from that for the days when the men were in bed.

F. Effect of bed rest. The effect of bed rest on urinary riboflavin excretion was studied in 6 normal young men. Details of this study have been described previously[5] (Taylor et al., '45; Miller et al., '44). During the week preceeding the bed rest, the subjects went through a period of strenuous physical conditioning. The period of bed rest ranged from 7 to 28 days. When the men were in bed, their caloric intake was reduced from a level of 4800 Cal. per day to 2500 with a reduction in protein from 75 gm per day to 55 gm; the intake of riboflavin also dropped from 1.93 mg per day to 1.62 mg. Despite the reduced dietary intake, the excretion of riboflavin increased. The average increase over that during the pre-bed-rest period was 487 μg per day (table 6). There was some individual difference in the day-to-day excretion of riboflavin. Some men showed a constantly high excretion during the bed-rest period, while others showed considerable fluctuation. In all cases, however, the excretion during bed rest was greater than during the pre-bed-rest period.

The caloric intake was adjusted throughout this study to maintain constant body weight. In spite of the constancy of their body weights, the men lost an average of 12.1 gm of protein per day during bed rest. Although the excretion of riboflavin was elevated during the period of negative nitrogen balance, the correlation between these two values is insignificant as shown by the 7-fold variation in the ratio of riboflavin to nitrogen excretion (table 6).

Within a day or so after the men got out of bed, the urinary excretion of riboflavin decreased to pre-bed-rest values whereas nitrogen was retained for a week or more.

There are a number of reports implying that the increase in urinary riboflavin excretion during periods of negative nitrogen balance (e.g., starvation, bed rest, trauma, etc.) results from the breakdown of body tissue (Oldham et al., '47; Pollack and Bookman, '51; Roderuck et al., '46; Dean and Holman, '50). At best, the correlations between the increase in riboflavin and nitrogen excretion are only suggestive of such a relationship. The present study raises some doubt as to whether the excretion of nitrogen and riboflavin are causally associated. This doubt is based on (1) the reduced urinary excretion of riboflavin seen on the third day of starvation: the reduction occurred even though the men continued to lose weight at a constant rate; (2) the great variability in the ratio of riboflavin to nitrogen excretion seen during bed rest and (3) the low riboflavin ex-

[5] Miller, E. V., O. Mickelsen, W. W. Benton and A. Keys 1945 The effect of bed rest on mineral and nitrogen balances. Federation Proc., 4: 99 (abstract).

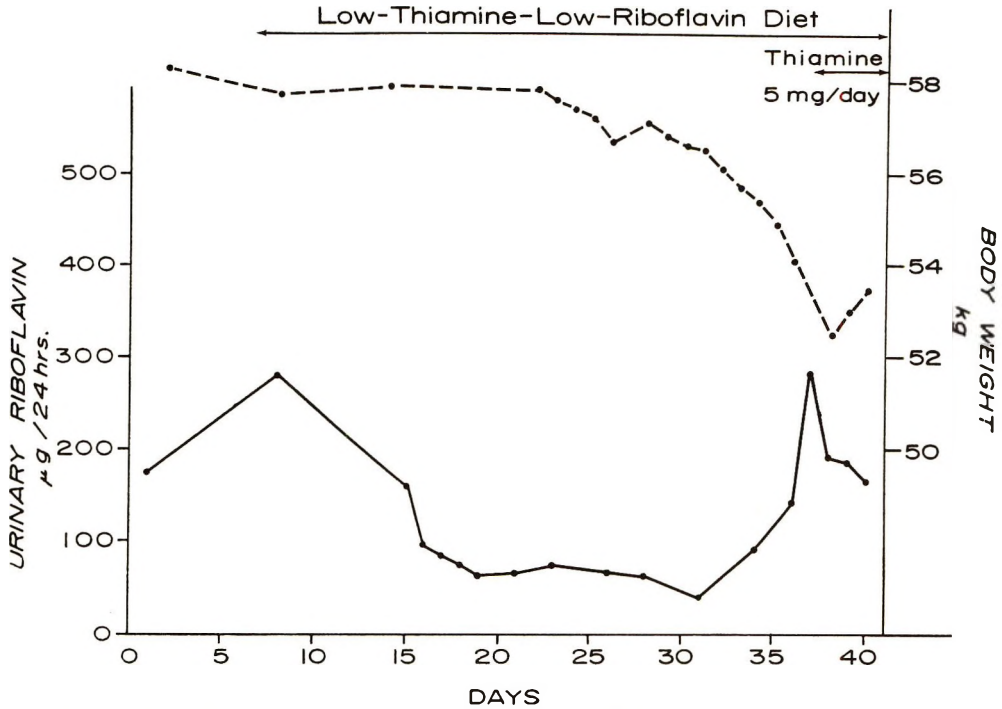

Fig. 6 Effect of deprivation of thiamine and riboflavin on body weight (broken line) and excretion of riboflavin (solid line), mean data (exp. 1).

cretion during acute thiamine deficiency when there is a marked loss of body weight.

G. Relation of riboflavin to thiamine. Experiment 1. Three normal young men received a purified diet supplemented with the synthetic vitamins except thiamine, riboflavin and niacin (Keys et al., '45). Excretion of thiamine, if not already negligible, decreased within a few days to zero, and no thiamine was excreted until some time after the vitamin had been returned to the diet. The 24-hour excretion of riboflavin decreased over 14 days to values of 15 to 100 μg per 24 hours (fig. 6). After about 4 weeks the excretion of riboflavin started to increase but never approached the high levels seen in other conditions (e.g., starvation) associated with a comparable loss of body weight. During the period of acute deficiency, these three men lost an average of 5.5 kg, and showed typical symptoms of thiamine deficiency. Supplementation with 5 mg of thiamine per day produced an increase in weight as well as a decrease in the excretion of riboflavin.

The 4 control subjects were maintained with the same diet supplemented with 1 mg of thiamine, 1 mg of riboflavin and 10 mg of niacin per day. No weight change occurred in these men at any time, and they remained normal throughout the entire experiment. The urinary riboflavin increased throughout this period in all 4 men. One subject excreted almost 70% of his intake. Another subject never excreted less than 40% of his intake of riboflavin, and his excretion occasionally increased to as much as 90%. These two subjects had been ingesting 1.8 mg of riboflavin for the 6 months preceding the experiment. The other two men who received only 0.8 mg per day for the 6 months preceding the experiment also showed an increase in their urinary excretion of riboflavin; toward the end of this experiment they were excreting about 40% of their intake.

Experiment 2. In a second experiment, a diet devoid of thiamine (Anderson et al., '46) was fed to three young men who had been maintained for 6 months on an intake of 1 mg of thiamine and 2 mg of ribo-

flavin. Body weight had been kept constant during the 6 months by adjustment of caloric intake. This thiamine-free diet provided 2 mg of riboflavin per day. As the thiamine deficiency progressed, the excretion of riboflavin increased for the individual subjects from 592, 752 and 1010 μg per day during the week preceding the start of the deficient diet to 1860, 2662 and 3770 μg per day for the last 4 days of the deficiency. The deficient diet was fed to these subjects for 23, 22 and 17 days, respectively. During the thiamine deficiency, these men lost 7.3, 6.8 and 3.2 kg, respectively. As soon as thiamine was restored to the diet, the urinary excretion of riboflavin started to decrease. No urinary analyses of riboflavin were made on the control men receiving thiamine during this phase of the study.

It is difficult to correlate the excretion of riboflavin with any peculiarity of the low-thiamine diet or with the weight changes in the men. The diet supplied 48 gm of casein per man per day; about 30% of the calories were from fat. The most important question is why the excretion of riboflavin was so low in the group receiving no thiamine or riboflavin. In spite of considerable weight loss, urinary excretion did not increase to any great extent; therefore, the riboflavin coming from the muscles must have been catabolized or stored in other tissues. Perhaps some storage took place in the liver (Singher et al., '44).

SUMMARY

Urinary excretion of riboflavin was studied in normal men under a variety of environmental conditions. Diurnally, meals tended to increase and sleep to retard the rate of excretion. Short periods of hard physical work were accompanied by a decreased excretion of riboflavin which appeared to be related to decreased renal plasma flow. Diuresis, induced with water, was without effect on the excretion of riboflavin. Acute starvation increased the excretion of riboflavin approximately threefold when men performed hard physical work for three days and approximately 5-fold when starved for 7 days under sedentary conditions. Heat stress (49°C and 30% relative humidity 10 hours per day for 6 days) increased the mean excretion of riboflavin gradually from 443 μg per day initially to a maximum of 692 μg per day. Enforced bed rest increased the mean excretion of riboflavin by 487 μg per day in spite of a concomitant reduction in riboflavin intake of 0.31 mg per day. Thiamine deficiency alone led to an immediate increase in riboflavin excretion. In combined thiamine and riboflavin deficiency, the increased excretion of riboflavin was observed only after 4 weeks of deprivation. In both cases, the increased excretion of riboflavin was restored to normal when the subjects were given 5 mg per day of thiamine.

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

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

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

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