SERUM VITAMIN C OF IOWA SCHOOL CHILDREN AND ITS RELATIONSHIP TO DIET AND AGE [1]

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ONE FIGURE

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Vitamin C has been measured in the serum of children in various geographic areas of the United States (Moyer et al., '48; Clayton et al., '53; Babcock et al., '53; Williams et al., '51; Moschette et al., '52; Storvick et al., '51; Mack and Urbach, '48; Bessey and Lowry, '47). Serum concentrations of vitamin C considerably below those which indicate tissue saturation appear to be widespread. In controlled experiments, changes in intake of vitamin C are reflected rapidly by changes in the concentration of this nutrient in fasting blood samples. Less clear-cut, but significant, relationships between intakes and serum concentrations have been observed in the surveyed populations.

Information about the nutritional status of Iowa school children with respect to vitamin C has been obtained by a survey carried out from 1949 to 1951. The present report is concerned with children from 6 to 18 years of age in Iowa. Data from the 9-, 10-, and 11-year-old children also will be analyzed along with data from children of the same age group in Ohio and Kansas and will be published separately.

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EXPERIMENTAL PROCEDURE

Children were selected randomly by age and sex within elementary and junior and senior high schools which were sampled from two population groups in Iowa. Eleven elementary schools were located in cities with over 50,000 people and 26 elementary and 7 junior and senior high schools were located in cities and towns with a population of less than 50,000. The entire sample used in the study of serum concentrations consisted of 705 children from these 44 schools. Permissions were obtained from parents for the children to participate in the study. Refusals necessitated replacements but accounted for less than 10% of the sample. The children were requested to eat no fruit for breakfast on the day that their finger-tips were to be pricked. Blood samples were collected within two hours after the children arrived at school. Seven-day dietary records were obtained for 692 children, serum ascorbic acid concentrations for 655.

Vitamin C in the serum was analyzed by the microchemical procedure (Lowry et al., '45, and Bessey et al., '47) and the nutritional values of the diets were calculated with the use of the USDA Agriculture Handbook number 8 (Watt and Merrill, '50).

RESULTS AND DISCUSSION

Means, standard deviations and ranges by sex and age for the concentration of serum vitamin C are presented in table 1. Sex differences in serum vitamin C levels of these children were not apparent. A sequential range test (Hartley, '54) indicated that the serum concentration of vitamin C differed at successive ages. For boys, significant differences occurred from 8 to 9, 9 to 10, 11 to 12, 13 to 14 and 17 to 18 years. For girls, differences were significant from 10 to 11, 12 to 13, 13 to 14, 14 to 15, 15 to 16 and 16 to 17 years of age. While these differences from year to year were too large to attribute to reasonable sampling fluctuation there was no consistency as to direction as may be seen in table 1. For boys, however, there was a preponderance of significant negative differences

indicating a general decline in serum concentration of vitamin C with age (see below).

The mean intakes of vitamin C (table 1) remained the same or increased slightly with increasing age. Deviation of mean intakes have been reported by Eppright and others ('54b). It

TABLE 1

Average daily intakes and serum concentrations of vitamin C of Iowa children sampled from 44 schools in 1949–1951

AGE	NO.	MINIMUM	MEAN	MAXIMUM	STANDARD DEVIATION	MEAN INTAKE
yrs.		*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/day*
			BOYS			
6	21	0.30	0.91	2.18	0.57	78
7	26	0.16	0.83	1.79	0.46	62 (25)[1]
8	37	0.15	0.86	2.00	0.51	74
9	35	0.15	1.07	1.85	0.57	80
10	32	0.14	0.80	1.74	0.43	74
11	24	0.33	0.88	1.87	0.48	82
12	64	0.22	0.72	1.74	0.39	87 (62)
13	27	0.15	0.70	1.81	0.50	91
14	17	0.15	0.56	1.60	0.35	89 (16)
15	14	0.19	0.50	0.88	0.22	86
16	15	0.15	0.56	1.19	0.35	115 (14)
17	7	0.21	0.60	1.94	0.32	78 (6)
18	10	0.13	0.47	1.34	0.42	91 (12)
All boys	329		0.78			82 (325)
			GIRLS			
6	24	0.19	0.96	1.87	0.53	66
7	31	0.26	1.05	2.10	0.49	71
8	25	0.20	0.94	2.50	0.61	79 (24)
9	37	0.14	0.94	2.47	0.58	82
10	28	0.28	1.00	1.92	0.58	87
11	34	0.23	0.70	1.64	0.35	73 (33)
12	62	0.16	0.73	2.29	0.44	86 (58)
13	25	0.12	0.49	1.27	0.31	69
14	13	0.09	0.58	1.69	0.46	80
15	15	0.12	0.47	1.25	0.29	85
16	15	0.16	0.67	1.62	0.50	78
17	10	0.22	1.01	1.95	0.62	76
18	7	0.27	1.00	1.69	0.56	94
All girls	326		0.81			79 (320)

[1] Numbers in parentheses indicate difference in the number of dietary records available as compared to number of serum vitamin C determinations.

appears that beyond 11 or 12 years of age, mean serum vitamin C concentrations were frequently lower than 0.8 mg %, which many nutritionists believe is desirable, although mean intakes similar to the Recommended Allowances were associated with these blood values. During teen-age years, stresses accompanying physical changes may increase the need for vitamin C.

TABLE 2

Distribution of Iowa children according to classification by serum vitamin C levels

SEX	AGE	SERUM VITAMIN C: Poor Below 0.4 mg %		Fair 0.4–0.6 mg %		Good 0.7–1.0 mg %		Excellent 1.01 + mg %		TOTAL
	yrs.	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*
Boys	6,7,8	19	(23)[1]	15	(18)	17	(20)	33	(39)	84
	9,10,11	17	(19)	18	(20)	16	(18)	40	(44)	91
	12,13,14	28	(26)	30	(28)	28	(26)	22	(20)	108
	15 +	20	(43)	9	(20)	13	(28)	4	(9)	46
	Total	84	(26)	72	(22)	74	(22)	99	(30)	329
Girls	6,7,8	11	(14)	16	(20)	15	(19)	38	(47)	80
	9,10,11	18	(18)	20	(20)	29	(29)	32	(33)	99
	12,13,14	34	(34)	24	(24)	21	(21)	21	(21)	100
	15 +	16	(34)	11	(23)	6	(13)	14	(30)	47
	Total	79	(24)	71	(22)	71	(22)	105	(32)	326

[1] Figures within parentheses are percentages of the totals given in the last column.

The children in this study have been classified by serum vitamin C concentrations as suggested by Bessey and Lowry ('47) and the distribution is presented in table 2. More of the older boys and girls than of the younger were in the poor and fair categories. The greatest increase in the percentage of children falling into these two categories occurred from the 9-, 10-, and 11-year-olds to the 12-, 13-, and 14-year-olds. About 50% more boys and girls were classified as poor or fair in the latter group than in the former.

Since the reported value of vitamin C in serum is a concentration figure, the intakes of this nutrient were calculated

per kilogram of body weight in order to make some adjustment for differences in body size. Decreasing mean intakes per kilogram body weight and decreasing mean concentrations of vitamin C in the blood serum both accompanies increasing age (fig. 1). The vitamin C concentrations in the serum of girls older than 14 years of age were an exception to this generaliza-

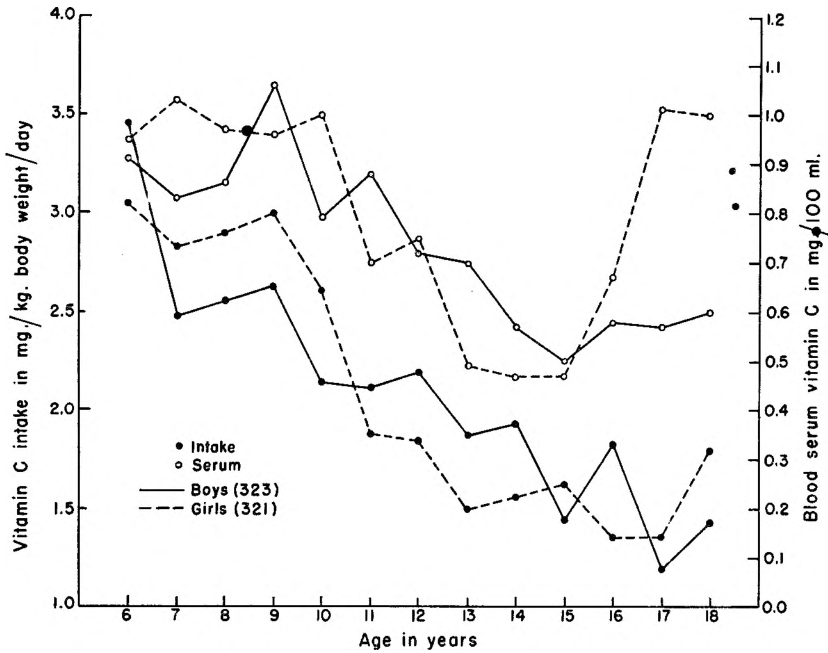

Fig. 1 Changes in mean intakes and mean serum concentrations of vitamin C with age for Iowa children sampled from 44 schools in 1949–1951 and for whom both serum values and dietary records were available.

tion and they have been handled separately in a subsequent analysis in this paper. The correlation coefficient (r) between intake per kilogram of body weight and serum concentration of vitamin C was 0.53 for boys and 0.47 for girls. When total vitamin C intake per day and serum vitamin C were considered, r equalled 0.39 for both boys and girls. Hence, the intake per kilogram of body weight was better than intake per day for predicting serum concentration of the nutrient.

When vitamin C from tomatoes, citrus fruits, raw cabbage, cantaloupes and some berries was considered, the correlation of this fraction of the total vitamin C intake and serum vitamin C was 0.4[illegible] for the boys in this study, essentially the same value as that when total intake of vitamin C was used. Correlation between vitamin C-rich foods and total vitamin C intake was 0.93, between "other" sources of vitamin C and total vitamin C intake, $r = 0.47$, and between vitamin C-rich foods and "other" sources of vitamin C, $r = 0.11$. These correlations may be interpreted to mean that "other" sources of vitamin C were a smaller and more variable fraction of the total intake than the vitamin C-rich foods, and that the change in intake of vitamin C-rich foods accounted for 86% of the change in the total vitamin C intake. "Other" sources of vitamin C are also more uncertain sources since they are usually exposed to cooking losses. Thus it appears that knowledge of the vitamin C-rich foods in the diet provided as much information as total vitamin C intake for predicting serum levels of vitamin C for the boys in this population, although neither was a precise predictor.

Regression equations have been calculated for the data from girls. Age and intake of vitamin C from fruits and vegetables per kilogram of body weight per day were considered as factors influencing the blood serum concentration of vitamin C. When Y = blood serum vitamin C, X_1 = vitamin C from fruits and vegetables per kilogram body weight per day, and X_2 = age in years, then, from 6 to 14 years of age $Y' = 63.2 + 0.18X_1 - 0.19X_2$, and for 15 years and above $Y' = 203.9 + 0.23X_1 + 1.22X_2$. These two age groups were chosen because an apparent change in relationship of intake to blood serum vitamin C was observed in figure 1. The coefficients for intake (0.18 and 0.23) were not different for the two age groups, but the coefficients for the age factor (− 0.19 and 1.22) were significantly different and changed from a negative to a positive relationship with age. It is possible that a similar change in trend of the concentration of vitamin C in the blood serum is associated with age among boys, too, but the apparent

change in direction may not occur until about 17 years of age and the number of observations in this survey beyond that age was inadequate for analysis.

Total dietary intakes were grouped into (1) those diets meeting all of the Allowances recommended by the National Research Council (2) those having some nutrient less than 100% of the Allowance but none less than 67%, and (3) those

TABLE 3

Serum and dietary ascorbic acid of Iowa survey children whose intakes of nutrients met all the National Research Council allowances or were lacking one-third of some single nutrient

AGE	GROUP I [1]			GROUP II [2]		
	No.	Serum	Intake	No.	Serum	Intake
yrs.		*mg/100 ml*	*mg/day*		*mg/100 ml*	*mg/day*
			BOYS			
6,7,8	33	0.96	90	21	0.62	46
9,10,11	25	1.07	102	25	0.68	52
12,13,14	19	0.92	137	43	0.60	68
15 +	4	0.90	158	15	0.35	61
			GIRLS			
6,7,8	23	1.22	102	18	0.81	46
9,10,11	14	0.94	104	29	0.64	57
12,13,14	9	0.72	116	48	0.56	62
15 +	2	0.56	149	33	0.66	77

[1] Group I includes children whose nutrient intake met or exceeded the National Research Council allowances and for whom serum vitamin C analyses were available.

[2] Group II includes children whose nutrient intake lacked one-third of some single nutrient when compared with the National Research Council allowances and for whom serum vitamin C analyses were available.

lacking 33⅓% of the Allowance for some single nutrient. In table 3, data for groups 1 and 3 are given. Group 3 was comprised of children whose intakes of vitamin C were about one-half that of group 1; at the same time, the serum level of vitamin C was reduced to two-thirds that of group 1. Calcium was the nutrient which most frequently caused the diets to be classified into group 3 (Eppright et al., '54a), and vitamin C was the second nutrient most apt to be lacking. However,

diets poor in one nutrient were likely to be poor in several nutrients.

Children were divided into groups on the basis of their blood serum concentrations of vitamin C. Group A were those children with blood serum concentrations within ± 1 standard deviation of the mean at each age, group B, those below 1 standard deviation, and group C, those above 1 standard deviation. Intakes for these groups were then computed and the data for the extreme groups only are given

TABLE 4

Vitamin C intakes of Iowa survey children classified outside one standard deviation of the mean serum concentration of each age

AGE	BOYS						GIRLS					
	Group B [1]			Group C [2]			Group B [1]			Group C [2]		
	No.	Serum	Intake	No.	Serum	Intake	No.	Serum	Intake	No.	Serum	Intake
		mg %	*mg/day*		*mg %*	*mg/day*		*mg %*	*mg/day*		*mg %*	*mg/day*
6	3	0.32	65	4	1.81	93	5	0.31	40	6	1.66	106
7	5	0.28	45	6	1.51	81	6	0.41	49	6	1.75	84
8	9	0.27	46	6	1.63	76	3	0.25	68	5	1.87	96
9	7	0.29	58	7	1.83	95	6	0.28	69	7	1.88	103
10	7	0.25	55	7	1.43	101	5	0.33	54	7	1.78	118
11	4	0.36	70	5	1.54	105	4	0.30	47	5	1.37	80
12	8	0.28	51	10	1.44	101	7	0.22	71	10	1.42	106
13	5	0.17	49	5	1.52	158	3	0.15	59	5	1.02	85

[1] Group B, children with serum vitamin C levels below 1 S.D. from the mean.
[2] Group C, children with serum vitamin C levels above 1 S.D. from the mean.

in table 4. The lower serum concentrations of vitamin C in group B were associated with lower intakes than group C. The serum of group B was usually less than one-fifth that of group C while the intake of group B was about one-half that of group C. The figures are given for children 6 to 13 years of age only because the numbers in the extreme groups beyond that age are small. It appears that classification by either intakes or blood levels produces differentiation in the other, but the degree of difference is smaller in the dependent variable.

SUMMARY

Data useful in defining the nutritional status with respect to vitamin C have been obtained from about 650 boys and girls in Iowa towns and cities. Decreasing concentrations of vitamin C in the serum (except girls over 14 years of age) and decreasing intakes of vitamin C per kilogram body weight were associated with increasing age. From 15 years of age, the concentration of vitamin C in the blood serum of girls increased although there was no comparable change in intake. More children over 12 years of age than children under 12 years of age were classified as poor or fair according to the categories suggested by Bessey and Lowry.

The calculated intake of vitamin C from vitamin C-rich foods only was as good as total vitamin C intake for predicting blood serum concentrations of this nutrient; intake per kilogram body weight was a somewhat better predictor of serum concentrations than was total intake per day. None of these measures of intake was a precise predictor of the vitamin C concentration in the serum. Mean intakes of vitamin C similar to the Recommended Allowances were associated with mean serum concentrations lower than 0.8 mg% in children beyond 11 or 12 years of age.

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FURTHER STUDIES ON THE EFFECT OF AUREOMYCIN ON THE APPARENT UTILIZATION OF VITAMIN A BY THE RAT

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ONE FIGURE

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In a previous paper it was postulated (Murray and Campbell, '55) that, while aureomycin increased the response to vitamin A as measured by the vaginal smear assay, it did not do so by increasing absorption of the vitamin from the intestinal tract. This theory was based on the assumption that all vitamin A had disappeared from the gut contents within 24 hours after administration of an oral dose. While this assumption seemed justified, more direct evidence was required. The purpose of this paper is to report (1) data on the absorption and distribution of doses of vitamin A in depleted rats and (2) further observations on the effect of aureomycin on vitamin A utilization.

METHODS

The vaginal smear technique and diets were those previously described (Murray and Campbell, '55). Chemical assays for vitamin A were carried out according to the method of Ames, Risley and Harris ('54). For organs in which only small amounts of vitamin A were expected, a known amount of vitamin A acetate was added, and then subtracted from the total found by analysis.

RESULTS

Distribution of an oral dose. Vitamin A-deficient female rats, 28 in number, were dosed orally with 0.1 ml of an oil solution containing 625 I.U. of vitamin A. Fourteen of these rats were fed a vitamin A-free diet, containing 66 mg of aureomycin per kilogram, during the depletion period. The remainder were given only the vitamin A-free diet. At intervals ranging

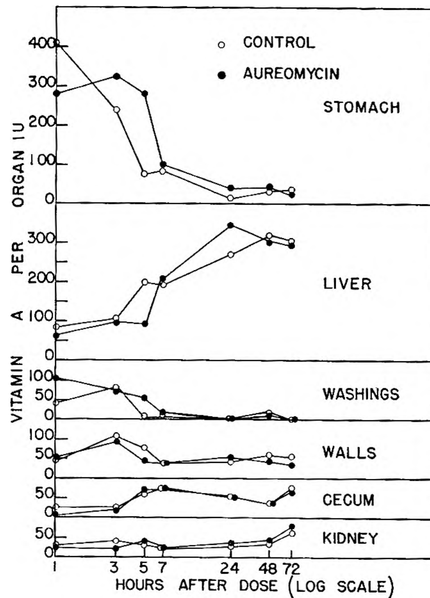

Fig. 1 Effect of aureomycin on the distribution of an oral dose of 625 I. U. of vitamin A.

from one to 72 hours after dosing, 4 rats, two of which had received aureomycin and two of which had not, were killed by decapitation. The stomach (and contents), intestinal contents, intestinal walls, caecum (and contents), liver and kidney were analyzed for vitamin A. Intestinal contents were obtained by flushing the intestine several times with 0.9% saline. The tissues of undosed, vitamin A-deficient rats were also analyzed and, where any vitamin A was found, the basal values were subtracted from those of the test animals. From the results

of this experiment, shown in figure 1, it is apparent that aureomycin had no measurable influence on the distribution of vitamin A. It is also evident that the intestinal contents were free from vitamin A within 24 hours after an oral dose. The assumption that aureomycin could not influence the absorption of vitamin A from the intestine when it was fed only 24 hours after dosing, was therefore supported. The much smaller doses used in the vaginal smear assay might be expected to disappear from the gut contents even more quickly. As has been noted previously by Popper and Volk ('48) the gut wall held a small amount of vitamin A for periods up to 72 hours.

Effect of aureomycin on subcutaneous doses. The use of subcutaneous doses of vitamin A offered another approach to the study of the mode of action of aureomycin. Two vaginal smear assays were conducted in which half the rats received aureomycin (66 mg/kg) in their diets from two days before dosing until the end of the test. Each assay comprised 60 rats and was identical to those described previously (Murray and Campbell, '55) except that the doses were administered by subcutaneous injection rather than orally. In one of these assays the doses were corn oil dilutions of vitamin A acetate while in the other the doses were made by diluting a commercial aqueous dispersion [1] of vitamin A. Aureomycin increased the response to vitamin A in each of the assays. The increase in response amounted to 18.2% (limits at $P = 0.05 = \pm 11.6\%$) when the doses were given in an aqueous dispersion, and to 16.3% (limits at $P = 0.05 = \pm 25.0\%$) when oil solutions of vitamin A were used. Aureomycin therefore increased the response to subcutaneous doses of vitamin A by about the same amount as has been reported (Murray and Campbell, '55) in the case of oral doses. This supports the theory that aureomycin does not affect the response to doses of vitamin A by increasing the proportion of the dose absorbed from the intestine.

[1] Aquasol A, U.S. Vitamin Corporation.

Distribution of a subcutaneous dose. Since the intestinal wall held a portion of an oral dose of vitamin A for as long as 72 hours, the possibility existed that aureomycin might have some influence over the mobilization of this portion of the vitamin from the intestinal wall. However, if it could be shown that vitamin A did not appear in the gut wall after a subcutaneous injection, this possibility would be eliminated. An experiment was therefore conducted in which doses of 1,250 I.U. of vitamin A were administered subcutaneously to vitamin A-deficient rats. Single rats were killed at intervals from three to 72 hours after receiving the doses. Table 1 shows the

TABLE 1

Distribution of vitamin A in single rats after a subcutaneous dose of 1250 I.U.

HOURS AFTER DOSING	VITAMIN A PER ORGAN (I.U.)					
	Liver	Kidney	Intestinal walls	Stomach	Intestinal contents	Caecum
3	2	10	0	43	0	0
5	97	0	0	78	0	0
7	113	65	0	8	0	0
24	570	0	0	9	0	0
48	910	10	12	13	0	0
72	1040	77	11	78	0	0

distribution of vitamin A in various organs. In this case, after 72 hours, the liver contained 81% of the dose while virtually none was found in the intestinal walls, intestinal contents or caecum. The very small amounts which are listed as appearing in the intestinal wall 48 hours after the dose are considered to be well within the limits of error of the assay method. The effect of aureomycin on the utilization of subcutaneous doses of vitamin A cannot be attributed, therefore, to mobilization of vitamin A from the gut wall or to increased absorption from the gut contents.

Influence of dietary changes on the aureomycin effect. It was of interest to determine whether changes in the diet had

any influence on the utilization of vitamin A in the presence of aureomycin. It was felt that this might furnish some further clue to the mechanism of the action of aureomycin. The following changes were made in the vitamin A-free diet: (1) starch was replaced by sucrose; (2) vitamin mixture, the composition of which is shown in table 2, was added; (3) folic acid was added at the rate of 8 mg/kg; (4) ascorbic

TABLE 2

The effect of changes in the vitamin A-free diet and of aureomycin on the utilization of vitamin A

DIET	NUMBER OF DAYS TO BECOME DEFICIENT AFTER AN ORAL DOSE OF VITAMIN A									
	A-free	A-free + aureomycin	Sucrose	Sucrose + aureomycin	Vitamin mixture [1]	Vitamin mixture [1] + aureomycin	Folic acid	Folic acid + aureomycin	Ascorbic acid	Ascorbic acid + aureomycin
Test										
1	18.3	19.3	19.1	19.3	17.3	18.8	19.1	19.4	18.8	18.3
2	16.7	17.5	16.1	16.6	16.9	17.4	16.6	16.8	17.0	16.6
3	15.9	16.6	15.4	15.1	16.6	17.2	17.0	17.0	16.4	15.7
4	15.6	17.0	15.3	15.3	16.4	17.5	16.5	16.8	15.7	14.5
Mean	16.6	17.6 [2]	16.5	16.6	16.8	17.7 [2]	17.3	17.5	16.9	16.5

[1] The composition of the vitamin mixture was as follows (amounts in milligrams per kilogram of diet): thiamine 20, riboflavin 40, niacin 80, pyridoxine 20, calcium d-pantothenate 80, inositol 800, choline 4000, folic acid 8, para amino benzoic acid 40, vitamin B_{12} 16.

[2] Significant at $P = 0.05$.

acid was added at the rate of 2 gm/kg. Each of these diets was fed to 10 vitamin A-deficient rats, and 10 others were given the same diets plus 66 mg of aureomycin per kilogram. After two days all rats were dosed with 100 I.U. of vitamin A and the number of days that elapsed until each rat again became deficient, was recorded. Deficiency was judged by an examination of the vaginal smears. This test was repeated twice with the same groups of rats after which the rats were distributed randomly to form the groups of tests 3 and 4. The results

of these tests are shown in table 2. Aureomycin increased the response to vitamin A to a small but significant extent when the regular starch diet, or the regular diet supplemented with a multivitamin mixture, was fed. It had no effect, however, when a diet containing sucrose, ascorbic acid or folic acid was fed. It appeared from these results that folic acid itself might have increased the utilization of vitamin A, but in another experiment in which two levels of folic acid were fed it was found to have no influence on the vitamin A assay.

DISCUSSION

The assumption (Murray and Campbell, '55) that vitamin A disappears from the intestine within 24 hours after an oral dose has been shown to be a fact. Thus the feeding of aureomycin, 24 hours after the vitamin A dose, cannot affect the absorption of vitamin A. This finding has been confirmed by the observation that aureomycin increases the response to subcutaneous doses of vitamin A although no part of these doses could be found in the intestinal wall or contents. The increase in response, which could be attributed to aureomycin, was of the same order as that found when oral doses were used. A somewhat similar observation has been made by Jones and Baumann ('55) and by Schendel and Johnson ('54) with respect to various B vitamins.

The significance of the vitamin A found in the stomach after a subcutaneous injection is not known. In the early hours of the experiment this could have been a result of the rat licking the site of injection, but this could not have been the case after 72 hours. The average amount found in the stomach of the injected rats was 37 I.U. greater than that found in undosed rats. However, there was considerable variation among both the dosed and undosed rats. Reifman, Hallman and Deuel ('43) have also commented on the variable and appreciable amounts of vitamin A found in the gastro-intestinal tract of undosed rats. In any case the small amount of vitamin A found in the stomach is not thought to have any bearing on the problem being discussed. If aureomycin were

to have any influence on vitamin A in the stomach, one would expect the effect to be much more marked after an oral dose than after a subcutaneous injection. This, however, was not the case. Furthermore, none of the vitamin A was found in the intestinal contents or walls.

The observation that dietary sucrose and ascorbic acid are capable of influencing the action of aureomycin has been made by others, although not with regard to vitamin A utilization. Stokstad, Jukes and Williams ('53) reported that aureomycin was more effective in promoting growth in chicks when the starch of the diet was replaced by sucrose. Daft and Schwarz ('52) found that either aureomycin or ascorbic acid prevented the appearance of deficiency symptoms in rats fed diets devoid of riboflavin or pantothenic acid. It is known (Monson et al., '54) that the intestinal synthesis of folic acid is increased in the presence of aureomycin. This may be the mechanism by which added folic acid eliminates the effect of aureomycin, but folic acid itself had no influence on the vitamin A assay. The fact that aureomycin had no influence on the vitamin A assay when sucrose, ascorbic acid or folic acid were added to the diet, suggests that intestinal bacteria are involved. Thus, aureomycin could increase the response to doses of vitamin A by eliminating bacteria which would otherwise destroy part of the dose, or by promoting the growth of bacteria which are capable of synthesizing vitamin A. Partial destruction of the dose can be ruled out because aureomycin was effective even when fed after the vitamin A dose had left the intestine. Intestinal synthesis remains a possibility. Luckey ('55) has recently presented evidence that dietary antibiotics stimulate the growth of germ-free chicks by direct action on the tissues. It is impossible to say what part, if any, such a mechanism has in the work reported here.

SUMMARY

Further evidence has been obtained which indicates that aureomycin does not exert its effect on the vaginal smear assay for vitamin A by increasing the absorption of the dose.

It has been shown that the intestinal contents are free of vitamin A during the period in which aureomycin is effective. Furthermore, aureomycin increased the response to subcutaneous doses of vitamin A even though no part of these doses appeared in the intestinal contents or wall.

Aureomycin had no effect on the utilization of vitamin A when ascorbic acid or folic acid were added to the diet, or when sucrose was substituted for starch. Possible mechanisms of the action of aureomycin are discussed.

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THE BIOLOGICAL UTILIZATION OF THE PALMITIC ACID ESTERS OF PANTOTHENIC ACID [1]

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INTRODUCTION

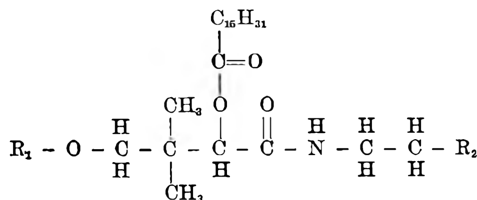
Poor utilization of orally administered pantothenic acid (Henderson et al., '42; Silber, '45) has been shown to be due to the ready excretion of the calcium and the sodium salts into the feces (Nelson et al., '47; Rubin et al., '48), and to the possible destruction of the vitamin in the gastric juice (Rubin, '48; Rubin et al., '48). Since the long-chain fatty acid esters of pantothenic acid (Sakuragi and Kummerow, '56) are soluble in fats, the absorption and the excretion of the pantothenic acid moiety would be different from those of the water-soluble forms. Improved stability of the active fragment may also be expected because of less contact of the fat-soluble esters with the gastric juice.

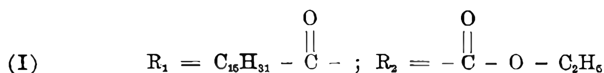
In the present study, the biological utilization of the palmitic acid esters of pantothenic acid was investigated with rats. So called fat-soluble derivatives of pantothenic acid such as the acetate, the p-nitrobenzoate or the carbobenzoxy derivative have been prepared (Stiller et al., '40; Harris et al., '41; Wooley, '45). Quantitative evaluation of the biological activity of these preparations, however, was not complete (Williams et al., '50; Robinson, '51; Sebrell and Harris, '54).

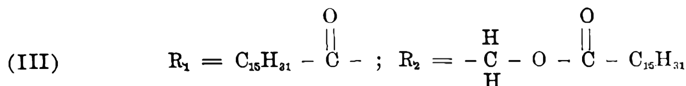
[1] This work was supported by research grant no. A-257 from the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

EXPERIMENTAL

Test compounds. The synthesis of ethyl dipalmitoxypantothenate (I), ethyl 2′-monopalmitoxypantothenate (II) and pantothenyl tripalmitate (III) has been reported elsewhere (Sakuragi and Kummerow, '56).







Feeding experiments with various pantothenate preparations. Thirty-five-day-old male rats which had been kept on a pantothenate-deficient diet (table 1) for two weeks were divided into various groups. The diets were supplemented with various levels of calcium pantothenate, ethyl dipalmitoxypantothenate (I), pantothenol or pantothenyl tripalmitate (III) with or without the incorporation of a sulfa drug[2] in the diet. The diets were fed ad libitum and the change in body weight was recorded over a period of three weeks. The pantothenate levels tested were 3.5, 7.0, 10.0 and 20.0 µg as *d*-calcium pantothenate per gram of ration. The calcium pantothenate and the pantothenol served as controls for the

[2] The sulfaguanidine was kindly supplied by the American Cyanamid Company, Fine Chemicals Division; the succinylsulfathiazole was a gift from the Paul Lewis Laboratories, Inc.

ethyl dipalmitoxypantothenate (I) and pantothenyl tripalmitate (III) respectively.

Growth response of the pantothenate-depleted rats to a single dose of various preparations. The male weanling rats which were used in this experiment had been kept for 4 weeks on a pantothenate-free ration (table 1, I). One per cent sulfa-

TABLE 1

Composition of the basal ration

	DIET I	DIET II [1]
Glucose [2]	68 gm	64 gm
Vitamin-free casein	18 gm	24 gm
Corn oil	10 gm	8 gm
Wesson salts	4 gm	4 gm
Vitamins per 100 gm ration		
Choline chloride	200.0 mg	100.0 mg
Inositol	100.0 mg	40.0 mg
Niacin	10.0 mg	2.0 mg
Riboflavin	0.9 mg	1.0 mg
Thiamine hydrochloride	0.45 mg	0.5 mg
p-Aminobenzoic acid	0.3 mg	1.0 mg
Pyridoxine hydrochloride	0.15 mg	0.5 mg
Menadione	...	0.5 mg
Folic acid	0.08 mg	...
Biotin	0.4 µg	...
Vitamin A	160 I.U./week	
Vitamin D	1.6 µg/week	
Vitamin E	259 µg/week	

[1] Composition of diet III was the same as diet II, except that it included 0.05 mg each of folic acid and biotin per 100 gm of ration.

[2] Cerelose.

guanidine or 2% succinylsulfathiazole was added to this diet at the expense of the glucose. The rats were then divided into 4 groups of 4 to 5 rats each, and supplemented with a single dose of *d*-calcium pantothenate, *dl*-ethyl 2′-monopalmitoxypantothenate (II), or *dl*-ethyl dipalmitoxypantothenate (I) equivalent to 500 µg of *d*-calcium pantothenate per rat. It has been reported that a single dose of calcium pantothenate at a level of 800 µg induces a marked weight gain in panto-

thenate-depleted rats (Stiller et al., '40). The calcium pantothenate was fed as an aqueous solution and the fatty acid ester was dissolved in 60 mg of olive oil. An equal amount of olive oil was fed to the animals which had been supplemented with calcium pantothenate. The gain in body weight was recorded over a period of 13 days.

Urinary excretion of pantothenic acid by rats following the administration of a single dose of various pantothenate preparations. Normal male rats weighing between 192 and 210 gm were divided into three groups of three rats each. The rats were kept on the synthetic diet (table 1, I) which had been supplemented with 10 μg of *d*-calcium pantothenate per gram of ration. The rats were then supplemented with 10 mg of *d*-calcium pantothenate or an equivalent amount of *dl*-ethyl dipalmitoxypantothenate (I) or *dl*-ethyl 2′-monopalmitoxypantothenate. The calcium pantothenate was fed as an aqueous solution with the aid of a calibrated medicine dropper, and the fatty acid derivatives were fed as solutions in olive oil. The urine was collected in three 12-hour periods over a total of 36 hours. The yeast microbiological assay of Atkin et al. ('44) was used to determine the amount of pantothenic acid in the urine samples.

Deposition of pantothenic acid in the livers of the pantothenate-depleted rats after administration of calcium pantothenate and of ethyl dipalmitoxypantothenate. Male weanling rats were kept on a pantothenate-deficient 1% sulfaguanidine diet (table 1, I) for 5 weeks; they weighed between 78 and 99 gm at this time.

Two of these rats were sacrificed, and the livers saved for pantothenate assay. The rest of the animals were fed *dl*-ethyl dipalmitoxypantothenate (I) in olive oil or an aqueous solution of *d*-calcium pantothenate at a level of 10 mg per rat. Two of the rats in each group were sacrificed two, 6, and 12 hours after supplementation. The fresh livers were weighed, pooled and homogenized. The homogenate was digested with

TABLE 2

Average weekly body weight gains of male rats on diets supplemented with various pantothenate preparations

	DIET						
	Vitamin level[1]	20.0 µg	10.0 µg	7.0 µg		3.5 µg	
	Sulfa drug[2]	SG	SG	—	SST	—	SST
	Diet number	III	I	II	III	II	III
		Period[3] A	Period A	Period A	Period B	Period A	Period B
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
Supple-	Calcium pantothenate	28.8 ± 3.0[4]	17.6 ± 1.2	..	..	14.8 ± 2.4	14.0 ± 2.2
ments[5]	Ethyl dipalmitoxypantothenate	29.0 ± 1.5	20.8 ± 1.6	20.4 ± 1.8	..	12.4 ± 1.9	11.0 ± 1.3
	Pantothenol	..	..	18.8 ± 1.7	26.6 ± 2.8	..	..
	Pantothenyl tripalmitate	..	..	18.8 ± 2.7	26.2 ± 2.1	..	..

[1] The vitamin level is indicated as *d*-calcium pantothenate, micrograms per gram of ration.

[2] SG: Sulfaguanidine, added at a level of 1% at the expense of the glucose. SST: Succinylsulfathiazole, added at a level of 2% at the expense of the glucose.

[3] Period A: An assay period of three weeks following conditioning for two weeks. Period B: An assay period of three weeks following period A.

[4] Standard error of the mean, $\sqrt{\Sigma d^2/n(n-1)}$.

[5] Six rats were used for each of the groups supplemented with 20.0 µg of the vitamin supplement. In the rest of the groups, 5 rats were used for each group.

"Clarase,"[3] and the pantothenic acid level was determined by the yeast microbiological assay of Atkin et al. ('44).

RESULTS AND DISCUSSION

The results of the feeding experiments indicated that the fat as well as the water-soluble derivatives of pantothenic acid were biologically active. Levels of pantothenate ranging between 3.5 and 20 µg per gram of ration, whether calcium

TABLE 3

Average growth responses of pantothenate depleted rats to a single dose of various pantothenate preparations

SUPPLEMENTS[1]	NO. OF RATS	NUMBER OF DAYS AFTER SUPPLEMENTATION				
		1	2	3	10	13
		gm	*gm*	*gm*	*gm*	*gm*
None (Control)	4	0.0 ± 0.0[2]	0.0	— 3.0	—[3]	—[3]
Calcium pantothenate	5	+ 6.8 ± 1.0	+ 10.4	+ 11.0	+ 15.6 ± 1.9	+ 13.2
Ethyl monopalmitoxy-pantothenate	5	+ 3.2 ± 0.9	+ 9.8	+ 11.2	+ 17.2 ± 1.2	+ 12.6
Ethyl dipalmitoxy-pantothenate	5	+ 3.6 ± 0.9	+ 9.4	+ 12.0	+ 14.2 ± 1.5	+ 13.0

[1] Supplementation was made so as to supply 500 µg as *d*-calcium pantothenate.

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

[3] No data were obtained due to the death of the animals.

pantothenate, ethyl dipalmitoxypantothenate, pantothenol or pantothenyl tripalmitate gave identical body weight gains in the presence or absence of the sulfa drugs (table 2). Thus the esterification of the water-soluble form of the vitamin with a long-chain fatty acid did not appear to diminish or to increase its availability.

The growth response test to a single dose of various pantothenate supplements indicated that the administration of cal-

[3] We are indebted to the Takamine Laboratory, Inc. for a supply of "Clarase."

cium pantothenate, ethyl monopalmitoxypantothenate or ethyl dipalmitoxypantothenate resulted in similar body weight gains (table 3). The gain in body weight reached a maximum at the 10th day. The growth response which was induced by calcium pantothenate after the first day appeared to be higher than the response observed in the other groups; the differences, however, were not statistically significant.

Esterification of the vitamin with palmitic acid increased the amount of pantothenic acid that appeared in the urine after the administration of a single large dose (table 4).

TABLE 4

Average urinary excretion of pantothenate[1] per rat following the administration of a single large dose of various supplements

SUPPLEMENTS[2]	HOURS AFTER ADMINISTRATION			TOTAL	% RECOVERY	AV. BODY WT.[3]
	0–12	12–24	24–36			
	μg	*μg*	*μg*	*μg*		*gm*
Calcium pantothenate	600	300	220	1120	11	203.3
Ethyl monopalmitoxy-pantothenate	1970	1030	260	3260	33	202.3
Ethyl dipalmitoxy-pantothenate	1270	800	370	2440	24	206.0

[1] The amounts are indicated as *d*-calcium pantothenate.

[2] Each rat was supplemented with a preparation at a level equivalent to 10 mg of *d*-calcium pantothenate.

[3] Each group consisted of three normal male rats. Analysis was made on a pooled sample from each group.

With calcium pantothenate, 11%, with the monopalmitate, 33% and with the dipalmitate, 24% of the pantothenic acid was recovered from the urine in 36 hours. In all of the three 12-hour collection periods, a higher excretion of pantothenic acid was observed after the palmitic acid esters were fed. During the first and the second periods supplementation with ethyl monopalmitoxypantothenate resulted in a higher excretion of the vitamin than was obtained with the dipalmitate. In the last period, however, the average excretion per rat fed the dipalmitate was higher than it was for those supple-

mented with ethyl monopalmitoxypantothenate, or 370 and 260 μg respectively. It thus appeared possible that a higher utilization of pantothenic acid is achieved when the vitamin is fed in the form of palmitate, and a longer retention in the body is maintained when the pantothenic acid is esterified with 2 moles rather than 1 mole of palmitic acid. This observation, however, was not reflected in the growth rates upon a single dose assay, as described.

It has been pointed out that the utilization of pantothenic acid, when administered orally, is surprisingly low (Henderson et al., '42; Silber, '45), and a considerable amount of the vitamin is excreted into the feces (Nelson et al., '47; Rubin et al., '48). The use of pantothenol has, therefore, been suggested, since the alcohol is more stable and has been shown to possess better biological utilization (Drekter et al., '48; Rubin, '48; Rubin et al., '48). The results obtained in the present experiments appear to indicate that esterification with a long-chain fatty acid may similarly improve the biological availability of the vitamin.

The vitamin level in the livers [4] of deficient rats was found to be 48 μg (as calcium pantothenate) per gram of the fresh tissue before supplementation with the pantothenate preparations. This value indicated that the animals were depleted of the vitamin, since a level below 50 μg per gram of liver has been considered to serve as evidence of pantothenate deficiency (Wright and Welch, '44). Striking increases in the pantothenate level in the liver was noted in two hours after 10 mg of the calcium pantothenate was administered. The level was 115 μg per gram of the liver, which represented, however, only a temporary accumulation of the vitamin as marked drops were observed after the 6th and 12th hours, or 73 μg and 68 μg respectively. When the rats were supplemented with ethyl dipalmitoxypantothenate, the level of the vitamin increased to 71 μg per gram of the fresh liver in two hours. This amount was 44 μg less than when calcium panto-

[4] The experimental rats weighed between 78.0 and 99.0 gm, and the fresh livers weighed between 3.6 and 4.9 gm.

thenate was used as a supplement. The 71 μg level of pantothenate, however, is within normal range for liver (Wright and Welch, '43, '44; Ford et al., '53; Everson et al., '54). During the course of the 12-hour experiment, it was noted that the amount of liver pantothenate tended to increase; the levels at the 6th and the 12th hour were 72 μg and 82 μg per gram of liver respectively. The results thus appeared to indicate that a gradual utilization of pantothenic acid took place when the dipalmitate of ethyl pantothenate was fed. However, a level of pantothenate equivalent to that of normal rat liver was noted within two hours after supplementation with the dipalmitate.

SUMMARY

The biological utilization of fat-soluble derivatives of pantothenic acid, ethyl dipalmitoxypantothenate and ethyl 2′-monopalmitoxypantothenate was compared with that of calcium pantothenate in rats. The over-all activity of the palmitic acid esters was equal to that of the water-soluble form as a supplement for pantothenic acid; this was proved by feeding experiments under various conditions. When a large single dose of the preparation was administered to the rats, the excretion of the pantothenic acid into the urine was markedly increased by esterifying the vitamin with one or two moles of palmitic acid. The biological utilization of the pantothenic acid moiety when present as an ester appeared to be slower than that of the free vitamin. The liver of pantothenate-deficient rats, however, contained a normal pantothenate level within two hours after the administration of ethyl dipalmitoxypantothenate. The activity of pantothenyl tripalmitate was found to be equal to that of free pantothenol.

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THE BENEFICIAL EFFECT OF PROGESTERONE ON PREGNANCY IN THE VITAMIN A-DEFICIENT RABBIT [1]

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It is known that progesterone is required to maintain pregnancy in the rabbit (Allen and Corner, '29; Pincus and Werthessen, '38). It is also known that a deficiency of vitamin A causes an impairment in reproduction in the rabbit (Lamming, '49; Lamming et al., '54). The latter workers suggested that exogenous progesterone may alleviate some of the impairment in reproduction induced by a vitamin A deficiency.

This is a report of experiments in which an attempt was made to increase the reproductive performance of vitamin A-deficient female rabbits by the injection of progesterone.[1]

EXPERIMENTAL

In the first experiment, 34 adult New Zealand white female rabbits were placed on a diet (Lamming et al., '54) containing no detectable carotene. After 4, 8 or 12 weeks on this diet, the females were mated to fertile males with one-half of the females receiving daily injections of 8 mg of progesterone in oil starting on the day of mating. This dosage of progesterone was selected to make it well in excess of the 4 mg minimum daily level required to maintain pregnancy in the rabbit (Allen and Heckel, '39). At the conclusion of this experiment the

[1] An abstract of this paper was presented at the 47th Annual Meeting of the American Society of Animal Production 1954.

question was raised as to whether the administration of a higher level of progesterone daily might show even a greater beneficial effect.

In the second experiment, 24 females were placed on the carotene-deficient diet for 13 weeks before mating. Beginning on the day of mating, 8 received daily injections of 12.5 mg of progesterone in oil, 8 received an excess of vitamin A acetate (100,000 I.U. weekly) and 8 served as controls.

The females were autopsied on the 28th day after mating and the numbers of corpora lutea, living young, resorptions and implantation sites were determined.

At autopsy, blood and liver samples were taken for vitamin A analysis. The procedure of Gallup and Hoefer ('46) was used for extraction of vitamin A from the liver and that of Kimble ('39) for blood plasma. Vitamin A was determined by the procedure of Sobel and Werbin ('45).

RESULTS AND DISCUSSION

The results are shown in table 1. The column "Dead" includes dead fetuses and placental remnants while the column "Sites" includes implantation sites which had no placentae. In all groups the number of living young was considerably below the normal 8.3 (Kendall et al., '50) for this breed. The average number of living young did not differ significantly in the does on the diet for 4 or 8 weeks, but the number of living young of the females on the vitamin A-deficient ration for 12 weeks before mating and not receiving progesterone was drastically reduced. Progesterone injections markedly increased the reproductive performance of the 14 females on the ration for 12 or 13 weeks prior to mating. The average number of living young was increased from 0.9 in the controls to 4.8. There was a highly significant difference in the living and in the dead young of the two groups. The fact that females treated with progesterone had an average of 7.8 living and dead young as compared to an average of 1.5 for the untreated group, indicates that the progesterone in-

jections enabled the females to carry the young for a much longer period of time.

The low number of corpora lutea in the untreated groups on the diet for 12 or 13 weeks was probably due to abortion or resorption early in gestation which has been shown to occur in rabbits on a carotene-deficient diet (Lamming et al., '54). If pregnancy is terminated in the early stages, the corpora lutea are not grossly discernible 28 days post coitum.

TABLE 1

The reproductive performance of New Zealand white female rabbits on a carotene-deficient diet

NO. OF FEMALES	WKS. ON DIET BEFORE MATING	TREAT-MENT	AV. YOUNG/FEMALE			AV. C.L.	AV. LIVER VIT. A	AV. PLASMA VIT. A
			Living	Dead	Sites			
							μg/gm	μg/100 ml
6	4	None	5.0	1.8	0.8	9.6	9.9	8.2
6	4	Prog.[1]	3.7	0.3	0.5	7.2	8.3	8.0
5	8	None	4.0	0.6	0.0	7.6	2.8	3.6
5	8	Prog.[1]	2.5	1.3	0.5	9.0	1.0	6.4
6	12	None	0.8	0.8	0.0	1.0	1.8	7.6
6	12	Prog.[1]	4.3	2.2	0.3	9.8	2.0	9.9
8	13	None	1.0	0.5	2.1	5.0	0.5	2.0
8	13	Prog.[2]	5.1	3.4	0.0	10.2	0.5	2.5
8	13	Vit. A[3]	1.2	2.9	1.0	5.5	787.0	40.7

[1] Daily injection of 8 mg.

[2] Daily injection of 12.5 mg.

[3] Weekly feeding of 100,000 I.U. vitamin A acetate.

As a result of this, the number of corpora lutea 28 days post coitum was not an accurate measure of the number of ovulations.

Since the levels of vitamin A found in table 1 were taken 28 days post coitum, they were undoubtedly higher at the beginning of pregnancy. It is not known whether the levels of vitamin A were sufficiently low during pregnancy in the 4- and 8-week groups to cause a decrease in the number of live young. However, feeding this carotene-deficient ration for 12 weeks caused a marked reduction in reproductive efficiency.

The vitamin A per gram of liver or total liver vitamin A is reduced below normal in the 4-week group. The liver vitamin A of rabbits on normal diets is 20 to 40 μg/gm (Lamming et al., '54). The vitamin A per gram of liver in the 8- and 12-week groups were essentially the same at autopsy. It is possible that the vitamin A of the 8-week group was sufficiently high during pregnancy to enable more young to survive than in the 12-week group even though the liver vitamin A levels were the same at autopsy. Progesterone injections had no apparent effect on the vitamin A levels in the liver or the plasma. In none of the rabbits were gross symptoms of vitamin A deficiency observable.

There is no apparent reason why vitamin A injections failed to improve the reproduction of vitamin A-deficient females unless the deficiency had progressed to such a state that either the females could not return to normal in time to maintain pregnancy or that permanent tissue damage had occurred.

The underlying causes for the observed progesterone effects are not readily apparent. Possibly the injected progesterone enables the female to mobilize nutrients which are not present in sufficient quantities to maintain pregnancy. This mobilization may occur not only in the case of vitamin A deficiency but also when there are other deficiencies in the diet as has been shown on a protein-free diet (Nelson and Evans, '54), or perhaps vitamin A is associated with the formation of progesterone. Also, it is possible that vitamin A and progesterone are involved in some physiological process essential to the maintenance of pregnancy and that injected progesterone may be substituted at least in part for vitamin A.

SUMMARY

Fifty-eight adult female rabbits were placed on a carotene-deficient diet for varying lengths of time and were then mated to fertile males. The average litter size of the 22 females on the diet for 4 or 8 weeks was well below the normal of 8.3 and injections of 8 mg of progesterone in half of them had no apparent effect on litter size. The 14 females on the diet for

12 or 13 weeks before mating averaged 0.9 living and 0.6 dead young at autopsy on the 28th day of pregnancy, while 7 comparable females injected with 8 mg of progesterone daily had 4.3 living and 2.2 dead young and the 7 females receiving 12.5 mg of progesterone had 5.1 living and 3.4 dead young.

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THE BIOLOGICAL VALUE OF OILS AND FATS

IV. THE RATE OF INTESTINAL ABSORPTION

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INTRODUCTION

Earlier experiments established that the increase in weight of young rats is dependent on the type of the dietary fat (Thomasson, '55a). These investigations further revealed that the different growth-action of the fats and oils investigated is actually due to a different food intake.

It is conceivable that food intake and growth are correlated with the rate at which the fat in question is absorbed. The literature in this field is very restricted, however. Steenbock et al. ('36) established that halibut- and cod-liver oil are absorbed significantly more rapidly than lard and maize oil. In addition, a number of fats and oils could be arranged in the order of decreasing rates of absorption as follows: linseed oil, olive oil, whale oil, soya-bean oil, groundnut oil, rancid lard, cottonseed oil, coconut fat, and palm oil. Deuel et al. ('40) observed no differences in the rates of absorption of cottonseed oil, butterfat, and coconut fat; they found, however, that rapeseed oil was absorbed at a much lower rate. In later investigations carried out in Deuel's laboratory it could be shown that maize oil (Bavetta and Deuel, '42) and lard (Crockett and Deuel, '47) possessed the same rate of absorption. In addition, Bhalerao et al. ('47) found that sesame oil, coconut fat, and butterfat are absorbed at the same rate, and safflower, groundnut and cottonseed oil slightly, although not significantly, slower.

In the present investigation the rate of absorption of a series of 18 different natural oils and fats, of vegetable and animal origin, has been studied.

EXPERIMENTAL PART

For the determination of the absorption rate a modification of Deuel's method (Deuel et al., '39, '40) was used. Male Wistar rats weighing 150 to 350 gm and fed a Sherman diet ($\frac{2}{3}$ wheat, $\frac{1}{3}$ milk powder and salts), were fasted for 48 hours (drinking-water was available during this period) the fat to be investigated then being administered by stomach-tube. After a certain test period the fat remaining in the gastro-intestinal tract was determined. Since, according to Deuel et al., ('40, '41) more constant results are achieved when the amount of absorbed fat is expressed per unit of body surface, a dosage of 400 mg of fat per 100 cm^2 surface was administered in all cases. The body surface was calculated from Lee's formula (Lee, '29). For the determination of the fat content in the gastrointestinal tract the animal was decapitated, the abdomen opened, and the contents of stomach, small and large intestine separated by means of ligatures. Subsequently, each of these three parts was flushed twice with 20 ml of diethyl ether, using a syringe with blunt needle, care being taken to ensure that all solid material in the tract was removed. The three extracts obtained in this way were dried overnight with Na_2SO_4, and filtered. The residue was washed with 100 to 125 ml of ether, the ether evaporated, and the residue weighed. Blank determinations carried out with fasted animals to which no fat had been administered, showed that an average correction of 5, 8 and 17 mg of fat must be applied to the values found for stomach, small and large intestine respectively.

As a criterion for comparing the fats and oils investigated the *A*(bsorption) *T*(ime)$_{50}$ was employed, i.e. the time after which 50% of the administered fat has disappeared from the entire gastrointestinal tract. This value was determined as follows: in two groups, each of 6 animals, the contents of the gastrointestinal tract were analyzed three and 6 hours re-

spectively after administering the fat. From these values it was calculated at what time 50% of the dosage would have disappeared from the gastrointestinal tract. The value so obtained (it varied from 5 to 11 hours) was applied to a third series of 6 animals.

When calculating AT_{50} it was supposed that, at any rate within the scope of the relevant observations, a linear relationship exists between time and the percentage of fat absorbed. AT_{50} was calculated as follows: $AT_{50} = \bar{x} + \frac{50 - \bar{y}}{b}$,

in which

x = absorption time = time interval between administering the fat and the analysis (in mins.);

y = percentage of fat recovered in the gastrointestinal tract after absorption time x;

$\bar{x}$ = the mean of all x values;

$\bar{y}$ = the mean of all y values;

$$b = \text{regression-coefficient of } y \text{ upon } x = \frac{\Sigma xy - \frac{\Sigma x \cdot \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}},$$

in which n = number of observations.

The 95% confidence interval of the AT_{50} value is:

$$\bar{x} + c.\frac{50 - \bar{y}}{b} \pm t_{(0.05)} \cdot s_{x_{50}},$$

in which

$s_{x_{50}}$ = standard deviation of the AT_{50}-value;

$t_{(0.05)}$ = critical value from Student-distribution for $P_2 = 0.05$;

c = correction factor for skewness $= b^2 : (b^2 - t^2_{(0.05)} \cdot s_b^2)$, in which s_b = standard deviation of the regression coefficient.

In the calculation of AT_{50} the values for the entire gastrointestinal tract have been employed. Actually, however, separate data for each of the three parts — stomach, small and large intestine — are available.

RESULTS AND DISCUSSION

The amounts of fat recovered in the entire gastrointestinal tract after administration of 400 mg per 100 cm^2 of body surface are shown in table 1 as percentages of the dosage. These

figures are for absorption times of three and 6 hours, as well as the time in which (as estimated from the absorption values after three and 6 hours), 50% of the fat would have been absorbed (d hours). Following the latter percentages the corresponding ''d hour''-value is shown in parentheses. Each percentage represents a mean of 6 determinations, with the exception of the values for rapeseed oil after 8 and 9 hours, which are based on 17 and 18 determinations respectively.

TABLE 1

Percentages of the fats recovered in the gastrointestinal tract, 3, 6 and d hours after administering 400 mg/100 cm², their AT_{50}-values and their relative order of growth-promoting ability [1]

CLASS	TYPE OF FAT	PERCENTAGE OF FAT RECOVERED AFTER			AT_{50}-VALUES (MINS.)		ORDER OF GROWTH-PROMOTING ABILITY
		3 h	6 h	d h	mean	95% limits	
I	Butterfat	63	41	55 (5)	305	272–349	2
II	Maize oil	81	47	49 (6)	350	329–378	7
	Cottonseed oil	75	49	49 (6)	352	328–385	4
	Beef tallow	78	47	56 (6)	354	327–394	5
	Coconut fat	73	48	50 (6)	354	310–436	13
	Soya-bean oil	77	47	53 (6)	359	321–429	8
	Sunflower oil	83	48	60 (6)	362	333–409	11
	Groundnut oil	69	48	56 (6)	365	318–487	9
	Olive oil	82	48	61 (6)	369	338–418	3
III	Sesame oil	79	60	54 (6)	392	349–486	14
	Lard	74	50	58 (6)	396	343–532	1
	Palm fat	79	62	56 (7) 31 (8½)	416	392–445	10
	Whale oil	79	59	47 (7½)	426	373–525	15
IV	Shea butter	76	64	40 (9½)	477	427–548	6
	Herring oil	79	60	52 (8)	487	423–619	16
V	Rapeseed oil	86	63	63 (8) 49 (9)	558	510–608	17
	Poppyseed oil	83	70	41 (11)	560	500–646	12
	Kapokseed oil	82	69	46 (10½)	588	523–690	18

[1] AT_{50} is the number of minutes after which 50% of the fat administered had disappeared from the gastrointestinal tract; d hours is the time at which, according to an estimation based upon the absorption values after three and 6 hours, 50% of the fat would have been absorbed.

In table 1 the AT_{50} values (= number of minutes after which 50% of the fat administered disappears from the gastrointestinal tract) calculated from these percentages, are also recorded. In addition, values are given for the 95%-interval representing the limits between which lie 95% of the AT_{50} values of the respective fats. From these statistical data the fats under investigation can be arranged in 5 classes of decreasing rates of absorption, although it is not certain whether sesame oil and lard belong to group II or to group III or form a separate group. The influence of these oils and fats on the increase in weight of new-born male rats is known from previous investigations (Thomasson, '55a). On the basis of these findings the fats have been arranged according to decreasing growth-action; these rank numbers are given in the last column of table 1. It appears that a correlation exists between the rate of absorption (AT_{50} values) and the growth-action ($\rho = 0.62$ with $P < 0.01$).[1]

Such a correlation suggests that the divergent growth-action of various fats and oils [which, as previously shown (Thomasson, '55), is in turn determined by the food intake], might be due to the rate at which these fats are absorbed. Some doubt regarding this supposition is justified, however, as certain fats do not conform to it. The growth-action of lard, shea butter, olive oil and poppyseed oil for example, is too favorable in respect of their relative slow rate of absorption, whereas coconut fat and maize oil have a poorer growth-action than would be expected from the favorable rates of absorption of these fats. However, rapid growth and a correspondingly rapid absorption need not necessarily be considered as favorable. It has been shown (Thomasson, '55b) that longevity on butterfat-containing diets is less than when this fat is replaced by rapeseed oil, in spite of the fact that with the former fat the growth rate and the rate of absorption

[1] This correlation was tested by numbering the fats investigated according to decreasing growth-promoting ability and according to decreasing rate of absorption, and applying the method of rank correlation to these two series of values (Dixon and Massey, '51).

are considerably greater. The reason why fats have different rates of absorption is unknown. It is plausible to suppose the rate of absorption is determined by the rate at which fats are hydrolyzed by lipase in the intestinal lumen. An investigation into the rate of hydrolysis of various types of fat by pancreas lipase is at present being carried out at this laboratory.

The mean amounts of fat recovered in the gastrointestinal tract after three hours varied from 63 to 86% of the dose administered (table 1). After 6 hours these values were from 41 to 70%, and after an absorption time of 8½–11 hours (= d hours) the percentage was between 31 and 63.

The distribution of these widely varying amounts of fat over stomach, small and large intestine is shown in table 2. In this table the amount recovered in the entire gastrointestinal tract is taken as 100% (this value actually varies from 31 to 86% of the dosage administered), and the amounts present in stomach, small and large intestine are shown as percentages of the amount recovered.

At the foot of table 2 the mean values found after three, 6 and d hours are given. Although these values show a certain constancy, they suggest that the percentage of fat in the stomach decreases on extending the absorption time (after three, 6 and d hours, averaging 74.9, 73.1 and 70.0% respectively) whereas the percentage of fat in the large intestine shows the opposite tendency (after three, 6 and d hours, averaging 2.2, 5.1 and 7.3% respectively). Application of the sign test (Dixon and Massey, '51) reveals that the differences in fat contents of the large intestine are, in fact, statistically significant after three and 6 hours, and after three and d hours. This is not the case, however, for the values found for the stomach, so that there is no conclusive evidence that the percentage of fat in the stomach decreases with an increase in the time of absorption.

Contrary to the differences observed in the percentage of fat present in the stomach and the large intestine, that of the small intestine is notably constant throughout the test period.

The mean values after three, 6 and d hours are 23.0, 21.9 and 22.7% respectively. This homogeneity prompted the calculation of the mean percentage from all the 56 observations: 22.5% with a standard error of 0.945%. Thus, this value

TABLE 2

Percentage distribution, over stomach, small and large intestine, of the recovered fat

TYPE OF FAT	DURATION OF ABSORPTION: 3 HOURS			DURATION OF ABSORPTION: 6 HOURS			DURATION OF ABSORPTION: D HOURS [1]			d
	stomach	intestine small	intestine large	stomach	intestine small	intestine large	stomach	intestine small	intestine large	
Butterfat	74	25	2	77	21	2	58	35	7	5
Maize oil	78	20	2	65	27	8	66	32	2	6
Cottonseed oil	84	16	0	74	24	2	68	28	4	6
Beef tallow	85	14	1	85	15	0	87	10	3	6
Coconut fat	74	22	4	75	19	6	63	27	10	6
Soya-bean oil	62	33	5	58	38	4	66	28	6	6
Sunflower oil	81	17	2	77	19	4	74	23	3	6
Groundnut oil	69	31	0	78	20	2	64	29	7	6
Olive oil	67	31	2	72	23	5	79	16	5	6
Sesame oil	70	29	1	67	29	4	77	20	3	6
Lard	78	22	0	73	21	6	82	14	4	6
Palm fat	87	10	3	77	19	4	58	32	10	8½
							71	19	10	7
Whale oil	72	25	3	76	21	3	72	21	7	7½
Shea butter	66	29	5	60	22	18	50	35	15	9½
Herring oil	71	29	0	60	34	6	71	25	4	8
Rapeseed oil	70	23	7	75	18	7	73	15	12	8
							58	19	23	9
Poppyseed oil	76	22	2	80	14	6	85	11	4	11
Kapokseed oil	84	16	0	86	10	4	78	15	7	10½
Mean	74.9	23.0	2.2	73.1	21.9	5.1	70.0	22.7	7.3	

[1] Time at which, according to an estimation based upon the absorption values after three and 6 hours, 50% of the fat would have been absorbed.

seems to be independent of the time of absorption and, independent, therefore of the total amount of fat present in the gastrointestinal tract. Probably the body has a mechanism at its disposal which attempts to maintain the percentage of fat in the small intestine at a constant level, namely 22.5% of

the total amount of fat in the gastrointestinal tract. Similarly, the fat contents of the stomach and the large intestine, although involving greater variations, also show a certain constancy, about 73 and 5%, respectively. Finally it is noteworthy that the distribution of the fat over stomach, small and large intestine seems to be independent of the nature of the fat administered.

Some practical objections can be raised to the dosage of 400 mg/100 cm² as applied in these experiments. In the case of fats which are absorbed at a slow rate, the AT_{50} value is especially unfavorable from a point of view of the normal

TABLE 3

AT_{50}-values of butterfat and rapeseed oil related to various dosages

DOSAGE mg/100 cm²	AT_{50}-VALUES (MINS.)	
	Butterfat	Rapeseed oil
100	194	258
200	204	336
300	270	442
400	305	558

[1] AT_{50} is the number of minutes after which 50% of the fat administered had disappeared from the gastrointestinal tract.

working hours in the laboratory. Consequently, a lower dosage is to be preferred. Table 3 records the AT_{50} values at various dosages of two fats: butterfat, which is rapidly absorbed and rapeseed oil as an example of an oil having a very low absorption rate. The table shows that on administering 200 mg/100 cm² of the slowly absorbed rapeseed oil the AT_{50} value is approximately 6 hours. This period is undoubtedly more convenient and for further investigations this dosage is therefore recommended.

SUMMARY

The rates of absorption of 18 natural oils and fats of vegetable and animal origin have been studied. It appeared that

these oils and fats could be divided into 5 groups according to a decreasing rate of absorption:

1. Butterfat.
2. Maize oil, cottonseed oil, beef tallow, coconut fat, soyabean oil, sunflower oil, groundnut oil and olive oil.
3. Sesame oil, lard, palm fat and whale oil.
4. Shea butter and herring oil.
5. Rapeseed oil, poppyseed oil and kapokseed oil.

A significant correlation appeared to exist between the rate of absorption and the growth-action of the oils and fats.

The fat recovered three to 11 hours after the administration of 400 mg per 100 cm^2 of body surface appeared to be distributed over stomach, small and large intestine with a certain constancy, averaging 73, 22.5 and 5% respectively.

The percentage of fat in the small intestine, seems to be independent of the absorption time and consequently of the amount of fat in the tract; that in the stomach showed a tendency to decrease while that in the large intestine increased when absorption time was extended.

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AMINO ACID IMBALANCE AS RELATED TO METHIONINE, ISOLEUCINE, THREONINE AND TRYPTOPHAN REQUIREMENT OF THE RAT OR MOUSE[1]

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Recent reports from this laboratory by Salmon ('54) and by Sauberlich and Salmon ('55) have been concerned with the production of an amino acid imbalance in the rat. The reduction in growth due to this imbalance could be corrected by dietary supplements of tryptophan, but not by niacin alone. From these studies it was revealed that the tryptophan requirement of the rat is not a constant factor, but is related to the diet employed and in particular to the protein or nitrogen level of the diet. Tryptophan is peculiar among amino acids because of its relationship to niacin. Therefore, it was of interest to determine whether or not similar imbalance conditions with other amino acids could be produced.

The present report is concerned with the production of methionine, isoleucine and threonine imbalances in the rat.

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Certain additional studies on tryptophan imbalance in weanling mice are also included. Methionine was selected for study because of its known interrelationship with vitamin B_{12}. Threonine and isoleucine, however, unlike tryptophan and methionine, represent amino acids that have not been demonstrated to possess unique interrelationships with vitamins. Part of the material in this paper has been previously reported in abstract form (Sauberlich, '52).

EXPERIMENTAL

Weanling rats of either the Sprague-Dawley strain (SD, male) or the Alabama Experiment Station strain (AES, mixed-sex), 45 to 55 gm in weight, were placed in individual wire-bottom cages. In two series of experiments, young adult male rats of the SD strain were used. Food and water were given ad libitum and the animals were weighed weekly. The animals were fed the basal diets (table 1) or modifications of them for a period of three weeks or longer as indicated. Food consumption records were maintained on nearly all animals. In one series of experiments, weanling male albino mice of the Rockland Swiss-Webster strain were used.

Heparinized blood samples were obtained by heart puncture under light ether anesthesia from certain series of animals at the termination of the experiments. Protein-free filtrates of the plasma were prepared immediately from the blood samples by essentially the procedure of Hier and Bergeim ('45).

The amino acid analyses of the plasma samples were performed with the use of previously employed microbiological assay methods (Sauberlich and Baumann, '46; Steele et al., '49). Hemoglobin values were obtained by the oxyhemoglobin method.

RESULTS

Effect of methionine imbalance on growth of the rat

An amino acid imbalance of methionine was successfully produced in weanling rats with the use of peanut meal-ox-

TABLE 1

Composition of experimental diets
(gm/kg of diet)

INGREDIENTS	DIET NO.[1]													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Casein (extracted)[2]	..	..	..	..	..	..	..	..	60	60	60	100	100	..
Oxidized casein[3]	..	200	..	200	..	..	..	..	..	..	..	..	..	..
Lard	..	..	..	..	..	..	..	..	190	190	190	..	..	..
Corn oil	40	40	40	40	25	25	8	8	..	..	..	40	40	40
Peanut meal (extracted)[2]	420	420	350	350	..	..	..	..	..	..	..	..	..	..
Hemoglobin[4]	..	..	..	..	..	150	..	200	..	..	..	..	..	..
Yellow corn (ground)	..	..	..	..	750	750	700	700	..	..	..	..	..	..
Corn grits	..	..	..	..	..	..	..	..	400	400	400	..	..	..
Sucrose	485	283	553	351	159	9	224	24	295	264	226	805	736	908
Cod liver oil	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Salts[5]	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Choline	2	2	2	2	2	2	2	2	2	2	2	2	2	2
L-Cystine	3	3	3	3	3	3	3	3	3	3	3	3	3	..
DL-Methionine	..	..	..	..	..	..	..	..	..	3	5	..	5	..
DL-Tryptophan	..	2	..	2	1	1	1	1	..	1	2	..	2	..
DL-Isoleucine	..	..	..	..	0.5	0.5	2	2	..	5	10	..	10	..
L-Lysine·HCl	..	..	2	2	5	5	6	6	..	5	10	..	10	..
L-Histidine·HCl	..	..	..	..	1	1	..	..	..	4	8	..	8	..
DL-Threonine	..	..	..	..	2	2	2	2	..	..	..	..	..	..
DL-Phenylalanine	..	..	..	..	..	..	..	..	..	5	10	..	10	..
L-Arginine·HCl	..	..	..	..	..	..	..	..	..	4	8	..	8	..
L-Leucine	..	..	..	..	..	..	..	..	..	4	8	..	8	..
DL-Valine	..	..	..	..	2	2	2	2	..	..	8	..	8	..
Vitamin B_{12} (μg)	..	..	60	60	60	60	60	60	60	60	60	60	60	60

[1] All diets were supplemented with the following vitamins (mg/kg of diet): α-tocopherol, 100; α-tocopherol acetate, 100; 2-methyl-1,4-naphthoquinone, 5; inositol, 1000; niacin, 25; calcium pantothenate, 20; thiamine, 6; riboflavin, 6; pyridoxine, 6; biotin, 0.5; and folacin, 1.0.

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[3] Hove, E. L., D. H. Copeland and W. D. Salmon, J. Nutrition, *39*: 397 (1949).

[4] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Salmon, W. D., J. Nutrition, *33*: 155 (1947).

idized casein diets (table 1, diets 1 to 4). Results obtained with these diets are summarized in table 2. In experiments A and B (table 2), rats of the SD strain were used. In experiment A (table 2, groups 1 to 8), rats fed the 42% peanut meal diet

TABLE 2

Effect of methionine on the growth of weanling rats fed the methionine imbalance diet

(Weanling SD rats; 4-week experimental period)

GROUP NO.	BASAL DIET NO.	DIET [1]	NO. OF RATS	AV. GAIN/ RAT/WK.	AV. DAILY FOOD CONSUMPTION/ RAT	GM FOOD INGESTED / GM GAIN
				gm	*gm*	
		EXPERIMENT A				
1	1	42% PM	8	34.0	16.9	2.3
2	2	42% PM + 20% oxid. casein	4	17.1	13.7	4.9
3	2	Same as group 2 + M	4	21.3	11.7	2.8
4	2	Same as group 2 + B_{12}	4	27.3	13.8	2.7
5	2	Same as group 2 + B_{12} + M	4	31.7	15.1	2.4
6	1	42% PM + B_{12}	8	40.8	17.6	2.2
7	1	42% PM + M	4	38.5	16.6	2.4
8	1	42% PM + M + B_{12}	4	40.3	18.0	2.4
		EXPERIMENT B				
9	3	35% PM	4	36.5	14.0	2.5
10	4	35% PM + 20% oxid. casein	4	19.7	9.4	3.9
11	4	Same as group 10 + M	4	30.1	11.3	3.0
12	4	Same as group 10 + aureomycin [2]	4	21.2	11.3	2.6

[1] PM = extracted peanut meal (48% protein); M = DL-methionine, 5 gm/kg diet; vitamin B_{12} (where indicated in experiment A), 60 μg/kg diet.

[2] Aureomycin (chlortetracycline) added at level of 100 mg/kg diet.

gained in weight an average of 34.0 gm per week during the 4-week experimental period. However, when 20% of oxidized casein was added to the diet and supplemented with 0.2% of DL-tryptophan, the growth of the animals was reduced to 17.1 gm per week (table 2, group 2). The methionine imbalance also produced a marked increase in the amount of food consumed per gram of gain of the animals, as may be noted from the ratios of food consumption to body-weight gain.

Supplements of either methionine or vitamin B_{12} partly reversed the reduction in growth while a supplement of both methionine and vitamin B_{12} gave essentially normal growth and food conversion (31.7 gm per week). Supplements of vitamin B_{12} or methionine also improved somewhat the growth of rats fed the 42% peanut basal diet.

In experiment B (table 2, groups 9 to 12), the level of peanut meal used in the diet was reduced to 35% to restrict the methionine content more rigidly. The diet was supplemented with lysine, cystine, folacin and vitamin B_{12} (table 1). This diet permitted the same growth as that obtained with the 42% peanut meal diet unsupplemented with vitamin B_{12}. Again the addition of 20% oxidized casein to the 35% peanut meal diet caused a marked reduction in growth and rate of food conversion of the animals despite the presence of vitamin B_{12} in the diet (36.5 to 19.7 gm per week, respectively). Supplementation of the diet with 0.5% of DL-methionine almost completely corrected the imbalance (30.1 gm per week), whereas, the addition of aureomycin to the imbalance diet had little, if any, effect.

An amino acid imbalance of methionine was also readily produced in weanling rats of the AES strain when fed the diets employed in the above experiments. The imbalance condition was also corrected by methionine. Since the results were essentially the same as those obtained with the SD strain of rats, data were not presented. These experiments also revealed that rats of the SD strain were more efficient than rats of the AES strain in the conversion of food to gains

in body weight when fed the above diets. Rats of the SD strain ingested about 2.3 gm of food per gram of gain when fed the 42% peanut meal diet, whereas rats of the AES strain required 3.0 gm.

TABLE 3

Effect of isoleucine on the growth of weanling rats fed isoleucine imbalance diets
(Weanling SD rats; 4-week experimental period)

GROUP NO.	BASAL DIET NO.	DIET	NO. OF RATS	AV. GAIN/ RAT/WK.	AV. DAILY FOOD CONSUMPTION/ RAT	GM FOOD INGESTED / GM GAIN
				gm	*gm*	
		EXPERIMENT C				
1	5	75% Corn	12	8.7	7.5	6.0
2	6	75% Corn +15% hemoglobin	12	0.9	5.4	42.2
3	6	Same as group 2 + isoleucine [1]	8	20.8	10.3	3.4
		EXPERIMENT D				
4	7	70% Corn	7	16.3	8.7	3.7
5	8	70% Corn + 20% hemoglobin	4	1.2	6.3	37.1
6	8	Same as group 5 + isoleucine [2]	4	16.7	8.7	3.4
7	7	70% Corn + isoleucine [2]	3	15.8	8.8	3.9

[1] DL-Isoleucine added at a level of 5.5 gm/kg of basal diet.
[2] DL-Isoleucine added at a level of 4 gm/kg of the respective basal diets.

Effect of isoleucine imbalance on growth of the rat

The production of an amino acid imbalance of isoleucine was attempted by feeding weanling rats a corn-hemoglobin diet. Ground corn grain was employed as the source of protein in order to control the isoleucine content of the diet. Earlier studies in this laboratory had demonstrated that corn was deficient in isoleucine, tryptophan, lysine, threonine and valine

(Sauberlich et al., '53). Therefore, the basal diets were supplemented with these amino acids. Isoleucine was added in amounts sufficient to provide only a suboptimal level (table 1, diets 5 to 8). Hemoglobin, because of its low isoleucine content, was employed in an attempt to produce an imbalance condition.

From the results summarized in table 3, it may be noted that the addition of hemoglobin to the corn basal diet caused a very severe inhibition in growth. In experiment C, growth was reduced from an average gain of 8.7 gm per week to only 0.9 gm by the addition of 15% hemoglobin to the 75% basal corn diet (table 3, groups 1 and 2). Supplementation of the diet with 0.55% of DL-isoleucine readily reversed the depression in growth.

In experiment D (table 3), the content of corn in the basal diet was reduced to 70%, but the level of DL-isoleucine was increased to 0.2% to permit increased growth of the animals. However, when 20% of hemoglobin was added to the diet, a severe inhibition in growth was again noted (16.3 vs 1.2 gm per week, respectively). Deaths were noted when the animals were maintained on the imbalance diet for periods beyond 4 weeks. An increased addition of DL-isoleucine (0.4%) to the diet prevented death and depression in growth. The amount of food required per gram of gain in body weight was also markedly increased, but was returned to normal with an increased supplementation of isoleucine to the diet.

Effect of threonine imbalance on growth of the rat

When a series of amino acids was added to a 6% casein-corn grits basal diet (table 1, diets 9 and 10), growth of weanling rats was very markedly reduced. For example, in experiment E (table 4, groups 1 to 3), growth of the rats fed the 6% casein-corn grits basal diet was reduced from an average weekly gain of 19.6 gm to only 7.3 gm by the addition of the amino acids to the diet. However, the depression in growth was prevented by supplementation of the diet with

0.4% of DL-threonine. It is evident, then, that an amino acid imbalance of threonine could be produced in the rat by supplementation of the diet with certain amino acids. Imbalances of lysine, histidine, isoleucine or leucine were not produced when similar respective combinations of amino acids were added to the 6% casein-corn grits basal diet (table 4, groups 4 to 7).

In experiment F (table 4, groups 8 to 11), the amino acid supplement used to produce the threonine imbalance was twice that employed in experiment E, and also included valine. Again the observed depression in growth could be prevented by the addition of threonine to the diet. The addition of only tryptophan and methionine to the basal diet did not produce an inhibition in growth. Supplementary experiments also revealed that the addition of the other amino acids singly and in certain pairs to the basal diet effected no reduction in growth.

The imbalance effect was also observed when the level of casein was increased to 10% and the corn grits omitted from the diet (table 4, experiment G). Again threonine reversed the effect. In each experiment, it may be noted that the imbalance condition also increased the amount of food required per gram of gain in body weight. This was readily reversed by supplements of threonine to the diet.

Effect of amino acid and protein deficiencies upon adult rats

Recent studies in this laboratory (Sauberlich and Salmon, '55) revealed that a tryptophan imbalance in the rat caused an increased urinary excretion of amino acids, including that of tryptophan itself. When the imbalance condition was corrected by the addition of increased supplements of tryptophan to the diet, the urinary excretion of amino acids returned to normal. The increased loss of amino acids produced by the tryptophan imbalance may have reflected an attempt

TABLE 4

Effect of threonine on the growth of weanling rats fed threonine imbalance diets
(SD rats; 4-week experimental period)

GROUP NO.	BASAL DIET NO.	DIET	NO. OF RATS	AV. GAIN/ RAT/WK.	AV. DAILY FOOD CONSUMPTION/ RAT	GM FOOD INGESTED / GM GAIN
				gm	*gm*	
		EXPERIMENT E				
1	9	6% Casein	5	19.6	9.0	3.1
2	10	6% Casein + amino acids	6	7.3	3.8	3.7
3	10	Same as group 2, + threonine [1]	5	18.0	5.9	2.3
4	10	Same as group 3, lysine omitted	5	19.0	8.5	3.1
5	10	Same as group 3, histidine omitted	5	22.5	7.4	2.3
6	10	Same as group 3, isoleucine omitted	5	18.0	6.4	2.5
7	10	Same as group 3, leucine omitted	5	24.3	7.8	2.3
		EXPERIMENT F				
8	9	6% Casein	4	18.4	7.9	3.0
9	9	6% Casein + T + M [2]	4	18.2	8.1	3.1
10	11	6% Casein + amino acids	5	10.4	6.8	4.6
11	11	Same as group 10, + threonine	4	23.8	8.3	2.4
		EXPERIMENT G				
12	12	10% Casein	4	22.4	9.1	2.8
13	13	10% Casein + amino acids	4	12.9	7.2	3.9
14	13	Same as group 13, + threonine	3	22.6	8.6	2.7

[1] DL-Threonine, supplemented at a level of 4 gm/kg diet.
[2] T = DL-tryptophan, 1 gm/kg diet; M = DL-methionine, 3 gm/kg diet.

TABLE 5

Effect of amino acid and protein deficiencies upon adult rats

(4 SD rats/group)

GROUP NO.	BASAL DIET NO.	DIET [1]	AV. INITIAL WEIGHT	AV. WEIGHT CHANGE AFTER		AV. DAILY FOOD CONSUMPTION FOR	HB VALUES AT [2]		SURVIVAL AT
				4 weeks	12 weeks	12 weeks	6 wk.	11 wk.	16 wk.
			gm	*gm*	*gm*	*gm*	*gm/100 ml*		
EXPERIMENT H									
1	14	Basal (protein-free)	250	— 68	— 128	9.2	13.2	11.8	0/4
2	14	Basal + 3% urea	266	— 74	— 137	9.0	12.9	12.1	0/4
3	14	Basal + 3% glycine	261	— 73	— 121	9.0	12.0	14.3	0/4
4	14	Basal + 20% gelatin	261	— 59	— 123	8.8	13.9	12.5	0/4
5	14	Basal + 20% zein	250	— 53	— 100	9.3	14.4	13.4	3/4
6	14	Basal + 20% oxidized casein	253	— 61	— 114	8.6	14.0	12.8	1/4
7	14	Basal + 20% oxidized casein + M [3]	270	— 74	— 140	8.3	14.6	13.0	0/4
8	14	Basal + 20% casein	250	+ 28	+ 58	12.9	14.6	14.3	4/4

[1] Supplements were added to the protein-free basal diet at the expense of sucrose.

[2] Hemoglobin content determined by the oxyhemoglobin method.

[3] M = DL-methionine, added at a level of 7 gm/kg of diet.

by the animal to remove the excess nnused amino acids. In so doing, tryptophan was also lost.

In an attempt to determine further the effect excess amino acids may have on the imbalance phenomenon, adult rats were placed on a protein-free diet (table 1, diet 14). Other groups of animals received this diet supplemented with proteins deficient in certain amino acids, or with glycine, or with urea. Under these more pronounced conditions, one could then, perhaps, determine whether or not excess amino acids exerted an effect by causing an increased urinary loss of amino acids limiting to the animal. Such losses of amino acids may then be reflected in an increased rate in loss of body weight and a reduced survival time of the animals.

Results of such a study are summarized in table 5. The data indicate that rats fed the diets supplemented with amino acid-deficient proteins, glycine or urea lost weight at no greater rate than animals fed the protein-free diet. Similarly, the excess amino acids or nitrogen did not reduce the survival time of the animals. The hemoglobin values of all animals remained near normal for a period of at least 11 weeks, even with the complete absence of protein in the diet. Food consumption of the animals also remained surprisingly high throughout the experiment.

Plasma amino acid levels of rats fed certain imbalance diets

Summarized in table 6 are levels of several amino acids in the plasma of rats fed certain imbalance diets. In experiment I, rats were fed the methionine-imbalance diets. From these experiments, it was noted that the methionine level in the plasma was not affected by the addition of 20% oxidized casein to the diet. The supplementation of the peanut meal-oxidized casein diet with 0.5% of DL-methionine increased the level of methionine from 10.6 to 20.6 μg/ml of plasma (table 6, groups 2 and 3). Only the L-isomer of methionine was measured in the microbiological method employed. The ad-

TABLE 6

Plasma amino acid levels of rats fed certain imbalance diets
(SD rats)

GROUP NO.	BASAL DIET NO.	DIET [1]	AMINO ACID CONTENT [2]			
			(μg/ml plasma)			
		EXPERIMENT I (4 weanling rats/group; 4-week experimental period)				
			Methionine			*Valine*
1	1	42% PM	10.4 ± 1.0			8.4 ± 1.9
2	2	42% PM + 20% oxid. casein	10.6 ± 0.3			24.6 ± 2.1
3	2	42% PM +20% oxid. casein + M	20.6 ± 2.8			27.6 ± 7.4
4	2	42% PM + 20% oxid. casein + B_{12}	15.2 ± 2.1			23.0 ± 5.0
5	2	42% PM + 20% oxid. casein + B_{12} + M	20.2 ± 1.3			33.3 ± 6.9
6	1	42% PM + B_{12}	13.5 ± 1.7			13.7 ± 1.1
7	1	42% PM + M	18.6 ± 1.2			15.0 ± 2.7
8	1	42% PM + B_{12} + M	21.8 ± 2.4			12.0 ± 3.7
		EXPERIMENT J [3] (4 adult rats/group; 3-week experimental period)				
			Methionine	*Proline*	*Tryptophan*	*Valine*
9	14	Basal + 20% casein [4]	19.0 ± 0.7	34.4 ± 8.8	32.3 ± 2.9	31.8 ± 3.9
10	14	Basal + 20% oxid. casein + T + M + C	12.7 ± 0.8	31.6 ± 4.4	29.1 ± 2.6	32.5 ± 1.3
11	14	Basal + 20% oxid. casein + C + M	10.7 ± 0.1	15.7 ± 0.9	14.1 ± 2.1	15.0 ± 1.3

[1] PM = extracted peanut meal (48% protein); M = DL-methionine (5 gm/kg diet); C = L-cystine (3 gm/kg diet); T = DL-tryptophan (3 gm/kg diet); vitamin B_{12} added at a level of 60 μg per kilogram of diet.

[2] Average ± standard error of the mean.

[3] Average initial weight of animals 280 gm; group 9 gained an average of 14.2 gm/wk.; group 10 gained an average of 3.2 gm/wk. and group 11 lost an average of 24.2 gm/wk.

[4] Proteins were added at the expense of sucrose to the protein-free basal diet.

dition of vitamin B_{12} to the imbalance diet increased the plasma level of methionine to 15.2 μg/ml. Supplementation of the basal peanut meal diet with methionine likewise produced a very marked increase in the concentration of methionine in the plasma (10.4 to 18.6 μg/ml). Vitamin B_{12} supplementation also produced some increase.

The valine level in the plasma, however, was increased nearly three-fold by the addition of oxidized casein to the basal peanut meal diet (8.4 to 24.6 μg/ml). Supplements of methionine or vitamin B_{12} to the diets had little effect on the level of valine in the plasma.

In experiment J, the methionine, proline, tryptophan and valine levels were determined in plasma from adult rats fed a 20% normal casein diet or a 20% oxidized casein diet supplemented with both tryptophan and methionine or only with methionine for a period of three weeks. The plasma amino acid levels of rats fed the casein diet or the fully supplemented oxidized casein diet were essentially the same, except for methionine. The methionine level was somewhat higher for rats fed the casein diet. This may be related to the level of DL-methionine added to the oxidized casein diet.

When tryptophan was omitted from the diet, the plasma amino acid levels were considerably lowered. This occurred despite the fact that the animals were consuming considerable quantities of amino acids, in addition to losing in body weight an average of 24 gm per week. Thus, the animals apparently were capable of removing from the blood stream considerable quantities of amino acids despite the dietary deficiency of tryptophan.

Effect of tryptophan imbalance on growth of the mouse

An amino acid imbalance was also produced in the mouse by the use of casein-oxidized casein diets. Results of these studies are presented in table 7. When mice were fed an 8% casein diet, growth of 8.4 gm was obtained in 4 weeks. The addition of 20% of oxidized casein to the diet, supple-

mented with 0.5% of DL-methionine, reduced the growth of the animals to only 1.1 gm in 4 weeks. This reduction in growth could be largely prevented by supplementation of the diet with 0.25% of DL-tryptophan (6.6 gm in 4 weeks). A higher level of supplementation may have improved the growth further, since the mouse utilizes the D-tryptophan with difficulty.

TABLE 7

Effect of tryptophan imbalance on growth of weanling albino mice[1]

DIET[2]	AV. GAIN IN 4 WEEKS
	gm
8% Casein	8.4
8% Casein + 20% oxidized casein + M	1.1
8% Casein + 20% oxidized casein + M + T	6.6
28% Casein	14.3

[1] Average initial weight 11.4 gm; five mice used per group.

[2] Diets employed were similar to those previously used (Sauberlich and Salmon, '55) except the level of casein was reduced to 8% and all diets were supplemented with 0.3% of DL-threonine. M = DL-methionine, added at a level of 0.5% of the diet; T = DL-tryptophan, added at a level of 0.25% of the diet.

DISCUSSION

The production of imbalances of methionine, isoleucine and threonine in the rat as described in these studies demonstrates that the amino acid imbalance condition is not peculiar to tryptophan alone. Previous studies have demonstrated that a tryptophan imbalance can be produced in the rat independently of niacin (Salmon, '54; Sauberlich and Salmon, '55), although it is true that under certain conditions niacin does demonstrate a corrective effect, the so-called "niacin effect" (Krehl et al., '45, '46; Briggs et al., '46; Singal et al., '48; Hankes et al., '49; Anderson et al., '51; Salmon, '54; Koeppe and Henderson, '55). In the present studies a similar relationship was observed for vitamin B_{12} with respect to the production of a methionine imbalance. Other studies have shown that vitamin B_{12} can exert a sparing-action on methionine under certain conditions (Patrick, '50, '52; Sunde et al., '50; Sauberlich, '54). Vitamin B_{12} also exhibited a spar-

ing action on methionine in the present experiments as was observed by the growth and corrective effects on the amino acid imbalance. When the methionine level in the diet was sufficiently reduced, an imbalance was produced even in the presence of vitamin B_{12}. The addition of methionine to the diet corrected the imbalance.

Tryptophan imbalances are similar to methionine in this respect (Salmon, '54; Sauberlich and Salmon, '55). When the level of tryptophan in the diet was reduced sufficiently, an imbalance condition was produced regardless of the presence of niacin. Under these circumstances tryptophan, but not niacin, corrected the condition.

The imbalances of isoleucine and threonine were produced without any apparent unique vitamin-amino acid interrelationships. These results suggest that the imbalance effect may be a general phenomenon associated with specified conditions for probably most of the essential amino acids and possibly even for some of the "non-essential" amino acids. Results of the present study emphasize again that the amino acid requirements of the rat are not constant factors but are related to the diet employed and in particular to the protein or nitrogen level of the diet. Such an effect was demonstrated previously in this laboratory for the tryptophan requirement of the rat (Salmon, '54; Sauberlich and Salmon, '55). The mouse also appears to be subject to the effects of an imbalance in tryptophan. Although determinations of the quantitative increase in the requirements for methionine, isoleucine or threonine under the imbalance conditions were not made in the present investigation, such increased requirements are evident.

Several possible explanations were previously offered for the increased demand for tryptophan (Salmon, '54; Sauberlich and Salmon, '55). Such explanations are also applicable to the increased requirements noted for methionine, isoleucine and threonine. For example, a possible explanation was that in the absence of sufficient tryptophan to permit a balance of the ingested amino acids for synthesis into tissue proteins,

the surplus amino acids must be excreted or disposed of in some other manner and that in this process there is a wasting of the tryptophan originally available. The tryptophan imbalance produced an increased loss of tryptophan and other amino acids in the urine and a marked lowering of the tryptophan level in the plasma. However, from the results of the present study such losses of amino acids do not appear to be the basic cause of the imbalance. When rats were fed diets deficient in protein or amino acids, excess amino acids did not appear to increase the loss of limiting amino acids as noted by weight loss and survival of the animals.

Although the imbalance in methionine did not produce a depression in the plasma level of this amino acid, it did appear to alter the ratio of methionine with respect to other amino acids. Such alterations in plasma amino acid concentrations may be an explanation for the imbalance phenemonon, since such a condition could very likely interfere with enzymatic ability and activity for protein synthesis. Thus in order for efficient and maximum tissue protein synthesis to proceed in an animal, the diet must furnish amino acids not only in sufficient quantity, but also in proper balance.

SUMMARY

1. Growth of weanling rats was depressed more than 50% when oxidized casein was added to a diet containing peanut meal as a protein source. This growth depression (or imbalance) could be corrected by additions to the diet of methionine or, under certain conditions, of vitamin B_{12}.

2. The addition of hemoglobin to a corn grain diet produced an imbalance that was corrected by supplements of isoleucine.

3. The supplementation of certain amino acids to a casein diet produced a marked reduction in growth. Threonine supplements, however, prevented the depression in growth.

4. The amino acid imbalances increased the amount of food required per gram of gain in body weight of the animals.

This was corrected by supplementation of the diet with the corresponding amino acids.

5. Plasma levels of methionine were not altered by the methionine imbalance. Tryptophan deficiency, however, caused a reduction in plasma amino acids.

6. The survival of adult rats fed protein-free diets was not influenced by supplements of certain amino acid-deficient proteins, glycine or urea.

7. An amino acid imbalance was produced in weanling mice fed a casein-oxidized casein diet. This imbalance could be largely prevented by supplements of tryptophan to the diet.

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THE INFLUENCE OF AMINO ACIDS AND OTHER ORGANIC COMPOUNDS ON THE GASTROINTESTINAL ABSORPTION OF CALCIUM[45] AND STRONTIUM[89] IN THE RAT

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TWO FIGURES

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INTRODUCTION

Calcium utilization and bone mineralization are greatly reduced when the dietary protein is low in either quality or quantity (McCance et al., '42; Desikachar and Subrahmanyan, '49; Frandsen et al., '54). Poor calcium utilization also occurs on diets deficient in one or more of the essential amino acids (Bavetta et al., '54 and Haggar et al., '55). These amino acid insufficiencies result in a narrowing of the epiphyseal cartilage plate due to the reduction of cartilage cells, and a marked osteoporosis in both the epiphysis and diaphysis of the femur. Similarly, structural changes in the intestinal wall or a reduction of pertinent enzymes resulting from severe protein lack, or both, may interfere with mineral absorption.

Short term interactions between proteins or protein derivatives and calcium are indicated from the studies of Lehman and Pollack ('41–'42). These investigators observed that α-amino acids increase the solubility of calcium salts and pro-

[1] On assignment from the United States Air Force Veterinary Corps.
[2] Under contract with the United States Atomic Energy Commission.

posed that the simultaneous presence of both factors in the gut would aid mineral absorption. The present study was concerned with the direct estimation of the effect of various amino acids on the absorption of ingested radiocalcium and radiostrontium by the rat. Also studied were other organic substances that have been reported to increase calcium absorption. Radiostrontium was employed to observe the comparative behavior of the two alkaline earths and because of contemporary interest in the hazards of radiostrontium.

MATERIALS AND METHODS

Young male (90 to 130 gm) Carworth albino rats, reared on a commercial stock diet, were used in most of these experiments. The schedule of treatment was as follows: (1) the rats were fasted for 22 hours with water available; (2) the rats were given, by stomach tube, 2 ml of a solution containing 10 μc of Sr^{89}, 20 μc of Ca^{45}, 10 mg of $CaCl_2$ carrier and 0.84 millimoles of the test substance; (3) the animals were killed 24 hours after dosage, having been maintained on the fast. The femurs were removed, ashed, dissolved in 3N HCl, and made to 50 ml volume. An external solution count was made on one aliquot with a conventional Geiger tube; a 55 mg/cm^2 absorber was inserted between the solution and tube to eliminate any Ca^{45} contribution. The counting rate was evaluated in terms of a standard and results were expressed as "percentage of Sr^{89} dose in the femurs." For the Ca^{45} determination, another aliquot of the ash solution was precipitated as the oxalate and radioassayed by procedures described by Comar ('55). The Ca^{45} content of the sample was determined by the usual methods for radioassay of mixtures using differential absorption (Comar, '55). The calcium-strontium ratios (Ca^*/Sr^*) were calculated by dividing the percentage of Ca^{45} dose in the femurs by the corresponding Sr^{89} value.

Commercial amino acids were used without further purification. Amino acids of low solubility were put into solution

with hydrochloric acid and all test solutions in each series were adjusted to equivalent acidities. Other reagents were C. P. grade.

RESULTS

Tables 1 and 2 show the effects of essential and certain non-essential amino acids on the accumulation of Ca^{45} and Sr^{89} in rat femurs following simultaneous ingestion. Since the percentage increase was similar for both Ca^{45} and Sr^{89}, a

TABLE 1

Effect of essential amino acids on the accumulation of Ca^{45} and Sr^{89} in rat femurs[1]

TREATMENT	Ca^{45}	Sr^{89}	AVERAGE INCREASE IN Ca^{45} AND Sr^{89}	Ca*/Sr* IN FEMUR
	(% of dose in femurs)	*(% of dose in femurs)*	*(% of control)*	
Control	4.6 ± 0.2	2.8 ± 0.1	100	1.63
L-Lysine	8.0 ± 0.4	5.4 ± 0.4	182	1.48
L-Arginine	7.4 ± 0.3	5.5 ± 0.4	176	1.33
L-Tryptophan	7.3 ± 0.4	4.5 ± 0.4	159	1.62
L-Leucine	6.6 ± 0.2	4.0 ± 0.3	142	1.62
L-Histidine	6.3 ± 0.2	3.3 ± 0.1	126	1.89
L-Methionine	6.0 ± 0.3	3.1 ± 0.2	119	1.96
L-Isoleucine	5.3 ± 0.2	3.1 ± 0.3	112	1.70
L-Valine	5.4 ± 0.2	2.9 ± 0.1	110	1.84
L-Threonine	5.1 ± 0.2	2.9 ± 0.2	107	1.78
L-Phenylalanine	5.3 ± 0.2	2.8 ± 0.2	105	1.91

[1] Values represent mean ± standard error of the mean; 8 animals per group; mean body wt. = 92 ± 1 gm; mean femur ash wt. = 184 ± 4 mg; dose contained 10 mg carrier $CaCl_2$ and 0.84 millimoles of amino acid.

single average value is given for brevity. It will be noted that lysine and arginine were most effective in promoting the appearance of the radioisotopes in the bone; these two amino acids almost doubled the Ca^{45} and Sr^{89} values in the femur. Tryptophan, leucine, and aspartic acid were also appreciably effective. The other amino acids studied had lesser or no significant effect. It should be noted that the administration of lysine resulted in an occasional diarrhea; this was not observed with any other amino acid.

TABLE 2

Effect of lysine and some nonessential amino acids on the accumulation of Ca^{45} and Sr^{89} in rat femurs [1]

TREATMENT	Ca^{45}	Sr^{89}	AVERAGE INCREASE IN Ca^{45} AND Sr^{89}	Ca*/Sr* IN FEMUR
	(% of dose in rat femurs)	*(% of dose in rat femurs)*	*(% of control)*	
Control	4.6 ± 0.5	2.5 ± 0.3	100	1.85
L-Lysine	8.5 ± 0.4	5.2 ± 0.3	198	1.64
L-Aspartic acid	6.3 ± 0.4	3.8 ± 0.3	146	1.67
Hydroxy-L-proline	6.3 ± 0.3	3.4 ± 0.2	136	1.73
L-Glutamic acid	6.0 ± 0.4	3.4 ± 0.2	135	1.86
DL-Tyrosine	5.8 ± 0.5	3.3 ± 0.4	130	1.75
L-Serine	6.0 ± 0.3	3.0 ± 0.3	126	2.00
Glycine	5.7 ± 0.2	3.0 ± 0.2	123	1.90
L-Proline	4.9 ± 0.2	2.6 ± 0.1	107	1.89
L-Alanine	4.8 ± 0.4	2.5 ± 0.1	104	1.94

[1] Values represent mean ± standard error of the mean; 8 animals per group; mean body wt. = 101 ± 2 gm; mean femur ash wt. = 212 ± 5 gm; dose contained 10 mg carrier $CaCl_2$ and 0.84 millimoles of amino acid.

TABLE 3

Comparison of amino acids with various substances on the accumulation of Ca^{45} and Sr^{89} in rat femurs [1]

TREATMENT	Ca^{45}	Sr^{89}	AVERAGE INCREASE IN Ca^{45} AND Sr^{89}	Ca*/Sr* IN FEMUR
	(% of dose in femurs)	*(% of dose in femurs)*	*(% of control)*	
Control	3.2 ± 0.3	2.0 ± 0.2	100	1.63
Lactose	7.5 ± 0.3	5.3 ± 0.2	252	1.43
L-Lysine	6.2 ± 0.3	4.1 ± 0.3	201	1.53
L-Arginine	5.8 ± 0.3	4.0 ± 0.3	193	1.46
L-Leucine	4.5 ± 0.3	3.0 ± 0.2	146	1.51
Na gluconate	5.0 ± 0.2	2.7 ± 0.2	146	1.86
Na lactate	4.1 ± 0.1	2.7 ± 0.1	130	1.52
B vitamin mixture	3.2 ± 0.2	2.0 ± 0.2	100	1.66
Na citrate	3.3 ± 0.4	1.7 ± 0.2	94	1.95

[1] Values represent mean ± standard error of the mean; 8 animals per group; mean body wt. = 129 ± 3 gm; mean femur ash wt. = 266 ± 6 mg; dose contained 10 mg carrier $CaCl_2$ and 0.84 millimoles of test substance. The B vitamin mixture contained 0.35 mg thiamine-HCl, 0.75 mg riboflavin, 2 mg calcium pantothenate, 0.70 mg niacin, 1.0 mg pyridoxine-HCl, 2 mg p-amino benzoic acid, and 0.25 mg folic acid per dose.

Table 3 summarizes a comparison of lysine, arginine and leucine with other organic compounds that have been reported to influence calcium absorption. It may first be noted that the results with the amino acids showed good agreement with other experiments (tables 1 and 2). Of particular interest was the action of lactose, which was more effective than either lysine or arginine, and which increased the radioisotope content of the femur by a factor of about 2.5. The lactose effect on calcium absorption and utilization has been generally accepted and recently emphasized by Fournier ('55).

TABLE 4

Effect of lysine on femur accumulation of oral versus parenterally administered Ca^{45} and Sr^{89} [1]

TREATMENT	METHOD OF Ca^{45} AND Sr^{89} ADMINISTRATION	Ca^{45}	Sr^{89}	AVERAGE INCREASE IN Ca^{45} AND Sr^{89}	Ca^*/Sr^* IN FEMUR
		(% of dose in femurs)	*(% of dose in femur)*	*(% of control)*	
Control	Oral	4.4 ± 0.4	2.5 ± 0.3	100	1.78
L-Lysine	Oral	8.0 ± 0.2	5.4 ± 0.1	199	1.47
Control	I. P.	8.2 ± 0.3	7.5 ± 0.3	100	1.10
L-Lysine	I. P.	9.0 ± 0.3	7.7 ± 0.1	107	1.17

[1] Values represent mean ± standard error of the mean; 6 animals per group; mean body wt. = 138 ± 4 gm; mean femur ash wt. = 229 ± 14 mg; dose contained 10 mg $CaCl_2$ and 0.84 millimoles of amino acid.

The sodium lactate and sodium gluconate showed only small positive effects; the vitamin B mixture and sodium citrate had no effect. The negative results with citrate tend to corroborate the findings of Antoni and Cremer ('55).

The appearance of ingested Ca^{45} and Sr^{89} in the bone can be theoretically related to absorption from the gut, and also to any other processes concerned with the removal of the radioisotopes from the blood; for example, exchange into extravascular spaces and excretion. When animals to be compared are under similar physiological conditions, it is generally accepted that the appearance of ingested Ca^{45} and Sr^{89} in bone is a reliable index of absorption of these radio-

isotopes from the gut. An experiment was performed, however, to show that the effect of lysine was predominantly upon gastrointestinal absorption rather than upon bone mineralization or other processes within the body. The experimental plan and results are presented in table 4. The conditions and quantities of test substances were identical with previously described experiments. In principle, a comparison was made of the effects of lysine on ingested versus parenterally administered Ca^{45} and Sr^{89}. Lysine again doubled the amount of the radioisotopes found in the bone when the minerals and amino acids were administered orally. Injected Ca^{45} and Sr^{89}, however, were deposited to about the same extent in lysine-treated and control rats. This supports the premise that the reported amino acid responses do indeed reflect absorption from the gut rather than other processes concerned with the disappearance of the radioisotopes from the blood. If one assumes that the same proportion of the absorbed and injected dose enters the skeleton, it appears that the control rats absorbed 54 and 33% of the Ca^{45} and Sr^{89} dose, respectively, while the comparable values for the lysine-treated rats were 88 and 70%.

An indication of the comparative metabolism of radiocalcium and radiostrontium can be gained from the Ca^*/Sr^* ratios that have been presented in tables 1 to 4. These ratios show that, in general, radiocalcium was preferentially absorbed over radiostrontium by a factor of about 1.7. This is in agreement with data for man (Harrison et al., '55). It is important to note that the substances that increased Ca^{45} absorption also increased Sr^{89} absorption. The Ca^*/Sr^* ratios indicate, however, that the test substances tended to promote Sr^{89} absorption more than they did Ca^{45} absorption. This is shown in figure 1, a scattergram including data from all of the experiments; the normalized values of the Ca^*/Sr^* ratios are plotted against the corresponding increase in absorption. The explanation for this behavior is not clear. It is certain,

however, that the calcium and strontium ions do not respond equally to the processes causing the increased absorption. This may be related to ionic characteristics, the presence of the carrier calcium, or discriminating processes in the absorption mechanism.

Figure 2 presents a dose-response curve for lysine and the Ca^{45} and Sr^{89}. The conditions of this experiment were the same as those previously described, with the exception that

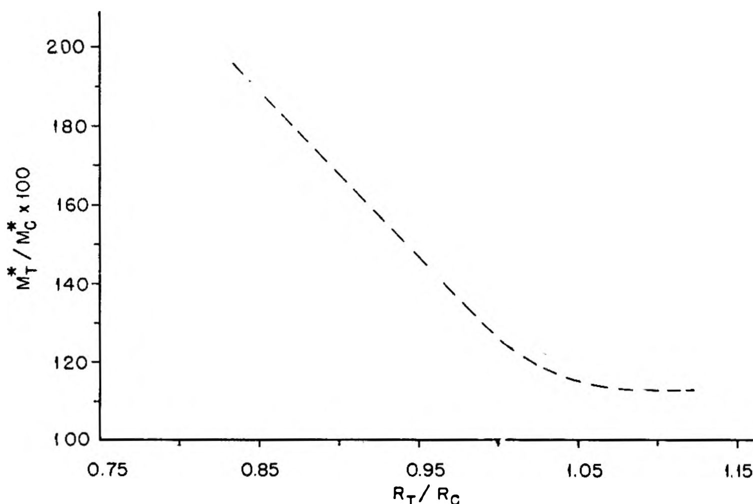

Fig. 1 Scattergram of the correlation between the Ca*:Sr* ratio and percentage increase in accumulated Ca^{45} and Sr^{89} in rat femurs. Mt* = average percentage dose of Ca^{45} and Sr^{89} in bones of treated group and Mc* = average percentage dose of Ca^{45} and Sr^{89} in control group. R_t/R_c = Ca*:Sr* ratio in treated group divided by Ca*:Sr* ratio in control.

the carrier $CaCl_2$ was increased to 60.8 mg to reduce the effect of endogenous calcium. The varying amounts of L-lysine and the molar ratios of L-lysine to $CaCl_2$ are shown in the graph. There appeared to be little effect at a lysine to calcium molar ratio of less than one half; the greatest increase in absorption occurred as the ratio increased from 1 to 2. This suggests that the action of lysine is other than that due to a vitamin-like stimulation; one would expect a much lower effective lysine to calcium ratio if this were true.

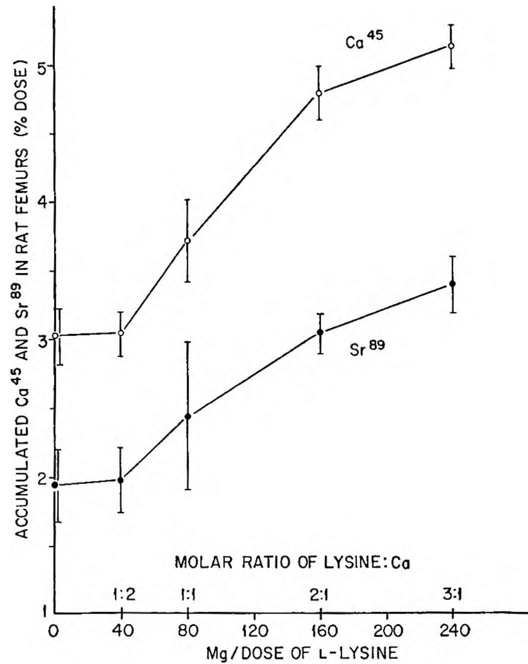

Fig. 2 Effect of various molar ratios of lysine to calcium on the accumulation of Ca^{45} and Sr^{89} in rat femurs.

DISCUSSION

The present study demonstrates that certain amino acids, notably lysine and arginine, promote the absorption of radiocalcium and radiostrontium. Other amino acids give little or no response. It seems unlikely that a stimulation of osteoid tissue synthesis or hormonal production would be involved as factors because of the short duration of these experiments. The correction of any of these disorders by administration of a single amino acid would be unexpected. Also, arginine and lysine were equally effective in promoting mineral absorption; arginine is considered to be only marginally required by the rat for growth as compared to the absolute need for lysine (Almquist, '51). In addition, one cannot conceive that such a nonspecific property as the energy

provided by the amino acid is primarily responsible for the increased mineral absorption.

Examination of the metabolic and physicochemical characteristics of these compounds as related to their effect on mineral absorption permits some limited interpretations concerning the mechanisms. The theory of Lehmann and Pollack ('41–'42), relating increased mineral absorption to the increased solubility of the calcium salts in the presence of α-amino acids, cannot entirely explain the present data. Glycine, which has a pronounced solvent action on calcium salts, was relatively ineffective in these studies. Except for the dicarboxylic amino acids, the other amino acids, especially the basic types, would probably be no more effective than glycine in the solution of calcium salts. Complex formation between the amino acid *per se* and the mineral also cannot alone account for the present observations. This is based on inferences from the data of Li and Doody ('52) in which it was shown that lysine and arginine form unstable complexes with the cupric ion, whereas glutamic acid forms a stable complex. Preliminary observations in this laboratory indicate that calcium and strontium are similar to copper in this respect. Since lysine and arginine (isoelectric points of 9.74 and 10.76, respectively) are in the cationic form under the pH conditions of the intestine, strong complex formation between these basic amino acids and calcium would be theoretically unexpected. As pointed out by Greenberg ('44), in the main, electrostatic forces can be considered as acting to prevent or retard the ionization of alkaline earth cations. From these considerations, it appears that the effectiveness of aspartic acid and glutamic acid may be related to complex formation, but one must look elsewhere to understand the mechanism of the stimulation by lysine and arginine.

The passage of the amino acid itself through the gut barrier is quite likely associated with its effect on mineral absorption. It has been shown with everted gut sacs *in vitro* that glycine and the L-isomers of alanine, phenylalanine, methionine, histidine, isoleucine, and proline are "actively" transported

against a concentration gradient; such transport did not occur with L-glutamic acid, L-aspartic acid, L-lysine, and L-ornithine (Wiseman, '55). Assuming that these data apply to *in vivo* conditions, it appears that there is no direct correlation between the ability of the amino acid to promote calcium absorption and its ability to undergo "active" transport. If the dicarboxylic acids were "actively" transported, a greater response from these amino acids might have been realized.

Inhibitor studies by Fridhandler and Quastel ('55), which were limited to the L-isomers of alanine, phenylalanine, and histidine, indicate that a phosphorylation process, possibly indirect, may be involved in their active absorption. Tuba and Dickie ('55) observed that phosphoproteins (casein and vitellin) and possibly single dietary amino acids increase the amount of intestinal alkaline phosphatase in fasted rats; this was taken as indicating that the active absorption of amino acids may involve the action of phosphatase. These recent reports suggest two hypotheses that may be applicable to the present data. The first would be that lysine and arginine in some manner interact with the mineral either in the gut lumen or the gut wall, resulting in increased mineral absorption. This interaction may involve the formation of an intermediate compound, such as a phosphorylated lysine or arginine, which would act as carrier for the mineral. Enzyme-resistant phosphopeptides isolated from milk protein have been shown to favor the absorption of calcium and iron (Mellander, '55), and, similarly, a phosphorylated amino acid may act likewise. Secondly, enzyme production might be stimulated, such as intestinal alkaline phosphatase, which aids, directly or indirectly, the absorption of Ca^{45} and Sr^{89}. These possibilities are highly speculative and further studies are in progress to confirm or deny these postulations. Also, the structural similarities of lysine and arginine with basic functional groups on the δ or ω carbon suggest some common denominator of activity for these compounds.

The effect of lactose on calcium absorption and utilization has not been definitively explained. Fournier ('55) suggested that lactose, galactase, and certain pentoses increase calcium utilization by a favorable metabolic effect on ossification; however, the foregoing studies were of longer duration than those reported in this paper and may not be entirely applicable. More classical explanations of lactose action involve the formation of a more acid condition in the gut, which in turn promotes calcium absorption (Maynard, '51). Gluconate and lactate probably increased the absorption of Ca^{45} and Sr^{89} by virtue of the greater solubility of the salts; little is known of the transport mechanism for either lactate or gluconate. Citrate, which forms a soluble complex with calcium, did not increase Ca^{45} or Sr^{89} absorption. This would suggest that complex formation is not necessarily a decisive factor in promoting mineral absorption. Other factors, such as the movement of the complex itself, must be considered. The lack of effect from the administration of vitamin B was expected since the test animals were not depleted of these nutrients under the present experimental conditions.

Although the practical implications of these results are not clear at this time, the data certainly offer an additional explanation for the favorable effect of protein or protein derivatives on calcium metabolism. Further studies are in progress to elucidate the mechanism of action, and to observe interrelationships with other factors, such as vitamin D and phosphorus.

SUMMARY

1. Eighteen amino acids, including those essential for the rat, were assayed for effect on the gastrointestinal absorption of Ca^{45} and Sr^{89}. The minerals and amino acid were ingested simultaneously; radioassay values for the femur obtained 24 hours after dosage were used as a measure of absorption.

2. L-Lysine and L-arginine were the most potent in promoting mineral absorption, approximately doubling the Ca^{45} and

Sr^{89} found in the femurs. L-Tryptophan, L-leucine, and L-aspartic acid also produced notable increases. The other amino acids were less effective or ineffective.

3. Lactose produced a greater response than L-lysine and L-arginine. The latter two were more effective in promoting mineral absorption than either gluconate, lactate, citrate, or a mixture of B vitamins.

4. Injection of Ca^{45} and Sr^{89} into lysine-treated rats resulted in no increase in radioactivity in the femur while ingestion of both the minerals and lysine produced the usual twofold increase in femur values.

5. The rat preferentially absorbed Ca^{45} over Sr^{89} by a factor of about 1.7.

6. Analysis of the Ca^*/Sr^* ratios in the femurs revealed that most substances were slightly more effective in promoting Sr^{89} absorption than Ca^{45} absorption.

7. A dose-response curve of lysine versus femur Ca^{45} and Sr^{89} values showed that a molar ratio of lysine:$CaCl_2$ between 1 and 2 was necessary for significant increases in mineral absorption.

8. These findings were discussed in terms of possible mechanisms of action for the stimulatory amino acids.

ADDENDUM

In an additional experiment, it was observed that D-lysine was as effective as L-lysine in promoting mineral absorption; therefore, the lysine response is apparently not stereo-specific.

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DIGESTIBLE ENERGY IN RELATION TO FOOD INTAKE AND NITROGEN RETENTION IN THE WEANLING RAT [1,2]

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The energy content of a ration appears to exert a considerable influence upon both food consumption and protein utilization. Several experiments with the chick have indicated that the productive energy content of the ration is a major factor in controlling feed intake. Hill and Dansky ('50, '54), and Dansky and Hill ('51), have pointed out the remarkable ability of the chick to compensate for reduced dietary energy level by increasing feed consumption. Similarly, Peterson et al. ('54) noted that feed intake increased to satisfy the energy needs of chicks, though when the rations were of a very low energy content the birds were unable to consume sufficient feed to satisfy their energy requirements.

Investigations relating to the effects of energy levels on food consumption, in species other than the chick, have not been widely developed. Hegsted and Haffenreffer ('49), working with rats, stated that, "the food intake of an animal is governed by means yet unknown at a certain percentage above its normal basal metabolism." In the same paper it is suggested that, "the mean daily calorie intake varied as the mean body weight raised to the 0.88 power." Cowgill ('28)

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[2] The authors are indebted to Hoffmann-La Roche Inc., Nutley, New Jersey, Lederle Laboratories Division American Cyanamid Ltd., Pearl River, New York, Merck and Co., Inc., Montreal, Canada and to Charles Albert Smith, Toronto, Canada for the vitamins used in this experiment.

reported that the feed intake of the adult dog appeared to be governed by the energy content of the feed.

Since Hoppe (1856) reported that carbohydrate ingestion lowered the nitrogen excretion of dogs, many workers have noted the sparing action of energy-rich foods on protein utilization. Bosshardt and Barnes ('46) indicated that protein utilization of growing rats and mice, as measured by the percentage of absorbed protein used for body nitrogen gain, increased with caloric intake when the animals were fed isocaloric diets ad libitum. These workers also noted that, "with each protein source there was a maximal caloric intake per unit body size." Among other works giving evidence of the protein sparing action of energy are those of Swanson ('51) who worked with rats, Rosenthal and Allison ('51) with dogs and Leverton et al. ('51) with young women.

METHODS

The experiment reported herein was designed to study the nitrogen retention and food consumption of the weanling rat when two different levels of bulk [3] were fed, while the nitrogen content of the rations was maintained at a constant level.

Four male and 4 female weanling rats of the Sprague-Dawley strain were alloted to each of the two rations listed in table 1, the rations varying in Alphacel content by a level of 10%. After a 7-day ration acclimatization period the rats were placed in metabolism cages for a further 7 days. Throughout the experiment the rats were fed ad libitum in individual cages.

The metabolism cages were sprayed with a hot 2% boric acid solution prior to the experiment. On the termination of the experiment the cages were rinsed with distilled water, the washings being added to the urine which was collected in 50% sulphuric acid. The total volume of urine and cage washings was brought to 500 ml with distilled water prior to analysis.

[3] Alphacel, a "non-nutritive cellulose" obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Feces were collected daily, dried in an air oven at 105°C. and then ground to a fine powder.

TABLE 1

Rations fed during acclimatization and metabolism periods

	RATION 1	RATION 2
Nitrogen source "A",[1] %	15.2	15.2
Sucrose, %	54.8	44.8
Alphacel,[2] %	20.0	30.0
Mazola oil, %	5.0	5.0
Salts, %[3]	4.0	4.0
Vitamin mix,[4] %	1.0	1.0
Analysis		
Gross energy, Cal./gm	4.22	4.23
Nitrogen content, gm %	2.07	2.08

[1] Nitrogen source "A." Casein 58.7 gm (~ 8 gm N), lactalbumin 68.8 gm (~ 8 gm N), DL-methionine 0.3 gm, L-histidine HCl 1.5 gm, DL-threonine 1.0 gm. Calculated to supply the amino acid requirements of the rat when expressed as a ratio to lysine = 1.0 (Rose, '38; Block and Bolling, '51).

[2] Alphacel "non-nutritive cellulose." Nutritional Biochemical Corporation, Cleveland, Ohio.

[3] Jelinek et al., '52.

[4] Vitamin mix modification of Jelinek et al., '52. Vitamin B_{12} at a level of 0.03 mg/kg of food replaced Wilson's whole liver powder.

RESULTS AND DISCUSSION

The data which follow refer solely to the 7-day metabolism period. Table 2 lists the principal mean values obtained during this experiment. The initial weight of the rats was the weight at the start of the 7-day metabolism period. Digestible energy consumption was determined by subtracting the total fecal energy from the gross energy intake. Digestible nitrogen consumption was calculated in a similar manner to digestible energy, while nitrogen retained was equivalent to nitrogen digested minus total urinary nitrogen. It should be noted that the term "digestible" refers to apparent and not true digestibility.

Ration 1 with an Alphacel content of 20% had a total digestibility of 78% while ration 2, containing 30% of Alphacel, had a digestibility level of 68%. That an increase of 10%

of Alphacel in the diet reduced the digestibility by 10% would indicate the suitability of Alphacel as a diluent in work of this nature.

In order to measure the influence on food consumption of the digestible energy content of the ration, an analysis of variance comparing food and digestible energy consumption of the two groups of rats was performed. No difference in either food or digestible energy consumption of the two ration groups was observed. However, a significant difference between the initial weights of the two groups of rats was observed, the difference being primarily a function of the superior growth-promoting effect of ration 1 during the 7-day acclimatization period. It was, therefore, considered advisable to adjust the two groups to a common initial weight by means of an analysis of covariance (Crampton, '34). The results of this analysis indicated that although food consumption between the two groups of rats was significantly different, there was no difference in digestible energy consumption. Table 3 contains a summary of the analyses of variance and covariance referred to above.

TABLE 2[1]

Mean values for data relating to the effect of digestible energy on food consumption and nitrogen retention during the seven-day metabolism period

Ration group	1 (20% Alphacel)	2 (30% Alphacel)
Number of rats	8 (4 males, 4 females)	7 (3 males, 4 females)
Initial weight, gm	79	66
Final weight, gm	116	100
Change in weight, gm	37	33
Food consumption, gm	81	83
Food digestibility, %	78	68
Energy digested, Cal.	273	248
Nitrogen digested, mg	1530	1514
Urinary nitrogen, mg	408	505
Nitrogen retained, mg	1122	1008

[1] As one male rat on ration 2 lost considerable weight during the experiment, data relating to this animal have been omitted from this report.

The results of the analysis of covariance indicate that the food intake of weanling rats, receiving rations containing approximately 2.07% nitrogen and varying in Alphacel content by 10% (20% and 30%), was significantly influenced by the digestible energy content of the food; that is, it would appear that the rats ate to satisfy their energy requirement. It is anticipated that the increase in food consumption, resulting from a reduction in the digestible energy content of the ration, could only occur within certain physiological limits; this having already been demonstrated for the chick (Peterson et al., '54).

TABLE 3

Analyses of variance and covariance of food consumption and digestible energy consumption between two rations differing in Alphacel content (non-nutritive cellulose)

ANALYSES	SOURCE OF VARIATION	NON-ADJUSTED		ADJUSTED FOR INITIAL WEIGHT	
		Degrees of freedom	Mean square	Degrees of freedom	Mean square
Food consumed	Between rations	1	12	1	263 [1]
	Within rations	13	79.4	12	43.6
Digestible energy consumed	Between rations	1	2307	1	124
	Within rations	13	700	12	284

[1] Significant at 5% level.

It was also desired to determine the influence which the digestible energy consumption may have had on the amount of nitrogen retained. The correlation between these factors was 0.928 for ration 1 (20% Alphacel) and 0.910 for ration 2 (30% Alphacel). The pooled correlation for both rations was 0.918. All these correlations were very large and highly significant indicating a strong association between digestible energy consumption and retained nitrogen.

Since initial weight might have influenced both digestible energy consumption ($r = 0.829$) and nitrogen retention ($r = 0.720$), it was necessary to remove the effects of initial weight from the correlation between digestible energy consumption and nitrogen retention. This was accomplished by calculating

the partial correlation between digestible energy consumption and nitrogen retention independent of initial weight. The resulting r value of 0.829 was significant at the 1% level and indicated that approximately 69% of the variation in nitrogen retention was associated with digestible energy consumption when the effects of initial weight were removed. A summary of the correlation coefficients is presented in table 4.

It has previously been pointed out that the rats on ration 2 (30% Alphacel) consumed a significantly greater quantity

TABLE 4

Correlation coefficients relative to nitrogen retention, digestible energy consumption and initial weight

VARIABLES CORRELATED	R VALUES [1]
Retained nitrogen:digestible energy consumption:	
Ration 1 (20% Alphacel)	0.928
Ration 2 (30% Alphacel)	0.910
Rations 1 and 2 pooled	0.918
Retained nitrogen:initial weight	0.720
Initial weight:digestible energy consumption	0.829
Partial correlation [2]	0.829

[1] All highly significant at 1% level.

[2] Partial correlation being the correlation between nitrogen retained and digestible energy consumed when the influence of initial weight is statistically controlled.

of food than did rats on ration 1 (20% Alphacel) when adjusted to a common initial weight by covariance. As the nitrogen content of the two rations was essentially the same, it is suggested that for each food nitrogen level there will be an optimum digestible energy level, when nitrogen retention is the criterion of measurement. The optimum digestible energy level will vary according to the quality and availability of the nitrogen source and with the species and stage of growth of the animal consuming the food. Further investigations to further clarify some of these relationships are in progress.

SUMMARY

It has been demonstrated that the food intake of two groups of weanling rats, whose rations contained respectively 20 and 30% of non-nutritive cellulose, was significantly influenced by the digestible energy content of the food. This would indicate that within physiological limits, as yet not determined, weanling rats eat to satisfy their energy requirements.

Digestible energy consumption has been shown to influence the nitrogen retention of the weanling rat. Approximately 69% of the variation in the nitrogen retention of the weanling rats used in this experiment was associated with digestible energy consumption when the effects of initial weight were removed.

It is postulated that within limits there is an optimum digestible energy level for each nitrogen level of a ration when the criterion of measurement is nitrogen retention.

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STUDIES IN CALCIUM METABOLISM. EFFECT OF FOOD PHYTATES ON CALCIUM[45] UPTAKE IN BOYS ON A MODERATE CALCIUM BREAKFAST [1,2,3,4]

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TWO FIGURES

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In an earlier study (Bronner et al., '54) it was reported that phytates significantly depressed the calcium uptake when the test breakfast contained approximately 85 mg of calcium (Ca) and 100 mg of phytic phosphorus. We are now reporting on

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[2] Partial support for this study came from grants by the Quaker Oats Company, by the Atomic Energy Commission, Contract AT(20-1)-952, and by National Institutes of Health.

[3] The data in this publication are taken from the dissertation presented (1952) by Felix Bronner to the Department of Food Technology, Massachusetts Institute of Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[4] Authorization for the use of restricted quantities of Ca^{45} in patients institutionalized for mental inadequacy was granted through the Subcommittee on Human Applications by the Isotope Division of the Atomic Energy Commission. The Ca^{45} was obtained on allocation from the Oak Ridge National Laboratory.

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the effect of phytates on the uptake of radiocalcium (Ca^{45}) in boys who were given a test breakfast which contained 80 mg of phytic P and 239 mg of Ca, an amount of calcium typical of that in the breakfast of many children in the United States.

In principle, the design and methods employed in this study were similar to those described in the previous report (Bronner et al., '54). A single test breakfast was employed to compare the effect on Ca^{45} uptake of a phytate-rich cereal (oatmeal) with that of a phytate-free cereal (farina). Milk served as the principal source of Ca and as the vehicle for Ca^{45} administration. At suitable intervals following the test breakfast, the concentration of Ca^{45} was determined in samples of blood, urine and feces.

A criss-cross design was employed. Individuals who had received oatmeal in the first experiment (experiment A) ate the farina test meal three weeks later (experiment B); those who had eaten the farina meal in the first experiment received the oatmeal in the second. Just prior to experiment B, the concentration of Ca^{45} in the serum, urine, and feces of the subjects was determined to be negligible.

EXPERIMENTAL

Subjects

Seventeen adolescent boys, who were institutionalized in a State school under uniform conditions because of subnormal intelligence, volunteered for these experiments.

Nine boys, subjects 19 to 27, were given the oatmeal breakfast first and the farina meal three weeks later. Their average age was 12.9 years, with a range of 10.5 to 15.5 years; their weight averaged 39.1 kg with a range of 28.6 to 55.4 kg; and their average mental age was 7.7 years, with a range of 7.0 to 9.8 years.

Eight boys, subjects 28 to 35, received the farina meal, but one of them did not participate in the oatmeal study three weeks later. The average age of these 7 boys was 13.3 years, with a range of 10.6 to 15.5 years; their average weight was 37.2 kg, with a range of 26.1 to 47.6 kg; their mental age

averaged 7.6 years, with a range of 5.0 to 9.8 years. If the 8th boy is included (subject 33) the respective group averages were 12.8 years, 40.2 kg and 7.1 years.

The subjects had been prepared for the study by receiving a daily supplement of one multivitamin tablet [6] and of one quart of milk, for a period extending from two weeks preceding the study period to the end of the experiment. Analysis of the diet served at the school revealed that it very nearly met the Recommended Daily Dietary Allowance of the National Research Council ('48) for children of this age group.

Experimental meals

Two breakfasts were given: an oatmeal breakfast (O) with phytate naturally present in the cereal, and a farina breakfast (F) which contained no phytate. Table 1 shows the composition of the breakfasts.

The radiocalcium ($Ca^{45}Cl_2$) was added to approximately 60 ml of the milk which was then mixed intimately with the cereal. The children drank the remainder of the milk as they ate the cereal.

Sample collection

Blood was drawn by venipuncture at about 2.5 hours postprandially. It was allowed to clot and, following centrifugation, the serum was analyzed for its content of Ca and Ca^{45}.

Urine was collected daily for 5 days. Ca and Ca^{45} analyses were carried out on the pooled samples collected during the first three days, and also on pooled samples of days 4 and 5 [7].

Feces were collected daily for 5 days and a pooled 5-day specimen was analyzed for its content of Ca and Ca^{45}.

Samples were preserved and handled in the manner described previously (Bronner et al., '54). The quantities of Ca^{45} given in these studies were so low that the level of

[6] Vi-Penta Perles Forte, generously donated by Hoffman-LaRoche, Inc.

[7] Because of the very low radioactivity of the urine samples collected on days 4 and 5, the analytical results are not reported, nor were they included in the statistical evaluation.

TABLE 1

Composition of experimental breakfasts

EXPERIMENT	TYPE OF FOOD	QUANTITY		SOLIDS	Ca	P	PHYTATE P	TOTAL Ca / TOTAL P	Ca^{45}	
		Wet	Dry							
			gm	*gm*	*mg*	*mg*	*mg*		*μc*	*cpm × 10⁻³*
				OATMEAL GROUP						
A	Milk + Ca^{45}	220 ml		25.6	229	200	0		0.87	185
	Oatmeal	180 gm	25.2 [1]	22.6	15	125	86			
	Total			48.2	244	325	86	0.75	0.87	185
B	Milk + Ca^{45}	220 ml		22.6	226	200 [2]	0		0.83	179
	Oatmeal	180 gm	21.7 [1]	19.4	12	108	74			
	Total			42.0	238	308	74	0.77	0.83	179
A + B	Average total			45.1	241	317	80	0.76	0.85	182
				FARINA GROUP						
A	Milk + Ca^{45}	220 ml		25.6	229	200	0		0.87	185
	Farina	210 gm	39.1 [1]	34.8	11	35	0			
	Total			60.4	240	235	0	1.02	0.87	185
B	Milk + Ca^{45}	220 ml		22.6	226	200 [2]	0		0.83	179
	Farina	210 gm	26.3 [1]	23.4	8	24	0			
	Total			46.0	234	224	0	1.04	0.83	179
A + B	Average total			53.2	237	230	0	1.03	0.85	182

[1] Dry weight basis: oatmeal 91.5% solids; farina 89.5% solids.
[2] Estimate, based on analysis of milk of experiment A.

radioactivity of the urine and stool specimens approached background in about 5 days and collections were therefore discontinued.

Analytical procedures

The analytical techniques have already been described (Bronner et al., '54). All counting data are reported as corrected to the time of ingestion of Ca^{45}. Decay corrections were made with the aid of suitable Ca^{45} standards used in all counting runs.

In the radiochemical determinations, experimental difficulties caused poor precision in some serum and urine samples. Serum samples in both the experiments, but particularly in experiment A, tended to gel during the preparative stage. Limitation in sample size often made repeat determinations impossible. The activities of many urine samples were so low that the error of replicate analyses in some cases reached 20 to 30% standard deviation (S. D.).[8] The error in the Ca^{45} analyses of replicate ash solutions prepared from stool specimens was always less than 10% S. D.

EXPERIMENTAL RESULTS

The average results of the analyses for content of Ca and Ca^{45} of the serum, urine and feces of each individual are presented in table 2. The group averages are shown in table 3.

Inspection of the serum data revealed no striking group differences. This was confirmed by statistical evaluation (see below). The specific activity of the serum averaged 0.023% of the ingested Ca^{45} per milligram of serum Ca at 2.5 hours following the ingestion of the test meal. This figure is lower than the comparable uptake figure (0.028%/mg) reported by us previously for somewhat older boys (Bronner et al., '54; see table 4).

[8] Percent standard deviation:

$$\frac{100(\Sigma x_i^2 - \bar{x}_i \Sigma x_i)^{\frac{1}{2}}}{[\bar{x}_i \ (n-1)]^{\frac{1}{2}}}$$

TABLE 2

Ca^{45} level in serum, and Ca^{45} output in urine and feces, of adolescent boys given 0.85 μc $Ca^{45}Cl_2$ orally

SUBJECT	SERUM [1]				URINE [2]				FECES [3]			
	Experiment A [4]		Experiment B [5]		Experiment A		Experiment B		Experiment A		Experiment B	
	Ca cont.	Ca^{45} cont.	Ca cont.	Ca^{45} cont.	Ca output	Ca^{45} output	Ca output	Ca^{45} output	Ca output	Ca^{45} output	Ca output	Ca^{45} output
	mg/ml	*% dose/mg* [6]	*mg/ml*	*% dose/mg* [6]	*mg*	*% dose/mg* [6]	*mg*	*% dose/mg* [6]	*gm*	*% dose* [7]	*gm*	*% dose* [7]
19	0.095	.... [8]	0.096	0.020	141	0.0040	70	0.0051	2.77	78.4	1.60	56.0
20	0.099	0.029	0.091	0.022	271	0.0039	243	0.0064	2.32	77.2	2.95	64.5
21	0.098	0.022	0.105	0.019	274	0.0044	128	0.0061	2.22	76.3	2.44	23.6
22	0.090	0.021	0.090	0.023	183	0.0040	191	0.0040	1.22	27.5	2.19	29.7
23	0.097	0.024	0.098	0.018	82	0.0041	77	0.0029	3.17	51.3	4.57	29.5
24	0.100	0.022	0.097	0.026	129	0.0034	99	0.0039	3.29	65.3	3.89	62.1
25	0.096	0.014	0.094	0.018	47	0.0057	18	0.0061	4.05	82.7	4.48	55.8
26	0.095	0.018	0.097	0.034	128	0.0046	131	0.0027	2.82	76.1	3.04	54.4
27	0.090	.... [8]	0.090	0.022	64	0.0032	74	0.0034	4.25	65.0	6.10	53.2
28	0.098	.... [8]	0.092	0.021	288	0.0032	283	0.0057	3.45	46.4	4.93	42.7
29	0.097	0.026	0.097	0.021	163	0.0047	115	0.0075	2.21	68.4	1.56	51.3
30	0.101	0.033	0.094	0.020	75	0.0056	55	0.0050	1.56	41.4	1.59	36.8
31	0.092	.... [8]	0.094	0.030	406	0.0035	382	0.0060	2.23	33.8	2.15	24.6
32	0.103	0.023	0.097	0.030	377	0.0040	219	0.0068	2.98	36.7	2.29	25.3
33	0.097	0.020	.... [5]	.... [5]	282	0.0044	.... [5]	.... [5]	1.23	39.5	.... [5]	.... [5]
34	0.104	0.020	0.091	0.024	103	0.0065	78	0.0050	2.40	56.4	2.82	55.7
35	0.097	0.023	0.094	0.025	73	0.0046	48	0.0044	3.12	40.0	3.47	30.9

[1] Serum samples obtained 2.5 hrs. post-prandially.
[2] Seventy-two-hour pooled urine samples.
[3] One hundred and twenty-hour pooled feces specimens.
[4] In experiment A, subjects 19 to 27 constituted the oatmeal group, subjects 28 to 35 the farina group.
[5] In experiment B, subjects 19 to 27 constituted the farina group, subjects 28 to 35 the oatmeal group; subject 33 excluded because of a broken arm.
[6] "% dose/mg" refers to "per cent of the ingested Ca^{45}/mg Ca."
[7] "% dose" refers to "per cent of the ingested Ca^{45}."
[8] Ca^{45} determination not carried out because of small sample size and experimental difficulties.

Both the urinary and fecal excretions of Ca (table 2) reveal wide intra- and interindividual differences. Interindividual differences are common, especially in growing subjects. Intraindividual differences can also be expected in these boys,

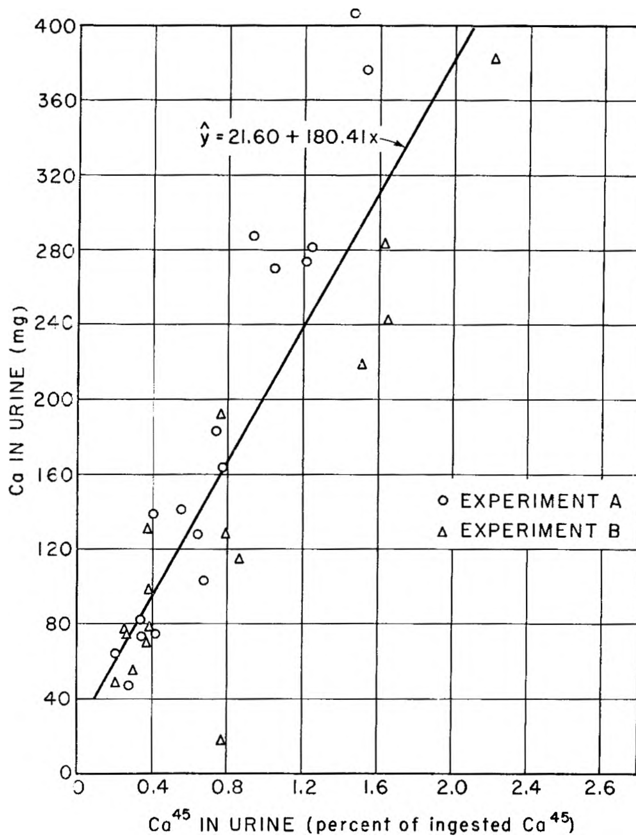

Fig. 1 Ca^{45} output in urine as a function of the output of calcium, using pooled 72-hour samples.

particularly when experiments cannot be conducted under conditions which permit absolute control of intake.

Figure 1 shows the highly significant linear relationship observed between the output of Ca and Ca^{45} in the urine. The slope of this regression line is the specific activity and is independent of variations in the Ca output in the urine.

This led us to use specific activity measurements in the evaluation of the urine data.

Because fecal Ca is made up of unabsorbed Ca and of endogenous Ca, one would expect the output of unabsorbed and

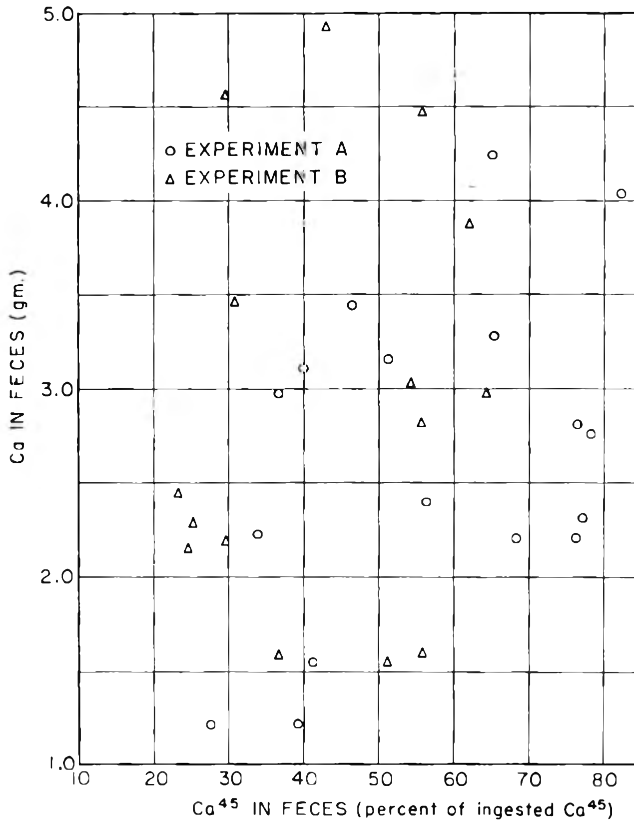

Fig. 2 Ca^{45} output in feces as a function of the output of calcium, using pooled 120-hour samples.

reexcreted Ca^{45} following ingestion of Ca^{45} to be fairly independent of the total Ca output in the feces over a period of several days. This appears to be the case (fig. 2), since no significant linear relationship can be shown to exist between the output of Ca and of Ca^{45} in the stools of these subjects.

Therefore the measurements of total Ca^{45} output were used in the evaluation of the fecal data.[9]

Table 2 shows that total Ca output had a wider range in the urine than in the feces. Excretion in the urine is a more direct measure of Ca metabolism than is excretion in the stool. On the other hand, fecal output reflected more directly the relatively uniform intake of Ca in the diet of these subjects.

STATISTICAL ANALYSIS

Paired comparison tests (Snedecor, '46) were done on the data for specific activity and output to see how differently the two diet groups had disposed of their Ca^{45}. Table 3 summarizes the results of these tests.

Because the data for serum were incomplete in experiment A, the paired comparison test was supplemented with a group comparison. This test again showed no significant effect attributable to the presence of phytate in the diet.

In general, the specific activity of the urines paralleled that of the sera. However, the mean specific activity of the urines, but not of the sera, of subjects 28 to 35 was higher after oatmeal than after farina. This difference was significant on a 10% probability level, but is not likely to have been caused by phytate, which would have depressed Ca^{45} uptake and, therefore, decreased the Ca^{45} output in the urine. A similar reversal was also observed for the data on fecal output (table 3).

Analysis of the data for the output of Ca^{45} in the feces showed that subjects 19 to 27 excreted significantly more Ca^{45} following the oatmeal than following the farina breakfast. On the other hand, subjects 28 to 35 excreted significantly more Ca^{45} after the farina than after the oatmeal breakfast.

[9] It has been shown (Bronner et al., '56) that the total quantity of absorbed Ca which is reexcreted within 5 days of its ingestion is relatively small and can probably be neglected in a first approximation.

When the data on fecal output of the subgroups were pooled, it appeared that the type of cereal eaten had no significant effect on the excretion of fecal Ca^{45}.

TABLE 3

Effect of oatmeal and farina test breakfasts on the distribution of Ca^{45}

SUBJECTS	SAMPLE	OATMEAL GROUP	FARINA GROUP	IS DIFFERENCE SIGNIFICANT [1] (PAIRED COMPARISON TEST)
19–27	Serum [2]	0.021	0.022	No
	Urine [3]	0.0041	0.0045	No
	Feces [4]	66.6	47.6	Yes
28–35	Serum [2]	0.024	0.025	No
	Urine [3]	0.0058	0.0046	No
	Feces [4]	38.2	45.3	Yes
All subjects	Serum [2]	0.023	0.023	No
	Urine [3]	0.0048	0.0045	No
	Feces [4]	54.2	46.6	No

[1] $P \leq 0.05$.

[2] Percentage of ingested Ca^{45} per milligram serum Ca 2.5 hours following test meal.

[3] Percentage of ingested Ca^{45} per milligram urinary Ca 72-hour urine pool.

[4] Percentage of ingested Ca^{45} in 120-hour feces pool.

DISCUSSION

These findings are interpreted to mean that phytates did not significantly affect Ca^{45} absorption under the conditions of these experiments. Perhaps difficulties of measurement obscured the findings, but not to the extent of hiding altogether a phytate effect, if it had been present.

It is difficult to explain why one group of subjects (19 to 27) excreted so much more Ca^{45} in their feces after the oatmeal breakfast than the other group (28 to 35) after a comparable oatmeal breakfast. Both samples were drawn from the same lot of rolled oats. However, the average output of Ca^{45} in the stools of these subjects is in good agreement with the quantity which comparable subjects excreted in a similar study (Bronner and Harris, '56, fig. 3).

The results obtained in this and in the preceding study of this series (Bronner et al., '54) are compared in table 4 which shows that phytate lowered Ca absorption at the lower, but not at the higher, level of Ca intake. Table 4 also shows that under comparable conditions an increase in the Ca intake caused a decrease in the *percentage* of Ca absorbed. Hansard and Plumlee ('54) have presented similar, but more extensive, data for rats and have come to a similar conclusion (also Holtz,

TABLE 4

Effect of two levels of calcium and of phytate intake on the distribution of ingested Ca^{45}

PHYTATE INTAKE		CALCIUM INTAKE	
		86 mg [1]	239 mg
gm			
0.1	Serum [2]	0.028 [3]	0.023
(Oatmeal)	Urine [4]	0.0058	0.0048
	Feces [5]	44.5	54.2
0.0	Serum [2]	0.046 [3]	0.023
(Farina)	Urine [4]	0.0065	0.0045
	Feces [5]	24.2	46.6

[1] Data adapted from Bronner et al. ('54).

[2] Percentage of ingested Ca^{45}/mg serum Ca at 2.5 hours following test meal.

[3] Serum data adjusted to mean body weight of 38.8 kg. Unadjusted figures: 0.020%/mg (oatmeal), 0.034%/mg (farina).

[4] Percentage of ingested Ca^{45} per milligram urinary Ca in 72-hour urine pool.

[5] Percentage of ingested Ca^{45} in 120-hour feces pool.

Popper and Silberman, '47). Recently Brine and Johnston ('55) analyzed the data in the literature and reported that the percentage of calcium absorbed by adults decreases when their intake increases.

The experiments reported here and previously (Bronner et al., '54), were designed to answer two related questions: (a) whether less Ca would be taken up from a phytate-rich than from a phytate-poor meal; and (b) what significance this might have in terms of practical nutrition. Table 4 shows

that when the intake of phytic P approximated that of Ca (weight basis), and the level of both was low, the uptake of Ca from the phytate-containing breakfast was reduced to nearly half that from the phytate-free breakfast. On the other hand, when the Ca intake was increased, the relative absorption decreased equally in the presence or absence of phytates.

It is probable that less than 25% of the total P in the average diet in the United States is in the form of phytic P. Therefore, the ratio of calcium to phytate phosphorus is seldom less than 2:1 or 3:1, if a daily intake of 0.5 to 0.8 gm of Ca is assumed. These conditions were approximated in the test meal used in the present study. We were unable to demonstrate a phytate effect under these conditions (see also Krebs and Mellanby, '43; Mellanby, '49; McCance and Widdowson, '49), and therefore consider phytates to have no nutritional significance in typical diets of the United States.

SUMMARY AND CONCLUSIONS

1. Seventeen boys were given two test breakfasts, one of oatmeal and one of farina, at an interval of three weeks, for the purpose of comparing calcium uptake in the presence and in the absence of food phytate. The boys had subnormal intelligence but were otherwise normal, were institutionalized under relatively uniform conditions. The two test breakfasts contained, respectively: 220 and 220 ml of milk; 241 and 237 mg of Ca., 80 and 0 mg phytic P; and 0.85 and 0.85 μc of Ca^{45}. Ca uptake was studied by determining the Ca^{45} contents of the serum samples taken 2.5 hours following ingestion of the test breakfast, and by measuring the Ca^{45} output in pooled urine and stool specimens collected for 72 and 120 hours, respectively, following ingestion of the Ca^{45}-labeled meals.
2. The specific activities of the serum and urine samples obtained from these subjects were not significantly changed when the cereal of the test breakfast was changed from oatmeal to farina, or the reverse.
3. For purposes of a criss-cross design the individuals had been divided into two subgroups. The fecal output of

Ca^{45} of each subgroup was affected significantly by changing the cereal of the test breakfast from oatmeal to farina. However, the direction of change was opposite for each subgroup. When the results of the fecal output of the two subgroups were pooled, the difference between the two test breakfasts was no longer significant.

4. The percentage of absorbed Ca decreased as the Ca intake increased.

5. It is concluded that phytates do not exert a significant effect on Ca^{45} absorption when the meal provides 239 mg of Ca and when the phytic P intake is 80 mg. Because this ratio of Ca to phytic P is typical of diets in the United States, it may be concluded that food phytates are of no nutritional concern in this country.

ACKNOWLEDGMENT

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TRYPTOPHAN-NIACIN METABOLISM IN ALLOXAN DIABETIC RATS[1]

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TWO FIGURES

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It has been shown that the rat's dietary requirement for niacin could be altered by changing the type of carbohydrate in the diet (Hundley, '49). Since carbohydrate metabolism is modified in diabetic animals, it appeared of interest to determine whether the presence of a diabetic state would influence the niacin requirement of rats. It was reported that the urinary excretion of N^1-methylnicotinamide (NMN) following test doses of nicotinamide was significantly lower in human diabetics than in control subjects (Lossy et al., '51), although later studies by Goldsmith et al. ('55) did not confirm this finding. Xanthurenic acid and 3-hydroxykynurenine were found in the urine of diabetic patients by Kotake and Tani ('53). Rosen et al. ('55) observed increased urinary xanthurenic acid in diabetics following oral tryptophan, indicating disturbances in tryptophan metabolism.

Reported here are experiments designed to study the effect of alloxan diabetes upon the ability of the rat to produce and excrete NMN following administration of various precursors.

[1] A preliminary report of this work has been published (McDaniel et al., '55).

[2] The American Cancer Society, New York.

EXPERIMENTAL

Adult female[3] Sprague-Dawley rats were made diabetic by a procedure similar to that described by Kass and Waisbren ('45). Animals were maintained for 48 to 72 hours without food or water, then injected intraperitoneally with 11 mg of alloxan monohydrate per 100 gm of body weight. The alloxan was administered as a freshly prepared 2% aqueous solution. A commercial stock ration[4] was fed prior to and for about 14 days after the administration of alloxan. Animals were housed individually in wire mesh cages and food and water offered ad libitum unless otherwise indicated.

Urine for determination of glucose and NMN was collected in glass bottles containing 2 ml of 10% sulfuric acid. When xanthurenic acid was to be determined toluene was used as the preservative in place of sulfuric acid. Determinations were made on 24-hour urine samples, which included water used to wash down the metal and glass metabolism cages. Urinary glucose was determined by titration with Benedict's ('11) quantitative reagent. The method used to determine urinary NMN was essentially that of Huff and Perlzweig ('47). Xanthurenic acid was determined by the method of Wachstein and Gudaitis ('52). A colorimetric procedure described by Eckert ('43), which measures both tryptophan and anthranilic acid, was used to determine other possible tryptophan metabolites in urine.[5]

Blood for determination of glucose was collected from the tail. Blood glucose was determined by the method of Haslewood and Strookman ('39).

[3] In the present study only female animals were used. It is not known whether sex would have influenced the findings.

[4] Hunt Club Dog Meal.

[5] Although the method used gave positive color reactions with both anthranilic acid and tryptophan, tests with pure compounds indicate that most but not all of the material measured in these experiments was anthranilic acid. For the purposes of this report, values shown for "other" metabolites will represent such compounds in urine as reacted positively to the color test cited. Since marked differences were observed between diabetics and non-diabetics, with respect to the excretion of such compounds following administration of tryptophan, the data were included in this report even though all the compounds included in "other" metabolites are not known.

In addition to the stock diet, three niacin-deficient purified diets were used in these experiments. Diet 9221 contained 80% sucrose, 8% vitamin-free casein, 8% hydrogenated cottonseed oil [6] and 4% Wesson ('32) salt mixture. Diet 9260 contained 75.85% sucrose, 9% vitamin-free casein, 3% gelatin, 0.15% L-cystine, 8% hydrogenated cottonseed oil and 4% Wesson salt mixture. Diet 9276 was similar to diet 9221, except that sucrose was replaced by fructose. Incorporated into each 100 gm of the purified diets were 1 mg each of thiamine HCl and pyridoxine HCl, 4 mg calcium pantothenate, 2 mg riboflavin, 1.25 mg folic acid, 200 mg choline Cl, 1 µg biotin, 0.4 mg menadione, 5 mg α-tocopherol acetate, 12000 USP units vitamin A and 2500 USP units vitamin D. In addition 12.5 µg vitamin B_{12} were added to diets 9221 and 9276.

RESULTS

In preliminary experiments it was observed that alloxan diabetic rats excreted lower amounts of NMN in the urine than did non-diabetic rats on similar diets. Administration of niacinamide or niacin resulted in marked increases in NMN in both normal and diabetic rats, but administration of tryptophan, which has been shown to be a precursor of NMN in the rat (Rosen et al., '46; Hundley and Bond, '49), produced marked increases only with non-diabetic rats. The low conversion of tryptophan to NMN in diabetic rats was observed whether the tryptophan was given with the diet, by stomach tube, or by intraperitoneal injection, and whether L- or DL-tryptophan was used (table 1).

It had been observed in earlier experiments that urinary NMN values often were higher during periods of fasting or limited food intake than during periods in which food was consumed ad libitum. Food intake of diabetic animals was much greater than for non-diabetics. However, it was shown by paired feeding and by fasting that the abnormally low NMN values observed for the diabetic rats given tryptophan were not the result of excessive food intake (table 2).

[6] Crisco.

TABLE 1

Effect of various supplements upon the urinary N^1-methylnicotinamide (NMN) of diabetic and non-diabetic rats (mg/day)[1]

SUPPLEMENT [2]	DIABETICS			NON-DIABETICS		
	No. rats	Av.	Range	No. rats	Av.	Range
None (F)[3]	4	0.05	0.03–0.08	6	0.16	0.05–0.23
None (NF)[3]	14	0.07	0.02–0.14	14	0.17	0.04–0.47
DL-Tryptophan (F)	19	0.47	0.05–0.90	19	1.39	0.36–3.12
DL-Tryptophan (NF)	22	0.21	0.08–0.44	23	1.68	0.39–3.09
L-Tryptophan (F)	5	0.26	0.05–0.63	5	1.18	0.53–2.22
Niacinamide (F)	17	2.12	0.37–4.85	18	1.66	0 52–4.03
Niacinamide (NF)	13	1.30	0.23–2.34	12	1.63	0.73–2.82
Niacin (F)	12	1.11	0.21–1.65	12	0.96	0.39–2.18
NMN (F)	15	2.66	1.57–3.53	15	2.52	2.11–3.25
Water intake (ml/day)	18	156	98–247	19	22	12–35
Food intake (gm/day)	18	19.4	12.0–29.4	19	13	7.5–15.6
Urine glucose (gm/day)	18	11.9	6.9–21.1	..	..	..

[1] Data shown in this table wore compiled from rats fed diets 9221 or 9260, and receiving supplements with the diet, by stomach tube, or by intraperitoneal injection. No significant differences in urinary NMN were observed with respect to diet or route of administration of the supplements.

[2] Supplements were given in the following dosages: 100 mg DL- or L-tryptophan; 3 mg niacinamide; 3 mg niacin; 3 mg NMN.

[3] (F) = fasting; (NF) = non-fasting.

TABLE 2

Lack of influence of excessive food intake on failure of diabetic rats to convert tryptophan to N^1-methylnicotinamide (NMN)

Diet 9221

TREATMENT	DIABETICS [1]		NON-DIABETICS [1]	
	Food intake	NMN	Food intake	NMN
	gm/day	*mg/day*	*gm/day*	*mg/day*
Ad libitum	21.3	0.06	10.5	0.07
Ad lib + 100 mg tryptophan [2]	21.0	0.14	9.3	2.16
Pair fed + 100 mg tryptophan	9.5	0.24	9.5	4.17
Fasting + 100 mg tryptophan	..	0.53	..	2.26

[1] Values shown are averages of two diabetic and two non-diabetic rats.

[2] Tryptophan was added to the diet during periods of feeding and given by stomach tube when fasted.

The possibility that glucose or other metabolites in diabetic urine might interfere with the determination of NMN was investigated. NMN was determined on urine from normal rats supplemented with tryptophan, and on this urine diluted with urine from diabetic rats containing known amounts of glucose, and after addition of known amounts of C. P. glucose to normal urines. It was observed that the amount of NMN determined was reduced both by dilution with diabetic urine and by addition of glucose. The reduction caused by the diabetic urine appeared to be due entirely to the presence of glucose. Urinary NMN values for normal rats given tryptophan were reduced by about 25% when measured in the presence of diabetic urines (sufficient diabetic urine was added to approximate a rat excreting 8 gm of glucose per day). An equivalent amount of C. P. glucose added to the normal urine resulted in essentially the same reduction. When the amount of glucose was doubled (equivalent to a rat excreting 16 gm of glucose per day), the NMN determined was reduced by about 40%. Although glucose in amounts often found in severe diabetes may lower the NMN value by as much as 40%, this reduction is not of sufficient magnitude to account for the very low NMN values observed following administration of tryptophan to diabetic rats. Furthermore, as is shown in tables 1 and 2, during periods of fasting when no glucose is present in diabetic urine, the apparent conversion of tryptophan to NMN is still abnormally low. To demonstrate further that the low NMN values observed for diabetics were not due to interfering substances in the urine, it was shown that NMN resulting from administration of niacinamide, niacin and NMN could be determined readily even in diabetic urines (table 1).

In earlier studies a marked individual variation was observed in the levels of urinary NMN among rats on similar diets either with or without supplementary niacin or tryptophan (Hundley, '47). In view of this known individual variation and to eliminate the possibility that the differences observed in the present experiments were merely the result of

such variation, normal rats were characterized as to NMN excretion on a niacin-deficient diet and following supplements of niacinamide and tryptophan. These rats were then treated with alloxan and similar studies repeated. It was observed that for all rats which became diabetic the conversion of tryptophan to NMN was greatly reduced, and that the conversion of niacinamide to NMN was moderately reduced in about half of the rats and greatly reduced in a few. Rats which did not become diabetic as indicated by normal water intake and negative urinary glucose, were still able to convert both niacinamide and tryptophan to NMN.

Since it had been shown that alloxan in large amounts will cause liver damage (Goldner and Gomori, '43; Palay and Lazarow, '46), and that liver is the site of conversion of niacinamide to NMN (Perlzweig et al., '43; Ellinger, '46a and '48), and that liver damage caused by a mixture of chloroform and carbon tetrachloride decreases the NMN excretion of rats given niacinamide (Ellinger, '46b and '47), experiments were conducted to determine the conversion of tryptophan to NMN with diabetic rats in which the symptoms of diabetes were alleviated by administration of insulin. A typical response is shown in figure 1. This rat was diabetic and converted abnormally low amounts of tryptophan to NMN, but the urinary excretion of NMN following single doses of either niacinamide or NMN was within normal limits. Administration of insulin was followed by a rapid decrease in water intake and urinary glucose, and a gradual increase in the NMN levels to within the normal range. The increase in NMN appeared to parallel the change in dosage of insulin which was given in increasing amounts to this rat. However, an immediate decrease in NMN to pretreatment levels followed withdrawal of the tryptophan, even though the insulin was continued, thus indicating that the increased NMN was derived from the administered tryptophan and was not merely an effect of insulin or the action of insulin upon some other mechanism.

It was observed that the rate of response to insulin (with respect to conversion of tryptophan to NMN) varied in different rats, even though the water intake and urinary glucose of all rats tested decreased rapidly after the insulin was started. In one such rat, which also converted abnormally low amounts of tryptophan to NMN, the response to insulin was much more rapid, with NMN increasing to a normal level

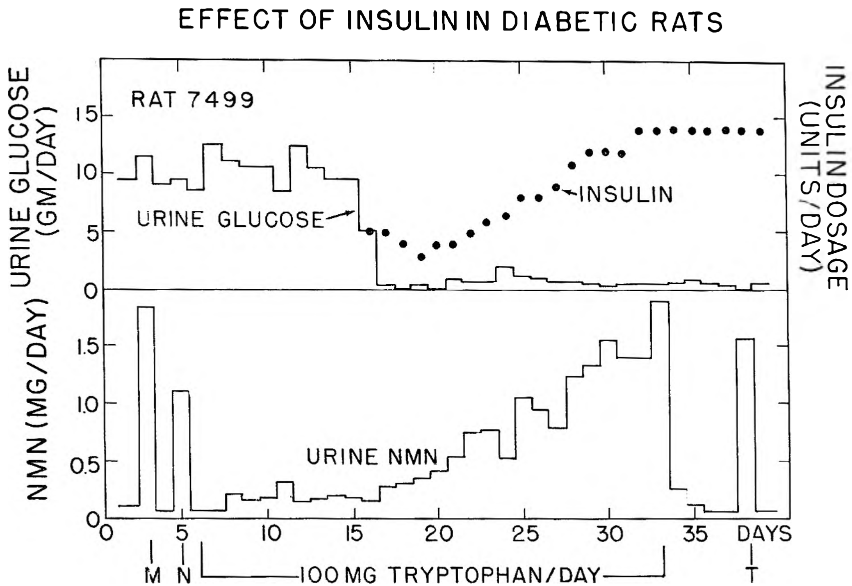

Fig. 1 Effect of insulin on conversion of tryptophan to NMN in diabetic rat. Diet 9221. T = 100 mg tryptophan; N = 3 mg niacinamide; M = 3 mg N^1-methylnicotinamide.

Niacinamide, NMN, and insulin were given intraperitoneally. Single doses of tryptophan were given by stomach tube, and added to the diet for daily feeding.

within 24 hours after the insulin was started. For this rat the insulin was discontinued after 4 days. The conversion of tryptophan to NMN remained relatively constant and in the normal range for 8 days then decreased gradually to a diabetic level in about 16 days after the insulin was stopped. In view of these observations further studies were made to determine the effect of insulin on the conversion of tryptophan and

niacinamide to NMN, and upon the excretion of administered NMN. Diabetic and non-diabetic animals were given single doses of tryptophan, niacinamide or NMN and the urinary NMN was determined. Insulin was then given in daily doses sufficiently large to clear glucose from the urine of the diabetics, with non-diabetics receiving similar doses. The single doses of tryptophan, niacinamide or NMN were then repeated [7]. The administration of insulin was not effective in increasing the urinary NMN response to these single doses in either the diabetics or non-diabetics. Diabetic animals, for which the conversion of single doses of tryptophan to NMN was not increased by insulin, were continued on insulin treatment and fed approximately 100 mg of DL-tryptophan daily. Excretion of NMN increased gradually from an average of 0.31 mg per day to 1.32 mg per day in about 19 days, indicating that insulin was effective in restoring the conversion of tryptophan to NMN after a period of time. This response was very similar to that shown in figure 1, and suggests the possibility of an indirect or delayed response to the insulin treatment. It was further observed that for non-diabetic rats in which the conversion of tryptophan to NMN was normally low, administration of insulin and tryptophan was not effective in increasing this conversion, even after prolonged periods.

Since it was evident that tryptophan was not being metabolized in the normal way by these diabetic rats, the possibility that part of the tryptophan was being converted to glucose was considered. Conversion of C^{14} labeled tryptophan to glucose in phlorhizin diabetic rats has been demonstrated by Sanadi and Greenberg ('50). It has been shown by Chernick and Chaikoff ('51) that liver slices of alloxan diabetic rats oxidize fructose at normal rates but that glucose oxidation is depressed. It has also been shown that fructose is utilized normally by depancreatized dogs (Pletscher and Hess, '51)

[7] Determinations after insulin were usually begun the day following the first 24-hour period during which the amount of urinary glucose was no longer significant.

and by diabetic humans (Craig et al., '51; Miller et al., '52). Sarett and Snipper ('54) have shown that alloxan diabetic rats fed fructose diets consume much less water and excrete less carbohydrate in the urine than do rats fed glucose diets. In the present experiments substitution of fructose for sucrose

TABLE 3

Effects of dietary fructose in alloxan diabetic rats [1]

CATEGORY OF INTEREST	DIET 9221 (Sucrose)	DIET 9267 (Fructose)
Basal urinary NMN (mg/day)	0.08 (0.06–0.12)	0.07 (0.07)
Urinary NMN/100 mg tryp (mg/day)	0.22 (0.17–0.26)	0.18 (0.13–0.24)
Urinary glucose (gm/day)	11.1 (8.0–14.9)	7.1 (6.8–8.5)
Water intake (ml/day)	155 (135–194)	107 (94–123)

[1] Three diabetic rats were used in this experiment. Values in the second column were obtained from the same three rats after 8 to 24 days of fructose feeding. Ranges of values are shown within the parentheses.

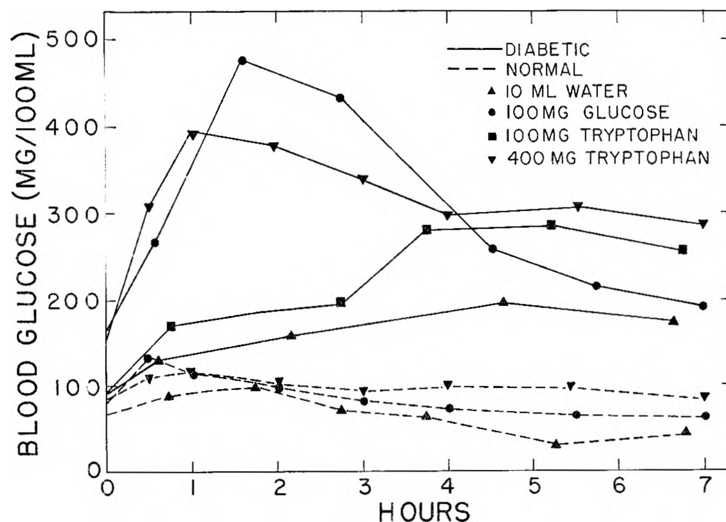

Fig. 2 Effect of tryptophan on blood glucose curves. Both rats on diet 9260. Glucose and tryptophan were given by stomach tube following a 16-hour fast.

in the diets of diabetic rats did lower the water intake and urinary glucose somewhat, but was not effective in restoring the ability to convert tryptophan to NMN, as shown in table 3.

To investigate further the possible conversion of tryptophan to glucose, the effect of tryptophan on the blood glucose levels of fasting rats was investigated (fig. 2). It was observed that oral administration of 400 mg of tryptophan resulted in marked increases in blood glucose in diabetic rats but had no marked effect in normal rats. Tryptophan appeared to be about one-fourth as active as glucose in elevating the blood glucose curves of diabetic rats. However it was observed that tryptophan would give a positive test for glucose by the method used in these experiments. To eliminate the possibility that tryptophan instead of glucose was being measured, it was shown by fermentation with bakers' yeast that 90 to 95% of the material determined as glucose following administration of tryptophan was fermentable. Although these data indicate that tryptophan does increase blood glucose in fasted diabetic rats, they do not necessarily mean that tryptophan was being converted to glucose. It is possible that tryptophan promoted the formation of glucose in some indirect manner.

The effect of very large doses of tryptophan upon urinary NMN, xanthurenic acid, and other metabolites [8] was determined. Three diabetic rats, in which the conversion of tryptophan to NMN was low, and three non-diabetic rats, in which the ability to make the conversion ranged from low to normal, were given doses of 100 and 400 mg of tryptophan (table 4). It was observed that the higher dose of tryptophan resulted in increased amounts of NMN in all rats tested, although the increase was less marked for the diabetic rats. The amounts of other metabolites and xanthurenic acid found in the urine following doses of 100 mg of tryptophan were low and no significant differences were observed between diabetics and

[8] See footnote 5, page 408.

non-diabetics. However, when 400 mg of tryptophan were given, both xanthurenic acid and other metabolites increased markedly in the urine of all rats. Xanthurenic acid was increased much more in diabetics than in non-diabetics, while for the other metabolites the reverse was true.

TABLE 4

Effect of tryptophan upon the urinary excretion of various tryptophan metabolites in diabetic and non-diabetic rats [1]

	RAT NO.	AFTER 100 MG TRYPTOPHAN [2]			AFTER 400 MG TRYPTOPHAN [2]		
		NMN	XA [3]	"Other" [4]	NMN	XA [3]	"Other" [4]
			mg/day			*mg/day*	
Diabetics	8690	0.05		14	0.23	32	70
	8709	0.10	2 [5]	19	0.52	50	62
	8675	0.18		19	0.88	65	82
Non-diabetics	8720	0.76		13	2.28	14	133
	8731	3.12	2 [5]	14	5.12	12	117
	8750	0.54		14	1.30	16	101

[1] Five diabetic and two non-diabetic rats not listed above were given 200 mg L-tryptophan. Average urinary values for the above compounds were as follows:

Diabetics 0.76 mg NMN; 25 mg XA; 13 mg "Other"

Non-diabetics 3.23 mg NMN; 8 mg XA; 70 mg "Other"

Unsupplemented rats (diabetics and non-diabetics) excreted averages of 3.5 mg/day of XA and 3.2 mg/day of "other" metabolites.

[2] Tryptophan was given by stomach tube during periods when animals were otherwise fasted. When 400 mg tryptophan were given it was necessary to use the more soluble L-form. No marked differences in the conversion of L- and DL-tryptophan to NMN have been observed.

[3] XA = xanthurenic acid.

[4] "Other" indicates compounds excreted in the urine which give a positive reaction in the Eckert ('43) test, and expressed as mg/day using tryptophan as a standard. Anthranilic acid, tryptophan, possibly kynurenine and other metabolites will react in this test.

[5] Values shown for xanthurenic acid (XA) following 100 mg tryptophan are averages of two diabetic and two non-diabetic rats not listed above.

DISCUSSION

It is evident that in the alloxan diabetic rat there is some disturbance in the metabolism of tryptophan which results in a marked impairment in converting tryptophan to niacin and then to NMN. The results of the present study suggest that

the primary difficulty is in the conversion of tryptophan to niacin, rather than an inability to methylate or excrete the metabolite, since administration of niacin, niacinamide and NMN each resulted in marked increases in urinary NMN. Conversion of niacinamide to NMN was reduced somewhat in about half of the diabetic animals and greatly reduced in a few. It is not known whether this reduction in methylation of niacinamide is a result of the same defect responsible for the reduced conversion of tryptophan to niacin. The data suggest, however, that there are two different mechanisms involved as shown by the fact that in all diabetic rats the conversion of tryptophan was greatly reduced while many were able to methylate and excrete niacinamide in a normal manner. The failure in methylation could possibly result from liver damage or some other effect of the alloxan, rather than from the diabetes itself. It was observed that for rats in which the conversion of niacinamide to NMN was greatly reduced, the survival time was usually short. Rats in which only the tryptophan to NMN conversion was reduced usually appeared relatively healthy and survival was good.

It is not known at present whether a similar abnormality exists in other species or in other types of diabetes. It is interesting that abnormal tryptophan metabolites have been found in the urines of diabetic patients by Rosen et al. ('55) and by Kotake and Tani ('53).

The increase in blood glucose levels resulting from administration of tryptophan to diabetic rats suggests that these rats may convert tryptophan to glucose in an attempt to supply energy rather than converting it to niacin. The lack of any beneficial effect of dietary fructose upon the conversion of tryptophan to NMN is against such an assumption. It is possible that tryptophan promotes the formation of glucose in some indirect manner.

Cataracts were observed quite frequently in diabetic rats fed purified diets. It is known that tryptophan deficiency results in the development of cataracts in rats (Curtis et al., '32; Totter and Day, '42; Albanese and Buschke, '42). Ribo-

flavin deficiency in rats has been shown to cause abnormal tryptophan metabolism (Henderson et al., '51; Mason, '53) and also results in development of cataracts (Day et al., '31; Day and Langston, '34; Bourne and Pyke, '35). The cataracts resulting from riboflavin deficiency closely resembled those due to tryptophan deficiency, according to Albanese and Buschke ('42). Whether there is a relationship between abnormal tryptophan metabolism and development of cataracts remains to be determined.

It is of interest that for some diabetic rats, in which the conversion of trytophan to NMN was low when 100 mg doses were given, increased amounts of NMN could be excreted if very large doses (400 mg) of trytophan were given. This suggests that, although the mechanism for conversion appears to be greatly impaired, the real difficulty may result from shifts in the primary metabolic pathways of tryptophan rather than from the complete absence of the proper mechanisms. In addition, the marked difference between diabetics and non-diabetics, with respect to the relative amounts of urinary xanthurenic acid and other metabolites following large doses of tryptophan, also suggests modification of the pathways of tryptophan metabolism in diabetic rats.

The rate of response to insulin, with respect to conversion of tryptophan to NMN, varied with different rats. Although the water intake and urinary glucose decreased rapidly after insulin was started, the response with respect to NMN excretion ranged from one to 19 days. The delayed responses observed following administration or withdrawal of insulin suggest that the process which permits increased conversion of tryptophan to NMN may result indirectly from the action of insulin upon some other mechanism.

Data obtained by chromatographic and chemical procedures indicate that ability to excrete anthranilic acid and 3-hydroxyanthranilic acid in the urine following administration of tryptophan is markedly reduced in diabetic rats, and that kynurenic acid is present in the urine of both diabetic and non-diabetic rats. Tryptophan may be converted to niacin

via the route outlined by Umbreit ('52), i.e., tryptophan — kynurenine — 3-hydroxykynurenine — 3-hydroxyanthranilic acid — niacin. In this scheme anthranilic acid and 3-hydroxyanthranilic acid are formed from kynurenine and 3-hydroxykynurenine by removal of the respective alanine side chains. Kynurenic acid and xanthurenic acid result from kynurenine and 3-hydroxykynurenine respectively by ring closure. The increased amounts of xanthurenic acid and the presence of kynurenic acid in the urine of diabetic rats following doses of tryptophan, indicate that there is no difficulty in the conversion of tryptophan to kynurenine then to 3-hydroxykynurenine. It appears possible therefore that an inability to effect removal of the alanine side chain from 3-hydroxykynurenine to form 3-hydroxyanthranilic acid may be a contributing factor in the reduced conversion of tryptophan to niacin in the alloxan diabetic rat.

SUMMARY

It has been shown that there is a marked reduction in the conversion of tryptophan to NMN in the alloxan diabetic rat. The primary failure appears to be in the conversion of tryptophan to niacin rather than in the methylation or excretion of the metabolite, since administration of niacin, niacinamide or NMN did result in increased urinary NMN.

The failure to convert tryptophan to niacin appeared to result primarily from the diabetes *per se* rather than from some more direct effect of the alloxan, since the defect was corrected in some rats by administration of insulin, and alloxan treated rats which failed to become diabetic converted tryptophan to NMN as did normal rats.

In some diabetic animals the conversion of niacinamide to NMN was also reduced, but to a lesser extent than was the conversion of tryptophan to NMN.

Administration of tryptophan elevated the blood glucose in fasting diabetic rats, but not in normal controls, suggesting that part of the tryptophan may be converted into glucose in an attempt to meet energy requirements. However, the lack

of a beneficial effect of dietary fructose in correcting the low conversion of tryptophan to NMN, indicates that conversion to glucose is not the entire explanation.

Some diabetic animals in which the conversion of tryptophan to NMN was greatly impaired did excrete increased amounts of NMN when very large doses of tryptophan (400 mg) were given, indicating that the defect may be due to changes in the primary metabolic pathways of tryptophan rather than to absence of the proper mechanisms.

Diabetics excreted much more xanthurenic acid than did non-diabetics, following large doses (200 to 400 mg) of tryptophan.

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THE EFFECT OF AGE ON THE LEVEL AND METABOLISM OF FLUORINE IN THE BONES OF THE FLUORIDATED RAT [1,2]

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Fluorine deposition in the skeleton may occur by means of actual incorporation into the bone salt molecule, or by ionic exchange as suggested by Klement ('37), Neuman et al. ('50) and Megirian and Hodge ('51). A growing animal would therefore incorporate fluorine by both means while the preformed bones of the adult would deposit skeletal fluorine in large measure only by ionic exchanges and periosteal growth. Exostoses or newly formed spicules of bone salts in cancellous bones under the influence of excess dietary fluorine would increase the fluorine content of the resulting bone deposits of the animal regardless of the age factor.

A reverse of the ionic exchange reaction apparently takes place in the reduction of the total fluorine content in the skeleton. For a reduction in the total skeletal fluorine to occur,

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the F^- of fluoroapatite must be replaced at a velocity greater than that of the deposition of new fluorine.

In the studies reported herewith an attempt has been made to determine the relationship of age to the storage and loss of fluorine from the skeleton of the fluoridated rat. Three studies were made as follows: (1) the effect of age upon the concentration, deposition and mobilization of femur fluorine; (2) the effect of age and time upon femur F^- saturation; and (3) the effect of age upon femur fluorine of rats subjected to repeated exposure and recovery periods.

EXPERIMENTAL

Female albino rats were used in the first and third experiments. Since femur fluoridation was sought, no efforts were made to measure fluorine[4] intake; hence food and water were fed ad libitum. The basal ration used was normal with respect to its fluorine content and averaged 5 p.p.m. of F or less. It was composed of ground whole wheat 29 parts, ground yellow corn 25, powdered skim milk 12, soybean oil meal 10, linseed oil meal 10, alfalfa meal 8, butter oil 5, calcium carbonate 1 and iodized salt 1 part. During fluoridation periods 0.10% of NaF (452 p.p.m. of F) mixed in the diet was used as the source of fluorine.

All analyses for fluorine were made by the Willard and Winter method ('33) as modified by the Aluminum Company of America Research Laboratories ('47), and the results were calculated on the femur ash basis.

Experiment 1

Three groups of 24 white rats each were selected at three weeks, 7 weeks, or 6 months of age. They were fed the basal ration to which was added 0.10% of NaF (0.045% of F) for an initial period of 40 days. This level of NaF feeding has been used repeatedly on many occasions in this laboratory and is known to be non-lethal but growth retarding. The

[4] F = elemental fluorine, atomic wt. 19.

animals were then transferred to the basal ration only (without added fluorine) for periods up to 300 days. The preliminary feeding period was for the purpose of rapidly fluoridating the skeletons of these rats of various ages. At the close of the fluoridating period rats from each age group were sacrificed, the femurs removed and analyzed for their fluorine content. Four rats were removed at intervals of 15, 30, 60, 90 and 120 days post-fluoridation and femur fluorine determined as indicated. Four rats (6 months age group) were sacrificed at 300 days.

The results obtained are summarized in table 1. The data indicate rapid and heavy fluorine deposition in the femurs of the three week age group to near the skeletal saturation levels (16,000 to 20,000 p.p.m.), while the 7 weeks (young adult) and 6 months old rats (adult) stored, respectively, approximately 50 and 25% as much fluorine as the weanlings.

The data also show clearly that the fluorine concentration and total fluorine of the femurs of the young dropped rapidly following the removal of the added dietary fluorine. These results are similar in pattern and magnitude to those obtained earlier by Miller and Phillips ('53), and they are in line with the observations reported by Savchuck and Armstrong ('51). The total fluorine content of the mature bone as indicated by milligrams of fluorine per femur did not decrease during the post-fluoridation period although there was a slight decrease in femur fluorine concentration, expressed as parts per million. This observation is believed to be the result of the dilution effect of new bone growth. It is remarkable that the fluorine varied only about 600 p.p.m. between the various lots at 120 days post-fluoridation.

Experiment 2

This experiment was designed to determine the femur concentrations of fluorine in young rats under continuous exposure to 0.10% of dietary NaF from weaning up to periods of 18 weeks. Three groups of 18 white rats were selected and

TABLE 1

The effect of age upon deposition and mobilization of fluorine in the femurs of fluoridated white rats

DAYS POST-FLUORIDATION	ANIMALS PER LOT	TOTAL FLUORINE PER FEMUR (MG) INITIAL AGE			CONCENTRATION OF FEMUR FLUORINE (P.P.M.) INITIAL AGE		
		3 wks.	7 wks.	6 mo.	3 wks.	7 wks.	6 mo.
				Experiment 1			
0	4	1.23 ± 0.05	1.26 ± 0.04	0.88 ± 0.15	17750 ± 750	8500 ± 420	4460 ± 360
15	4	0.93 ± 0.12	1.24 ± 0.13	0.95 ± 0.23	8600 ± 660	8050 ± 460	3860 ± 420
30	4	0.90 ± 0.11	1.23 ± 0.09	0.88 ± 0.14	6400 ± 680	6900 ± 420	3980 ± 160
60	4	0.77 ± 0.06	1.44 ± 0.16	0.90 ± 0.18	4300 ± 100	5800 ± 140	4200 ± 280
90	4	0.85 ± 0.09	1.27 ± 0.04	0.75 ± 0.08	4030 ± 170	5400 ± 140	3420 ± 530
120	4	0.81 ± 0.03	1.28 ± 0.21	0.85 ± 0.18	4660 ± 570	4730 ± 450	3940 ± 150
300	4	..	..	0.87 ± 0.18		..	3860 ± 300

Experiment 2

WEEKS OF ADDED FLUORINE		INITIAL AGE			INITIAL AGE		
		3 wks.	9 wks.	5 mo.	3 wks.	9 wks.	5 mo.
6	6	1.60 ± 0.14	1.55 ± 0.19	1.30 ± 0.15	15500 ± 900	7700 ± 920	4900 ± 690
9	4	2.40 ± 0.10	2.20 ± 0.09	1.50 ± 0.19	15200 ± 680	10200 ± 350	5100 ± 330
12	4	2.80 ± 0.08	2.60 ± 0.23	1.70 ± 0.19	15500 ± 1050	10800 ± 2400	6000 ± 600
18	4	3.35 ± 0.47	3.50 ± 0.51	2.30 ± 0.15	15000 ± 740	12100 ± 740	7800 ± 450

distributed as follows: lot 1, weanlings three weeks old; lot 2, young adults 9 weeks old; and lot 3, adults 5 months old. All rats were fed the basal ration used in experiment 1.

The results obtained from femur fluorine analyses (ashed basis) of the rats of the various ages show that the femurs of the young weanling rats were fully fluoridated (p.p.m.) after 6 weeks' exposure to the dietary fluorine, while the femur fluorine concentration of the two groups of adult rats continued to increase for the entire period of 18 weeks (table 1). At the close of the experimental period the young adult, 9 weeks old rat femurs averaged 80% as much fluorine in parts per million as the weanling rats. Likewise the adult rats (5 months old at the beginning of the experiment) had femur fluorine concentrations approximately half those of the weanling rat. The total fluorine content of the femurs increased with the length of the exposure period. The increase, which may be accounted for by the growth of the bone, cannot be completely explained on this basis since the adult group, lot 3, would seem to have started with a fully mature femur at 5 months of age and yet the total fluorine deposited in the femurs between 12 and 18 weeks was greater than during earlier periods.

Experiment 3

This experiment was conducted to determine the effect of re-exposure to dietary fluorine upon the deposition of fluorine in the femur, as related to age when first exposed. Thirty-two white rats representing one of three age levels were used per lot. Lot 1 was composed of weanlings three weeks old, lot 2, young adults 9 weeks old, and lot 3, adults 5 months of age when they were first exposed to added dietary NaF (0.10%). The ration used was the same as that used in the previous experiments. Each lot started with 26 rats for the first fluoridation period. In addition, 6 unfluoridated rats served as controls and were fed the basal ration only until their lot mates were ready for refluoridation. Four of these were then fluoridated and thus received only a single 6 weeks exposure

to dietary fluorine when they were approximately 5, 7 and 10 months of age. Two rats were used from this control group to check fluorine levels of the adult femur before fluoridation of these animals.

The results of experiment 3 are summarized in table 2. These results show that the weanling rat (lot 1) immediately fluoridated at that age, could subsequently mobilize a portion (66 to 70%) of its femur fluorine during a 60- or 120-day low-fluorine intake post-fluoridation period, a response which did not occur in the fully mature rat. The rats of intermediate age (lot 2, initial age 9 weeks) retained the ability to mobilize fluorine, but to a lesser degree. Attention is called to the fact that the concentrations of fluorine in the femurs of this lot was only half that in those of the weanling rat. The weanling rat (lot 1), when fluoridated a second time after a non-fluoridating or recovery period lasting 60 or 120 days, lost its ability to deposit the original high concentration of fluorine which was observed at the earlier age. The femur fluorine deposition then behaved very much like that seen in the older mature animals, which indicated that the rats of lot 1 were "mature" at the time of refluoridation.

In terms of total femur fluorine, mobilization of fluorine occurred which reached its maximum during a 60-day low-fluorine feeding period and which was not further affected by doubling the recovery period to 120 days. Upon re-exposure to added dietary NaF (0.10%) whether at the end of the 60- or the 120-day respite from supplemental fluorine, again the total femur fluorine increased. The amounts deposited in the femurs of lots 1 and 2 were similar while those of lot 3 mobilized less and redeposited less fluorine upon a second dietary exposure to the added fluorine.

The rats unfluoridated at the first exposure, as measured by two rats sacrificed at the end of either the 60- or the 120-day recovery period, had femurs that contained normal femur fluorine, or less than 0.2 mg total femur fluorine and under 600 p.p.m. of F. The 4 remaining rats were then exposed to the NaF supplementation for 6 weeks similarly to those in

TABLE 2

The effect of age upon femur fluorine deposition, mobilization and re-fluoridation in the rat

TREATMENT	NUMBER ANIMALS PER LOT	LOT 1 (weanling) FLUORINE		LOT 2 (9 wks.) FLUORINE		LOT 3 (5 mo.) FLUORINE	
		mg/femur	*p.p.m.*	*mg/femur*	*p.p.m.*	*mg/femur*	*p.p.m.*
1st fluoridation	6	1.58 ± 0.14	15500 ± 900	1.54 ± 0.19	7700 ± 950	1.28 ± 0.18	4900 ± 690
60 day recovery — F	4	1.14 ± 0.09	5250 ± 670	1.50 ± 0.15	5900 ± 280	1.16 ± 0.13	4070 ± 460
Re-fluoridation (after 60 day — F)	4	1.90 ± 0.16	7780 ± 870	1.93 ± 0.15	7000 ± 510	1.36 ± 0.15	4700 ± 290
120 day recovery — F	4	1.13 ± 0.09	4370 ± 470	1.36 ± 0.04	5000 ± 370	1.18 ± 0.16	4065 ± 390
Re-fluoridation (after 120 day — F)	4	1.90 ± 0.08	7520 ± 220	2.00 ± 0.39	6950 ± 620	1.52 ± 0.15	5410 ± 410
Controls [1] fluoridated at approximately		*18 wks*		*24 wks*		*35 wks*	
	4	0.91 ± 0.06	3640 ± 150	0.78 ± 0.03	3090 ± 80	0.95 ± 0.17	3200 ± 480
Controls [1] fluoridated at approximately		*24 wks*		*32 wks*		*43 wks*	
	4	0.98 ± 0.15	3560 ± 330	0.98 ± 0.04	3340 ± 460	0.98 ± 0.04	2950 ± 260

[1] Unfluoridated rats of similar ages had 0.2 mg F/femur and concentration of less than 600 p.p.m.

the refluoridated groups, lots 1, 2, and 3. All of these rats while differing in actual initial age, as indicated, were all mature rats when they were given their only exposure to the dietary fluorine. It is quite evident that these mature rats were able to deposit only about half the total femur fluorine whether measured as milligrams of total fluorine or in parts per million in comparison to their lot mates previously fluoridated.

The data in these experiments also show that there was a marked difference in the rate of femur fluorine deposition which was correlated with age. The femur of the weanling rat fluoridated quickly to saturation with progressively slower rates of fluorine concentration with increase in age.

DISCUSSION

The weanling rat has a tremendous capacity to concentrate fluorine into its skeletal structures. There appear to be two routes by which this accumulation occurs, and presumably these are the incorporation of fluorine into the bone salt molecule in the process of its formation and by ionic exchange. The present studies further indicate that in the young rat, fluorine deposited in the bone is subject to a rapid metabolic turnover. The inability of the older rats, whether fluoridated for the first time or re-fluoridated, to store fluorine at a rate equivalent to that of the young rat would suggest that in the aged-mature bone fewer ionic exchange sites were available to F^-. Hence the saturation range of the young rat was not attained by the older animals during the time period used in these studies.

The rat possesses the ability to mobilize or catabolize a portion of its femur fluorine during periods of low fluorine intake following a period of fluoridation. This phenomenon was observed in the adult rat and more dramatically so in the young rat. Apparently the catabolic activity of the bone changes in the young rat are such that many of the re-fluoridation sites in the femur were unavailable for a second fluoridation with F^-. This appears to be associated with the process of bone

maturation. The explanation of these observations is not clear at the present time.

SUMMARY

An effect of age upon the deposition and retention of femur fluorine has been demonstrated. Fluorine was deposited in greater amounts in the femur of the young rat than in that of the mature rat; however, both the young and mature rat femurs continued to concentrate fluorine progressively with time. Femur fluorine in the rat was mobilized from this bone during periods of low fluorine intake. Again age affected the rate and extent of such femur F^- catabolism. The mobilization of femur fluorine of the weanling rat accompanied by an increase in age caused bone changes in the femur which closed a portion of the available fluorine deposition sites to subsequent re-fluoridation.

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INDICAN EXCRETION BY RATS FED RAW SOYBEAN OIL MEAL

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INTRODUCTION

Although there have been occasional publications concerning indican (potassium 3-indoxyl sulfate), the attitude toward research on this subject has been aptly described by Meiklejohn and Cohen ('42): "For nearly a quarter of a century, there has been a general absence of interest in the significance of the urinary excretion of indole derivatives. The doctrine established by tradition that urinary indoles are derived from putrefactive processes in the intestines apparently has made further investigation of this subject unprofitable." The authors are aware of no direct proof that indican arises from intestinal putrefaction or more particularly from tryptophan degradation although such may be presumed from available data. Underhill and Simpson ('20) reported that indican was increased by a meat diet and that only a trace of indican was excreted on a gelatin diet. On the other hand, Sherwin and Hawk ('14) reported that indican excretion continued in a dog which was fasted for 117 days. The *in vivo* formation of indoxyl by means other than putrefaction and from compounds other than indole or its immediate derivatives has

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been suggested by Houssay et al. ('35), Bohm ('39) and Stoppani ('45). The compounds studied were mainly derivatives of the o-nitrobenzyl group. Wellers ('35) found that feeding indole to rats on a 6% casein ration resulted in growth depression. However, feeding indole plus cystine or methionine, supposedly to furnish adequate sulfur for detoxication, resulted in normal growth.

An indication that indican formation involves the action of intestinal bacteria is afforded by reports that antibiotics reduce indican excretion. Muller ('50) found that indican disappeared after oral administration of large doses of streptomycin to children. Feeding 0.05% of terramycin to rats was found by Makino and Umezu ('52) to result in a low excretion of indican even after the intestinal bacterial count had risen. Bacterial isolates from such antibiotic-fed rats did not produce indole in vitro.

Indican has been found to be excreted in amounts approximately three to 10 times greater by rats fed raw soybean oil meal than by rats fed autoclaved meal (Borchers and Mohammad-Abadi, '55). Quantitative data supporting these observations are presented in the present paper. Inasmuch as rats fed raw soybean oil meal rations grow at a slower rate than rats fed autoclaved soybean, indican (or its precursors) may be the cause of the growth depression resulting from the feeding of raw soybean oil meal. The following experiments were undertaken to investigate a possible causal relationship between indican and growth depression.

PROCEDURES

Weanling rats of the Sprague-Dawley strain were used in these investigations. Litter mates of the same sex and approximate weight were paired between experimental groups. Animals were housed in screen-bottom cages with food and water available ad libitum. The feeding period was for 20 days, results are expressed as average gain per day, in grams. The rations fed contained per 100 gm: 25 gm raw or autoclaved soybean oil meal (45.5% protein, N $\times$ 6.25), 0.6 gm DL-methio-

nine,[2] vitamins (including 2 μg vitamin B_{12}), minerals, starch and fat (for details, see Borchers and Ackerson, '51). Urine samples, collected under mineral oil, were obtained on alternate two-day periods beginning on the 4th day of feeding. Indican was determined by a quantitative modification of the Obermayer test as described in detail by Zacherl ('33). Results are expressed as milligrams of indican excretion/day/100 gm body weight. Because of wide variations in excretion by the same animal in successive periods, data for progressive changes with time in indican excretion are not presented.

EXPERIMENTS AND RESULTS

Rats fed raw soybean oil meal gained 2.93 gm/day and excreted 3.83 mg of indican/100 gm body weight/day; those fed autoclaved soybean oil meal gained 4.15 gm and excreted 0.89 mg of indican. These results are itemized in table 1, experiment 1.

In the second experiment, 5% of crude trypsin powder was added to each ration. A previous report (Borchers and Ackerson, '51) established that the addition of 5% of crude trypsin equalized the growth rate of rats fed autoclaved versus raw soybean oil meal (addition of 5% of casein was ineffective). In experiment 2, growth was similar for autoclaved and raw soybean oil meal plus trypsin. However, indican excretion on the raw soybean ration plus trypsin continued at a high level as in experiment 1.

Wellers ('53) reported that dietary indole did not depress growth provided adequate cystine or methionine was included in the ration. His publication gave no data on indican excretion. Hence, it was necessary to establish the effect of indole on rats fed soybean rations and to determine the actual level of indican excretion after indole feeding. In experiment 3, indole was fed at levels of 0.05 and 0.1% in an autoclaved soybean ration. These levels did not affect the growth rate. Indican excretion on 0.05% of indole was approximately equal

[2] DL-Methionine, courtesy of The Dow Chemical Company, Midland, Michigan.

TABLE 1

Gain and indican excretion of rats fed autoclaved or raw soybean oil meal plus trypsin, indole or streptomycin

EXP. NO.	SOYBEAN [1]	ADDITION	NO. OF RATS	GAIN [2]		NO. OF SAMPLES	URINARY INDICAN
				gm/day ± *SE*	*gm/gm food*		*mg/100 gm rat/day* ± *SE* *(range)*
1	Autoclaved	none	8	4.15 ± 0.18	0.38	32	0.89 ± 0.06 (0.50–1.80)
	Raw	none	8	2.93 ± 0.23	0.31	32	3.83 ± 0.10 (2.96–5.20)
2	Autoclaved	5% trypsin [3]	8	4.20 ± 0.12	0.38	32	1.10 ± 0.11 (0.46–1.92)
	Raw	5% trypsin	8	4.02 ± 0.17	0.37	32	4.02 ± 0.30 (2.34–5.61)
3	Autoclaved	none	8	3.80 ± 0.21	0.40	16	0.76 ± 0.08 (0.30–1.30)
	Autoclaved	0.05% indole	8	3.98 ± 0.12	0.42	16	4.00 ± 0.40 (0.61–6.26)
	Autoclaved	0.1% indole	8	3.63 ± 0.15	0.39	16	8.86 ± 0.58 (3.22–13.34)
4	Autoclaved	0.1% streptomycin [4]	10	3.92 ± 0.12	0.41	24	0.54 ± 0.09 (0.13–1.54)
	Raw	0.1% streptomycin	10	3.03 ± 0.14	0.31	24	0.59 ± 0.08 (0.16–1.67)

[1] Raw soybean oil meal was prepared with a minimum of heat treatment by the hexane process according to the manufacturer's statement. Autoclaved meal was prepared from this by autoclaving thin layers of the meal at 15 p.s.i. for 30 min.

[2] Average for the 20-day feeding period.

[3] Crude trypsin powder (1–110), Pfanstiehl Chemical Co., Waukegan, Illinois.

[4] Streptomycin sulfate, assay 745 μ/mg.

to, and on 0.1% of indole twice that of rats fed raw soybean oil meal in experiments 1 and 2. Similar results were obtained when 0.1% of indole was added to a raw soybean oil meal ration. The average amount of indican excreted at either the 0.05 or 0.1% level accounted for approximately 40% of the indole intake. Whether the remainder was not absorbed or was metabolized in some other manner was not determined in these experiments. Feeding of indole above 0.1% has resulted in some depression of the growth rate which was due, in part, to reduced food consumption.

If indican arises from bacterial action in the intestine, suppresion of bacterial growth should reduce indole production and indican excretion. Furthermore, if indican or its precursors or bacterial action in general are the cause of growth depression, suppression of intestinal bacteria should not only decrease indican excretion but should as well increase the growth rate of rats fed raw soybean oil meal. Since earlier experiments in our laboratory with antibiotics fed at moderate levels had shown no effects on growth of rats fed autoclaved or raw soybean oil meal rations, streptomycin sulfate was fed at a level of 0.1%. In this experiment, the usual difference in growth rate between animals fed autoclaved and raw soybean oil meal was observed. However, indican excretion was reduced for both the autoclaved and raw soybean rations; the excretion was approximately equal for the two rations as shown in experiment 4 of table 1. The 0.1% level of streptomycin has, in some experiments, stimulated the growth rate of rats fed raw soybean oil meal. A more detailed report of these feedings will be published later.

DISCUSSION

These investigations were conducted to determine whether a causal relationship existed between the increased excretion of indican and the reduced growth rate of rats when raw soybean oil meal was fed. If the assumption is made that possible intestinal putrefactive compounds, such as indole,

are toxic; that is, toxic to the extent of reducing the growth rate; then the conclusion might follow that the feeding of raw soybean oil meal reduced the growth rate because of increased intestinal putrefaction as indicated by increased indican excretion. An examination of the data presented in experiments 2 to 4 does not, however, support such a conclusion.

First of all, the addition of crude trypsin powder to the rations did not change the level of indican excretion although the growth rate of the animals fed the raw soybean oil meal was increased to the rate of those fed the autoclaved meal. This indicates that the sequence of events culminating in indican excretion was still operating but that these events were without effect on the growth rate. That the effect of the crude trypsin on the growth rate was not due to an increased protein level *per se* was established in a previous publication (Borchers and Ackerson, '51) where it was shown that the addition of 5% of casein was without effect on the comparative growth rates of rats fed autoclaved versus raw soybean oil meal. However, the effect of the crude trypsin may have been accomplished by unidentified factors in this material which protected the animal from or aided the animal in combating the effects of possible intestinal putrefactive compounds, such as indole.

Furthermore, and in confirmation of the work of Wellers ('53), the feeding of indole was without effect on the growth rate of rats under the conditions employed. Since the levels of indican excretion attained after indole feeding were in excess of those observed with the raw soybean oil meal rations, it is unlikely that indole produced by bacterial putrefaction has an effect on the growth rate. However, indole may not represent the only putrefactive product which is detoxified and excreted as indican. Such other compounds may have pronounced effects on the growth rate.

Finally, the suppression of bacterial putrefactive activities, accomplished by streptomycin feeding and indicated by a

marked decrease in indican excretion, was without effect on the comparative growth rates of rats fed autoclaved and raw soybean oil meal. Since the reduced growth rate with raw soybeans persisted while indican excretion was markedly reduced, it seems unlikely that events leading to the excretion of indican have any effects on the growth rate. Or, the factors which reduce the growth rate of rats fed raw soybean oil meal are operating in the absence of indican excretion. Therefore, the reduced growth rate and increased indican excretion following raw soybean oil meal feeding are unrelated phenomena; neither may be regarded as a cause or a result of the other.

The question as to the actual source or cause of the increased indican excretion remains unanswered. The assumption that bacterial putrefaction is a factor seems warranted in view of the suppression of indican excretion after streptomycin feeding. In this connection, Carroll et al. ('52) have concluded, on the basis of chromic oxide marker experiments, that a greater proportion of the nitrogen reached the cecum when rats were fed raw soybean oil meal than when fed autoclaved meal. These authors then reasoned that much of this cecal nitrogen must be absorbed from the cecum, presumably as putrefactive products. Such cecal absorption would then account for the generally observed similar digestibility values for raw and autoclaved soybean oil meal. However, similar marker studies in our laboratory (Borchers, '53) failed to substantiate the basic observations of Carroll et al. ('52), thus vitiating their reasoning that cecal absorption was an important factor in raw versus autoclaved soybean digestibility studies.

SUMMARY

Rats fed raw soybean oil meal were found to excrete about 4 times as much indican and to grow at about three-fourths the rate of rats fed autoclaved meal. Addition of crude trypsin powder to the rations equalized the growth rate without affecting indican excretion. Addition of indole to rations

markedly increased indican excretion without affecting the growth rate. The feeding of streptomycin greatly depressed indican excretion without affecting the growth rate. Each of the two conditions under study, namely indican excretion and growth inhibition, was produced independently of the other. Therefore, it is concluded that no causal relationship exists between increased indican excretion and the decreased growth rate which result from raw soybean oil meal feeding.

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