NITROGEN BALANCE AND HEMOGLOBIN OF ADULT RATS FED AMINO ACID DIETS LOW IN L- AND D-HISTIDINE [1]

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ONE FIGURE

(Received for publication February 22, 1954)

A diet devoid of histidine apparently does not cause negative nitrogen balance in adult human subjects (Rose, '49; Rose et al., '51) or the adult white rat (Burroughs et al., '40). According to Benditt et al. ('50), however, histidine is essential for the maintenance of nitrogen balance in the adult rat. This apparent discrepancy has not yet been explained but may very well be related to one or more of the following possibilities: (a) the histidine requirement of the adult is probably quite low, (b) a histidine deficiency is slow to manifest itself in nitrogen balance studies, and (c) the breakdown of hemoglobin may liberate relatively large quantities of histidine. Sebrell and McDaniel ('52) demonstrated that histidine is extremely important for regeneration of hemoglobin in the rat that has been made anemic by hemorrhage. The experiments described below represent an attempt to shed more light on these problems.

METHODS

Four groups of adult male albino rats of the Wistar strain were used. Each group initially included 14 to 16 rats but

[1] This work was supported by grants from the Office of Naval Research, and the Fluid Research Fund of the School of Medicine and Dentistry, University of Rochester.

owing to some deaths, illnesses, and errors in feeding or in the collection of excreta, the number of animals in any single experiment varied from 8 to 14.

At the beginning and after a 48-hour fast, the mean body weights for the 4 groups ranged from 217 to 275 gm. The maximum difference between the lightest and heaviest animals within a single group was 30 gm. The energy allowance of 121 Cal./day/$kg^{3/4}$ was adequate to maintain a relatively constant body weight. The energy intake for a 250 gm rat, for example, was 42.7 Cal./day. Each single experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet, one week; amino acid diet, containing approximately half of the maintenance requirement of total nitrogen (half-N), one week; amino acid diet, containing twice this amount of total nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each amino acid mixture investigated. These diets, except the maintenance diet, were fed by stomach tube in two equal portions daily, and each rat received the same amount of diet each day.

The "complete" amino acid mixture simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by the unnatural isomers of 6 essential amino acids plus sufficient L-glutamic acid to make up the same total amount of nitrogen.[2] This "complete" amino acid mixture comprised 4.17% of a basal diet which is described elsewhere (Anderson and Nasset, '48; Nasset, Anderson, and Siliciano, '51; Nasset and Ely, '53). The nitrogen-free diet was compounded by omitting the amino acid mixture and substituting for it an equal weight of sucrose.

It is the custom in this laboratory to observe changes in nitrogen balance which are brought about by a stepwise re-

[2] All amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

duction of the concentration of one essential amino acid at a time. This process simulates the consecutive testing of a series of proteins of different biological values. At some point in this stepwise reduction, a concentration is usually found which limits the efficient utilization of the total dietary nitrogen. In the present series of experiments, the concentration of histidine was varied from 1/5 to 1/32 of the concentration of L-histidine present in the "complete" amino acid mixture. Two concentrations of D-histidine were also investigated. In one experiment histidine was omitted from the diet.

Hemoglobin was determined by the cyan-hematin method of King and Gilchrist ('47). The working standard of cyan-hematin was checked against a sample which was standardized by iron analysis. Each rat of series 380 and 420 was bled weekly from the tip of the tail on the last day of each particular feeding period.

RESULTS

Table 1 contains the average results of all experiments with the rats of series 420. Experiments I and V of table 1 represent the initial and final 5-week feeding cycles in the whole program of 25 weeks' duration. The diets in these experiments contained the complete amino acid mixture and the results obtained on feeding it conform very closely to many others previously obtained in this laboratory under identical conditions. The noteworthy features of the results with the complete amino acid mixture are: (1) the absence of a rise in urinary nitrogen when the nitrogen intake is doubled (compare half-N with full-N data), (2) the attainment, essentially, of nitrogen equilibrium at very low intake of total nitrogen. These results indicate that the increment of the complete amino acid mixture, represented by the difference in ingested nitrogen between the half-N and the full-N periods, is completely utilized in the improvement of the nitrogen balance. A numerical expression of this fact is given by K′ in table 1, in which unity indicates complete

TABLE 1

Average data for rats receiving amino acid mixture diets

Experiment number	SERIES 420 RATS				
	I	II	III	IV	V
Amino acid mixture	Complete	No histidine	1/50 D-histidine	1/5 L-histidine	Complete
Number of rats	12	13	14	12	13
Body weight (kg)	0.250	0.250	0.253	0.257	0.265
Metabolic body size ($kg^{3/4}$)	0.353	0.353	0.357	0.360	0.369
	N balance data (mg N/day/$kg^{3/4}$)				
Half-N period:					
N intake	73	72	72	71	67
Fecal N	36	38	35	39	33
Urinary N	111	129	98	104	89
N balance	−73	−97	−61	−72	−55
Full-N period:					
N intake	141	137	138	140	132
Fecal N	34	31	30	30	31
Urinary N	107	137	125	106	92
N balance	+1	−31	−18	+4	+8
K'[1]	1.08 ± 0.05	1.11 ± 0.06	0.66 ± 0.06	1.11 ± 0.04	0.98 ± 0.05
NI_e[2]	142 ± 2	165 ± 6	171 ± 7	137 ± 3	124 ± 2

[1] K' is the slope of the line joining the Half-N and Full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

utilization. K′ is the slope of the line joining the half-N and the full-N points when nitrogen balance is plotted against nitrogen intake. The last item in table 1, NI_e, is the total nitrogen intake, in the form of a specific amino acid mixture, computed to be required for the attainment of nitrogen equilibrium. This item is the point of intersection of the line represented by K′ with the line representing nitrogen equilibrium. It is computed for each animal separately from the nitrogen balance data of the half-N and the full-N periods and this accounts for the fact that the mean values of NI_e do not agree exactly with the values obtained by subtracting the mean nitrogen balance from the mean nitrogen intake. The reduction in NI_e between control experiments I and V is quite characteristic. In these and many other experiments NI_e on the complete amino acid mixture progressively and uniformly becomes smaller over a period of at least 6 months; its lower limit has not been established. From a consideration of experiment II, table 1, it is perfectly clear that the omission of histidine from the dietary amino mixture results in a relatively severe negative nitrogen balance. If 1/50 of the amount of histidine in the complete mixture is supplied as D-histidine, as in experiment III, table 1, the nitrogen balance is improved but not restored to the control value (experiments I and V). In experiment IV (table 1) 1/5 of the L-histidine found in the complete mixture was sufficient to permit attainment of nitrogen equilibrium (full-N period) and NI_e is only 7% greater than expected on the assumption that NI_e for the complete amino acid mixture declines at a uniform rate over a period of 6 months. It is evident that 1/5 L-histidine is adequate for maintenance of nitrogen equilibrium. This concentration provides 6.9 mg of histidine/day/$kg^{3/4}$ or 2.4 mg/day/250 gm rat.

Table 2 contains the average results of all experiments with the rats of series 380. The nitrogen balance and NI_e values obtained with the complete amino acid mixture in experiment I are different from the corresponding values for series 420 (table 1). The animals in the latter series seemed

TABLE 2

Average data for rats receiving amino acid mixture diets

Experiment number	SERIES 380 RATS				
	I	II	III	IV	V
Amino acid mixture	Complete	1/16 L-histidine	1/16 D-histidine	1/32 L-histidine	Complete
Number of rats	10	14	9	9	9
Body weight (kg)	0.233	0.229	0.218	0.230	0.234
Metabolic body size ($kg^{3/4}$)	0.335	0.331	0.319	0.332	0.336
	N Balance data (mg N/day/$kg^{3/4}$)				
Half-N period:					
N intake	70	73	74	71	71
Fecal N	38	37	37	29	31
Urinary N	133	124	123	106	108
N balance	— 101	— 88	— 87	— 65	— 68
Full-N period:					
N intake	141	138	145	141	139
Fecal N	37	31	31	29	30
Urinary N	145	129	130	124	97
N balance	— 40	— 22	— 16	— 11	+ 12
K'[1]	0.85 ± 0.04	1.01 ± 0.04	0.99 ± 0.04	0.75 ± 0.03	1.18 ± 0.06
NI_e[2]	189 ± 7	161 ± 3	162 ± 3	157 ± 3	129 ± 2

[1] K' is the slope of the line joining the Half-N and Full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

to adapt themselves more quickly and completely to the amino acid diet. At the end of 6 months of the experimental regime, however, both series of rats attained positive nitrogen balance and the computed values for NI_e are nearly identical (experiment V, tables 1 and 2). These different rates of adaptation in nitrogen balance must be taken into account when considering the effects of diminished histidine intake.

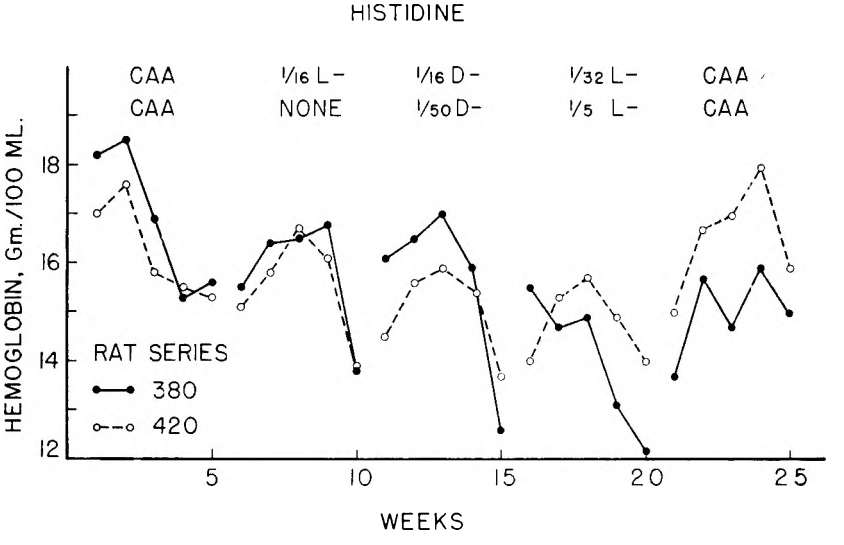

Fig. 1 In each 5-week feeding cycle shown in the figure, the first and second points represent the first and second week, respectively, of feeding the maintenance diet; the third point refers to the N-free diet; the 4th and 5th points were obtained after feeding the half-N and full-N amino acid diets, respectively.

The first and second lines refer to the experiments done with rats of series 380 and 420, respectively; CAA = complete amino acid mixture. Other designations = fraction of histidine in the amino acid mixture, as compared with CAA, and also the isomer used.

The results of the hemoglobin analyses are shown graphically in figure 1. The points obtained in any 5-week feeding cycle are joined by lines. The breaks are simply an aid in considering a single feeding cycle as a unit and do not represent any lapse of time between experiments. The number of animals represented by each point may be ascertained by reference to tables 1 and 2.

DISCUSSION

The essentiality of histidine for the efficient maintenance of nitrogen balance in the adult rat is most clearly demonstrated by the data in table 1. In experiments I and V the complete amino acid mixture contained the same proportion of histidine as is present in the proteins of whole egg. The animals fed these diets were in positive nitrogen balance. The computed requirements of nitrogen for equilibrium, NI_e, as usual, is appreciably smaller for experiment V than for experiment I. If histidine is removed from the amino acid mixture, as in experiment II, the nitrogen balance is decidedly negative and NI_e is significantly different ($P < 0.0001$) from the corresponding adjacent control value. It is impossible to assess the physiological significance of NI_e computed under these circumstances. The rats were not fed the histidine-free mixture in larger amounts to determine whether merely increasing the intake of nitrogen in the form of this mixture would compensate for the absence of histidine. Bothwell and Williams ('51) were able to maintain weanling rats in positive nitrogen balance for two weeks by force feeding a histidine-free diet of relatively high total nitrogen content.

The unnatural isomer of histidine can be utilized to improve nitrogen balance but the small amount fed in experiment III (table 1) was insufficient for the attainment of nitrogen equilibrium. The data of experiment IV show that positive nitrogen balance was achieved with only 1/5 as much L-histidine as was used in the complete amino acid mixture. NI_e for experiment IV would have been approximately 129 if the complete amino acid mixture had been fed; the actual value of 137 is only 6% greater and cannot be considered significantly different from the computed value for the complete amino acid mixture. Further evidence for the adequacy of the 1/5 L-histidine mixture is found in the constancy of urinary nitrogen output despite a doubling of nitrogen intake (compare half-N and full-N periods in experiment IV, table 1).

Table 2 shows the results of feeding histidine in amounts intermediate between 1/5 L-histidine, which is adequate, and 1/50 D-histidine, which is inadequate to achieve nitrogen equilibrium under these conditions. Values for NI_e in experiments II, III, and IV are nearly identical. On the valid assumption that NI_e for repeated trials with the complete amino mixture diminishes uniformly over a period of 6 months, the recorded values should be compared with what could be expected from such repetition. As computed for experiments II, III, and IV the values for NI_e are 161, 162, and 157, respectively (table 2); these should be compared with 174, 159 and 144, respectively, as expected values if the complete amino acid mixture had been fed in these experiments. According to this comparison 1/16 L- and 1/16 D-histidine are utilized as well as the complete amino acid mixture with possibly some advantage in favor of the natural isomer. Celander and Berg ('53) reported that the two optical isomers of histidine were equally effective for rat growth. The computed value of NI_e obtained in experiment IV is approximately 10% higher than expected and suggests that 1/32 L-histidine is inadequate to permit efficient utilization of the nitrogen of the diet.

A significant feature of the results is the high value of K′ which is the slope of the line joining half-N and full-N points when nitrogen balance is plotted against nitrogen intake. If $K' = 1.00$ it is obvious that the increment in nitrogen intake, represented by doubling the half-N intake, is completely utilized for the improvement of nitrogen balance. In experiment III, table 1 (1/50 D-histidine), K′ is significantly ($P < 0.001$) less than unity but still large enough to indicate fairly efficient usage of dietary nitrogen. Even in the absence of dietary histidine (experiment II, table 1), K′ is not diminished. These facts suggest that either the adult animal does not require preformed histidine for the utilization of dietary nitrogen, or any dietary deficit of histidine is made up from a readily available endogenous source of this amino acid.

Hemoglobin contains approximately 8% of histidine (Block and Bolling, '51) and might serve as an endogenous source of histidine. The average rat of 250 gm body weight has a blood volme of 19.2 ml containing 3.19 gm of hemoglobin or 16.1 gm/100 ml of blood (Drabkin, '50). The histidine content of the hemoglobin in a 250 gm rat is, therefore, approximately 225 mg. In experiment II, table 1, the average reduction in hemoglobin, resulting from the removal of histidine from the diet, was approximately 17% (from 16.7 to 13.9 Hb./100 ml). The total histidine contained in the hemoglobin that disappeared is 43 mg. If this histidine were available to meet metabolic needs the average supply from this source would have been 3 mg/day/250 gm rat over the two-week period. This is equivalent to 8.5 mg of histidine/day/$kg^{.74}$ and is greater than the intake of histidine in any of the experiments reported here, excepting the ones in which the complete amino acid mixture was fed.

The possibility of quickly drawing upon the histidine of hemoglobin, to meet acute metabolic needs for this amino acid, suggests that the apparent dietary requirement for histidine as determined by the nitrogen balance might be proportional to histidine intake. One might consider that the total reqirement could be met by the combined contribution from dietary and endogenous sources. For relatively short periods of observation the supplementing action of endogenous histidine might not be detected by the methods of nitrogen balance.

In table 3 the actual intake of histidine is compared with the computed requirements of dietary histidine for the attainment of nitrogen equilibrium. This comparison includes one experiment each with rats of series 300 and 340 which are not reported in tables 1 and 2. The histidine requirement is obtained by multiplying NI_e by the concentration of the amino acid being investigated. The amount of histidine fed is determined directly. The greatest intake of L-histidine was 6.9 mg/day/$kg^{.74}$ and the computed requirement is slightly less, reflecting the fact that these animals were in positive

nitrogen balance. The second greatest intake was 3.0 mg/day/kg$^{3/4}$ and the computed requirement precisely the same, indicating that these animals were in nitrogen equilibrium. For all lesser intakes of histidine, whether as L- or D-isomers, the computed requirements are only slightly greater than intakes. If the computed histidine requirement for nitrogen equilibrium is plotted against actual histidine intake, the 8 points experimentally determined all lie close to a straight line which passes through the origin with a slope of approximately 1.1.

TABLE 3

Apparent histidine requirement of adult rats

SERIES	AMINO ACID MIXTURE	NO. OF RATS	MG HISTIDINE/DAY/KG$^{3/4}$		
			Actual intake on Full-N	Computed requirement of isomers for N equilibrium	
				L-	D-
300	1/10 L-histidine [1]	8	3.0	3.0 ± 0.06 [2]	
340	1/15 L-histidine	12	2.5	3.2 ± 0.10	
380	1/16 L-histidine	14	2.2	2.5 ± 0.05	
	1/16 D-histidine	9	2.2		2.5 ± 0.05
	1/32 L-histidine	9	1.1	1.2 ± 0.03	
420	No histidine	13	0.0	0.0	0.0
	1/50 D-histidine	14	0.8		1.0 ± 0.04
	1/5 L-histidine	12	6.9	6.7 ± 0.15	

[1] Isomer and approximate amount of histidine as compared with "complete" amino acid mixture.

[2] Standard error of the mean.

The two series of rats represented in figure 1 exhibited similar fluctuations in hemoglobin concentration. The last point in each group of 5 is taken to represent the response to the previous two weeks of amino acid feeding. If this is a fair criterion then it is evident that the complete amino acid mixture is not only adequate for nitrogen equilibrium but also for maintenance of hemoglobin at normal levels. In series 380 the concentration of hemoglobin was 15.6 and 15.0 gm/100 ml in the initial and final complete amino acid (CAA) periods, respectively; in series 420 the corresponding values were 15.3 and 15.9 gm/100 ml. The average hemoglobin concentration

in the 22 rats of both series for the initial period of complete amino acid feeding (CAA in fig. 1; experiment 1 in tables 1 and 2) was 15.5 gm/100 ml of blood, precisely what it was 5 months later at the end of the final complete amino acid period. None of the fractional amounts of histidine was adequate to maintain hemoglobin at the control levels. These facts suggest that some concentration of histidine lying between that of the complete amino acid mixture and 1/5 of this amount (experiment IV, table 1) is required to maintain a normal hemoglobin content.

The discrepancy between adult rats and human beings with respect to histidine requirement may be due in part to the length of the feeding periods. A man lives approximately 25 times as long as a rat. Quite likely the feeding of a histidine-deficient diet for a week to a rat is not equivalent to feeding the same kind of a diet to a man for the same length of time. To get comparable results it may be necessary to feed the human subject for many weeks or even months.

It is interesting to note that in one human subject, for whom detailed data are available (Rose et al., '51), the elimination of histidine from the diet resulted in a drop of hemoglobin from 15.2 to 14.7 gm/100 ml and a reduction in the nitrogen balance from + 1.37 to + 0.96 gm/day for 8 days on a complete amino acid mixture and 8 days on a mixture devoid of histidine, respectively. This subject weighed 60.0 kg and probably contained 4.8 l of blood. Providing blood volume remained constant, a drop of hemoglobin of 0.5 gm /100 ml over a period of 8 days could release approximately 240 mg of histidine/day or 11.1/mg/day/kg$^{3/4}$. According to table 3, this is considerably more than is required for the attainment of positive nitrogen balance in the rat but probably not enough for the maintenance of a constant hemoglobin concentration.

SUMMARY

The nitrogen balance of adult, male Wistar strain rats was adversely affected by severe reduction of histidine in the amino acid mixture that supplied all of the dietary nitrogen.

Reduction of histidine resulted in a reduction of hemoglobin of as much as 20%. Certain intakes of histidine which were adequate for the attainment of positive nitrogen balance were inadequate for the maintenance of a normal hemoglobin concentration. It is suggested that histidine requirements are met normally by ingestion; temporary dietary shortages may be made good by the degradation of hemoglobin to supply relatively large quantities of histidine.

No obvious difference was noted between the optical isomers of histidine in the maintenance either of nitrogen balance or hemoglobin concentration.

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THE QUANTITATIVE LEUCINE REQUIREMENT OF THE SUCKLING PIG[1]

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ONE FIGURE

(Received for publication February 8, 1954)

Quantitative studies on the amino acid requirements of weanling pigs have been reported for tryptophan (Shelton et al., '51a), lysine (Brinegar et al., '50b; Shelton et al., '51b), isoleucine (Brinegar et al., '50a), methionine (Shelton et al., '51c; Curtin et al., '52b), valine (Jackson et al., '53) and threonine (Beeson et al., '53). The threonine requirement has also been studied for the suckling pig (Sewell et al., '53).

The indispensable nature of leucine for growing swine has been demonstrated (Mertz et al., '52). As nothing was known regarding amounts, this study was undertaken in an attempt to gain information on the quantitative L-leucine requirement of the suckling pig.

PROCEDURE

Two-day-old pigs were employed in two growth studies. These pigs were assigned to 5 groups according to litter and weight, and the groups randomly allotted to the dietary treatments. Each litter was equally represented in every dietary treatment. The pigs were fed a semi-synthetic "milk" diet, containing casein as the protein source, for the first two or three days to acquaint them with the method of bottle feeding

[1] These studies were supported largely through a grant from the Herman Frasch Foundation.

used in these studies. Then they were shifted to the experimental diets for a 7-day adjustment feeding period. Growth and efficiency of feed utilization were then measured over a 21-day experimental period. The pigs were individually fed essentially ad libitum.

TABLE 1

Composition of basal diet

INGREDIENTS	AMOUNT	
	%	*gm/kg of "milk"*
Casein	12.76	17.10
Amino acid mixture [1]	4.55	6.10
Ammonium citrate (dibasic)	9.58	12.84
Cerelose	38.53	51.63
Lard	25.86	34.65
Tween [2]	.26	.35
Minerals [3]	8.46	11.33
Vitamins [4]	+	+
Aureomycin [5]	+	+

[1] Contained the following crystalline amino acids: L-arginine · HCl, 17.66%; L-histidine · HCl · H_2O, 3.72%; DL-isoleucine, 7.37%; L-lysine · HCl, 12.30%; DL-methionine, 9.17%; DL-phenylalanine, 15.68%; DL-threonine, 22.24%; DL-valine, 8.78%; and DL-tryptophan, 3.07%.

[2] Composed of Tween 80, 50%; Tween 60, 50%. Supplied by Atlas Powder Company, Wilmington, Delaware.

[3] Salt mixture of Sewell et al. ('52).

[4] Vitamins added to each kilogram of liquid diet included: thiamine hydrochloride, 0.65 mg; riboflavin, 0.65 mg; niacin, 2.50 mg; inositol, 26.0 mg; choline, 260.0 mg; PABA, 2.60 mg; folic acid, 0.052 mg; pyridoxine hydrochloride, 0.65 mg; calcium pantothenate, 1.30 mg; alpha tocopherol, 1.0 mg; menadione, 0.28 mg; vitamin B_{12}, 10.0 μg; vitamin A, 2,000 I.U., and vitamin D, 200 I.U.

[5] Contained 150 mg/kg of dry matter. Supplied by Lederle Laboratories, Pearl River, New York.

The basal diet, shown in table 1, included casein as the base protein. Crystalline amino acids were used to raise the dietary content of each essential amino acid, other than leucine, to a level considered adequate for good growth. L-leucine was added to 4 of the diets at various levels to give 5 experimental diets of different leucine content. Ammonium citrate (dibasic) provided the balance of the dietary nitrogen,

equalizing all diets to contain sufficient nitrogen to be equivalent to 25% of the air-dry diet as protein.

In the second experiment body composition data were obtained in addition to growth and feed efficiency data. Three pigs, one from each litter, were slaughtered at the time the animals were placed on the experimental diets. At the close of the experimental feeding period all of the animals were killed for analysis, care being taken to save all the blood. Each animal, after removal of intestinal and stomach contents, was sealed in a polyethylene bag to conserve moisture, and frozen. The carcasses were chopped into small chunks with a meat cleaver and passed three times through a meat grinder, the blood being added back at this grinding stage. The grinding was done before the meat had time to thaw, keeping loss of moisture and fat at a minimum. A large aliquot of each ground carcass was then placed in a shallow pan and refrozen. These aliquots were dried from the frozen state in a vacuum desiccator. Analyses for nitrogen, ash, ether extract and moisture were carried out on the dried samples.

RESULTS AND DISCUSSION

Experiment 1

Originally 20 Yorkshire and 5 Yorkshire by Berkshire crossbred pigs were started on the experimental diets. Gastrointestinal disturbances were encountered during the 7-day transitional period, and one litter of 5 pigs was discontinued from the study at the end of this period. Of those continued on the study, one pig from the litter of 5 Yorkshire by Berkshire crossbred pigs died 8 days later from a cause diagnosed as intestinal obstruction with perforation. Three of the 4 remaining pigs of this litter did not perform normally during the experiment, and all 4 of them failed to make normal gains for the next month after the trial was completed. For these reasons, data from these pigs were not included in the analysis of the data. Thus, in all, two pigs were removed from each experimental treatment. The data reported here were

TABLE 2

Average data for pigs receiving various levels of leucine (experiment 1)

	SUPPLEMENTAL LEVEL OF L-LEUCINE				
	None	0.25	0.50	0.75	1.00
Total L-leucine (% of diet)	1.00	1.25	1.50	1.75	2.00
Total L-leucine (% of protein)	4.0	5.0	6.0	7.0	8.0
Number of pigs	3	3	3	3	3
Initial weight (kg)	2.14	2.39	2.23	2.26	2.47
Daily gain (gm)	136	169	173	193	195
Dry matter intake/kg gain (kg)	2.07	1.57	1.55	1.40	1.40
Plasma protein [1]	5.68	6.04	5.70	5.77	6.00

[1] Based on two pigs per lot.

TABLE 3

Average data for pigs receiving various levels of leucine (experiment 2)

	SUPPLEMENTAL LEVEL OF L-LEUCINE				
	None	0.25	0.50	0.75	1.00
Total L-leucine (% of diet)	1.00	1.25	1.50	1.75	2.00
Total L-leucine (% of protein)	4.0	5.0	6.0	7.0	8.0
Number of pigs	3	3	3	2 [1]	3
Initial weight (kg)	2.89	2.94	2.94	3.04	2.91
Daily gain (gm)	171	218	194	206	210
Dry matter intake/kg gain (kg)	1.57	1.24	1.40	1.28	1.27

[1] One pig died, see text.

from 15 Yorkshire pigs, three pigs per experimental treatment.

The data, presented in table 2, show the greatest average daily gains for the pigs receiving 1.75 and 2.00% of total leucine in the diet. The average daily gains for those receiving 1.25 and 1.50% of leucine were somewhat less, while those in the basal lot showed considerably lower gains. Feed efficiency followed a similar pattern. Analysis for plasma protein of two pigs in each lot failed to show any consistent differences between diets.

Experiment 2

The first experiment indicated that the level of L-leucine required for maximum growth and feed efficiency of a suckling pig fed a diet containing 25% of protein is probably not higher than 1.75% of the air-dry diet. However, the next two lower levels of leucine, 1.50 and 1.25% of the diet, gave rates of gain which were not much lower than for the 1.75% level, and some of the pigs on these two lower levels grew about as rapidly as those on the higher levels. With these indications that the requirement might be lower than 1.75% of the diet, it seemed desirable to repeat the first experiment.

Eighteen two-day-old Yorkshire pigs (6 pigs each from three litters) were used in the second experiment. One pig from each litter was killed for body composition analyses at the time the experimental diets were begun, the remaining 15 pigs being handled the same as in experiment 1. One pig died during the 7-day adjustment period from gastro-intestinal disorders. None of the other pigs became sick. The growth data, presented in table 3, show the greatest average daily gains for the pigs receiving 1.25% of L-leucine in the diet. The gains for the next higher level of leucine, however, were considerably lower (194 gm per day as compared to 218 gm per day). Just why this should occur is difficult to interpret. One pig on this diet accounted for a considerable part of this low value, but none of the pigs receiving 1.50% of leucine

TABLE 4

Average data for pigs receiving various levels of leucine (experiments 1 and 2 combined)

	SUPPLEMENTAL LEVEL OF L-LEUCINE				
	None	0.25	0.50	0.75	1.00
Total L-leucine (% of diet)	1.00	1.25	1.50	1.75	2.00
Total L-leucine (% of protein)	4.0	5.0	6.0	7.0	8.0
Number of pigs	6	6	6	5 [1]	6
Initial weight (kg)	2.52	2.66	2.58	2.57	2.69
Daily gain (gm)	153	194	183	199	203
Dry matter intake/kg gain (kg)	1.82	1.41	1.47	1.36	1.33

[1] See table 3.

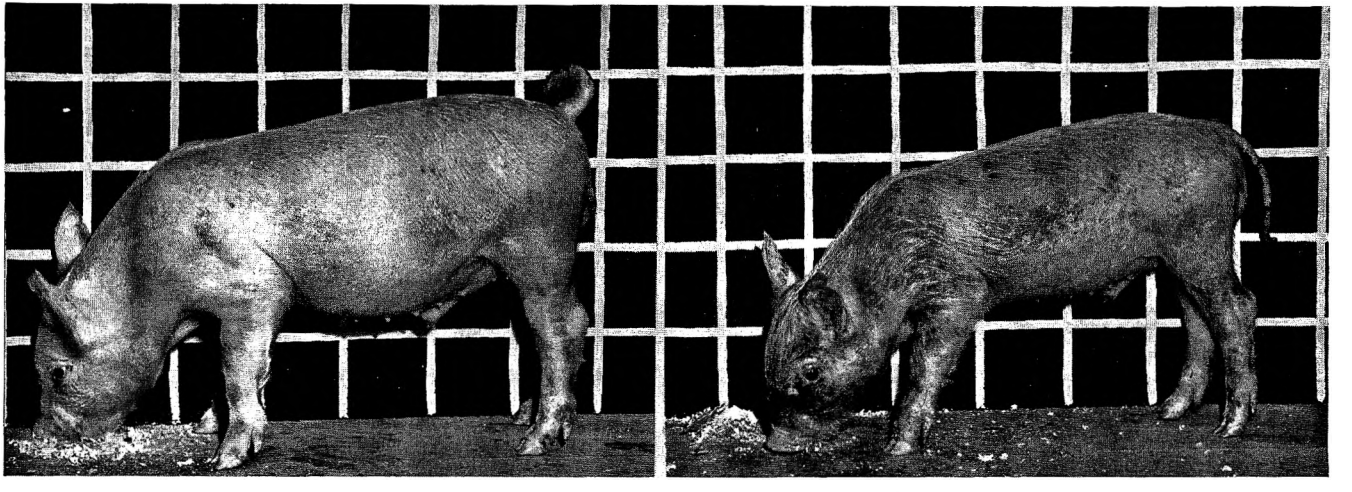

Fig. 1 The pig on the right was typical of those receiving 1.00% of the air-dry diet as L-leucine. The pig on the left was typical of those receiving 1.25% or more of the air-dry diet as L-leucine.

gained as well as the slowest gaining pig receiving 1.25% of L-leucine in the diet. The pigs receiving the two highest levels of leucine (1.75 and 2.00% of the diet) gained more rapidly than the pigs receiving the 1.50% level, but not as rapidly as those receiving the 1.25% level of L-leucine.

When the data from the two experiments are combined, as presented in table 4, it is readily apparent that the lowest level of leucine fed (1.00% of the diet) is inadequate for good growth. Daily gains for the pigs fed this diet are significantly lower ($P \leqq 0.01$) than when any of the other 4 diets are used. Differences in daily gains between the pigs receiving the diets containing 1.25, 1.50, 1.75 and 2.00% of L-leucine did not prove to be statistically significant, as measured by least significant difference analysis. Figure 1 shows a typical pig from the basal lot as compared to one typical of those fed higher levels of leucine.

The carcass composition study of the second experiment showed no very striking differences in the gross composition of the pigs for the various experimental treatments. The pigs receiving the lowest level of leucine tended to be somewhat lower in fat content, perhaps a reflection of their slower rate of gain. Using the composition values obtained from the three pigs slaughtered at the start of the experiment, it was possible to calculate the approximate storage of protein, fat and ash which was made by each pig during the growing period. A lower storage of all three was obtained for the pigs receiving only 1.00% of leucine in the diet, while at the next higher level (1.25% of the diet) there was the greatest amount of storage.

All of the data (growth, feed efficiency and storage of tissue protein) tend to show that 1.00% of leucine in the diet did not support optimum growth of the young pigs. It appears that the leucine requirement of baby pigs is more than 1.00 but not more than 1.25% of the air-dry diet containing the equivalent of 25% of protein. This corresponds to not more than 5.0% of the dietary protein as L-leucine, which is in good agreement with the estimated requirement of 4.6% of

the dietary protein as leucine based on carcass analyses (Curtin et al., '52a), or 0.91% of a diet containing 20% of protein (Williams et al., '54). This requirement is, however, considerably below the requirement of the chick for L-leucine, which is placed at 7.0% of the dietary protein (Almquist, '52).

SUMMARY

Two experiments were conducted to study the L-leucine requirement of suckling pigs fed simulated "milk" diets containing sufficient nitrogen to be equivalent to 25% of the air-dry diet as protein. The dietary nitrogen was supplied by casein, amino acids and ammonium citrate (dibasic). Growth and feed efficiency were used as criteria in studying the effects of adding the various levels of L-leucine in both experiments. In addition, carcass storage of protein, fat and ash were determined for all pigs in the second experiment.

The data indicate that the L-leucine requirement is more than 1.00%, but not more than 1.25% of the diet. This is equivalent to not more than 5.0% of the dietary protein.

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EFFECT OF THE LEVEL OF ANIMAL FAT IN THE DIET ON THE MAINTENANCE, REPRODUCTION AND LACTATION PERFORMANCE OF DOGS[1]

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ONE FIGURE

(Received for publication February 22, 1954)

In experiments reported previously (Siedler and Schweigert, '52), the effects of added stabilized animal fat in the diet on the rate of growth, general health and appearance, and food utilization of weanling Cocker Spaniel pups were studied. The results showed that the added fat was well utilized by the growing pups. It was of importance to extend these studies to measurements of the reproduction and lactation performances when animal fats were added to the ration. Deuel et al. ('47) found that the reproduction and lactation capacity of rats fed a semi-purified diet was increased by the addition of fat to the diet. Other workers (Nelson and Evans, '47, '47a) have reported adverse effects on the reproduction and lactation performances of rats fed a purified diet to which various levels of fat had been added. It may be concluded from these and other studies that the effects observed with additions of fat to the ration are closely related to the adequacy of the ra-

[1] Journal Paper No. 85, American Meat Institute Foundation.

A report of work done under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act. The contract is being supervised by the Eastern Regional Research Laboratory of the Bureau of Agricultural and Industrial Chemistry.

tion in other nutrients. Dilution of protein, minerals and vitamins by added fat may result in sub-optimal levels of these nutrients in the ration, and an inferior performance of the animals would be observed.

In the present studies, observations have been made on the maintenance, reproduction and lactation performance of registered Cocker Spaniel females which have been fed practical type rations, either with or without stabilized fat added.

EXPERIMENTAL

Registered Cocker Spaniel females were maintained in the same experimental feeding groups as reported previously for the growth studies (Siedler and Schweigert, '52). The experimental rations fed consisted of a basal ration composed of ingredients commonly used in dry meals either with or without 4 or 8% stabilized choice white grease (rendered pork fat) or 18% sucrose added at the expense of the ration. The sucrose was fed at a level to approximate the crude calories contributed by 8% fat. The fat was stabilized with an antioxidant mixture, as reported previously.

The dogs were weighed at weekly intervals throughout the experiment. Food intake was recorded for a three-week period when the dogs were approximately 6–7 months of age (prior to breeding) to determine the amount of the various rations needed for maintenance of body weight.

The dogs were examined bi-weekly for estrus, and at estrus were bred to one of the two studs retained from the previous experiments. The bitches were bred to the alternate stud at the estrus following birth of the first litter, whenever possible, but due to the failure of certain females to conceive after breeding or failure of females to come into estrus, it was impossible to achieve completely the objective of two litters per female during the two-year experimental period.

After parturition, the weight of the newborn pups was taken as a group. The pups were weighed individually from the first through the 7th week of age. Weaning of the pups

was begun at 5 weeks of age, and the pups were totally weaned at the end of the 6th week. The pups were fed the mother's diet from the 6th through 7th week of age, when they were removed from the experiment.

Food intakes of the lactating bitches were recorded through the first 4 weeks of lactation. Food efficiencies were then calculated from the food intakes and the weight change of the bitch and pups. Caloric efficiencies were also calculated from

TABLE 1

Maintenance data of females fed graded levels of fat
(3-week period)

RATION FED	NO. DOGS	AV. GAIN/ DOG/GROUP/ WK.	FOOD CONSUMPTION PER KG BODY WEIGHT/WK.	CALORIC CONSUMPTION PER KG BODY WEIGHT/WK.
		gm	*gm*	
Basal	7	7	222	792
Basal + 4% fat	7	107	218	824
Basal + 8% fat	6	20	190	755
Basal + 18% sucrose	5	30	205	747

these data and the caloric composition of the rations. The results are expressed as the grams gained by both the lactating bitch and the pups per unit of feed or caloric intake.

RESULTS AND DISCUSSION

The results obtained from the maintenance studies are shown in table 1. These results indicate that the maintenance performance of the dogs fed the basal ration plus 4 or 8% fat or 18% sucrose was equal to or better than that for dogs fed the basal ration. Since the group fed 4% added fat gained considerably more than the other groups during this period. it is difficult to make direct comparisons.

The reproduction data (table 2) show that the performance of bitches fed the basal ration plus 4% added fat was somewhat better than that for bitches fed the basal ration. The number of pups dead 24 hours after birth from the bitches fed the 4% added fat or the basal ration was negligible, and the average weights of the newborn pups were excellent from both groups.

The reproduction performances of the bitches fed the basal ration plus 8% fat indicate that dilution of other nutrients

TABLE 2

Reproduction and lactation performance of dogs fed different levels of fat

RATION FED		BASAL	BASAL 4% FAT	BASAL 8% FAT	BASAL 18% SUCROSE
Average weight at breeding, kg		8.4 ±1.2 [1]	9.2 ±1.5 [1]	8.0 ±0.5 [1]	7.6 ±1.4 [1]
Average weight gain pregnancy, kg		1.77±0.67	2.27±0.65	2.04±0.14	2.05±0.59
Average weight of newborn pups, gm		228	248	192	184
No. litters per group		11	11	8	5
Total no. pups born		54	51	49	27
Total no. pups dead 24 hrs.		4	2	9	8
Total no. pups weaned		43	47	38	14
Average weight pups 4 weeks, gm	♂	984±198	1,080±285	990±182	756±250
	♀	954±230	1,042±265	897± 92	967±140
Average weight pups 6 weeks, gm	♂	1,539±385	1,753±275	1,431±390	1,269±485
	♀	1,342±363	1,593±315	1,278±245	1,367±410
Average food efficiency [2]		0.25±0.06	0.30±0.07	0.33±0.07	0.22±0.06
Average caloric efficiency [3]		7.1 ±1.7	7.9 ±1.8	8.3 ±1.7	6.05±1.6

[1] Mean and standard error.

[2] Gram gain per gram of food consumed (see text).

[3] Gram gain per 100 crude calories consumed (see text).

may have become critical under this particular stress as indicated by the percentage of pups dead 24 hours after birth and the average size of the pups at birth as compared to those in the group fed the basal ration or basal plus 4% fat.[2] However, the average number of pups per litter was considerably larger than the average of the other groups tested, and this may have been a factor in these results. The reproduction capacity of the group fed the basal ration plus 18% sucrose was inferior to that observed for the other groups. Mortality of the newborn pups was quite high; however, sufficient data are not available to evaluate this group critically.

The lactation performances, including data on the number of pups weaned and the weights of the pups at 4 and 6 weeks are presented in table 2, and the rates of growth of the male and female pups from the first through the 7th week are shown in figure 1. The pups from the bitches fed 4% fat showed an increased rate of gain over those in any of the other groups. Those from the bitches fed 8% fat or 18% sucrose showed a slightly lower rate of gain than those whose mothers were fed the basal ration. One group of pups in the sucrose group was removed from the experiment at 6 weeks of age; therefore none of the data are presented for this group from the 6th to the 7th week. No differences were observed in the general appearance, health, and maintenance of body weight among the groups of females during lactation.

The differences in the rates of gain of the pups from bitches fed 4% added fat, as compared to those fed the basal ration, were evaluated statistically in view of the marked change in the slope of the two curves (fig. 1) from the 5th through the 7th week. A summary of this evaluation is shown in table 3. The rates of gain of pups from the second litters for the group fed 4% added fat were significantly greater

[2] A paper by Campbell and Phillips (Southwestern Vet., Winter Issue, p. 173, 1953) has come to our attention since submission of our manuscript in which these workers also observed an inferior performance of dogs fed rations containing higher levels of fat. From their work, the dilution of the ration by the addition of fat was corrected by methionine supplementation, indicating that the protein (methionine) level in the ration became limiting.

than those for pups from the basal group. The rate of gain of the second litter whose mother was fed the basal ration decreased as compared to the gain for pups from the first litter. The gains of the second litter from bitches fed 4% added fat were also greater than those from the first litter.

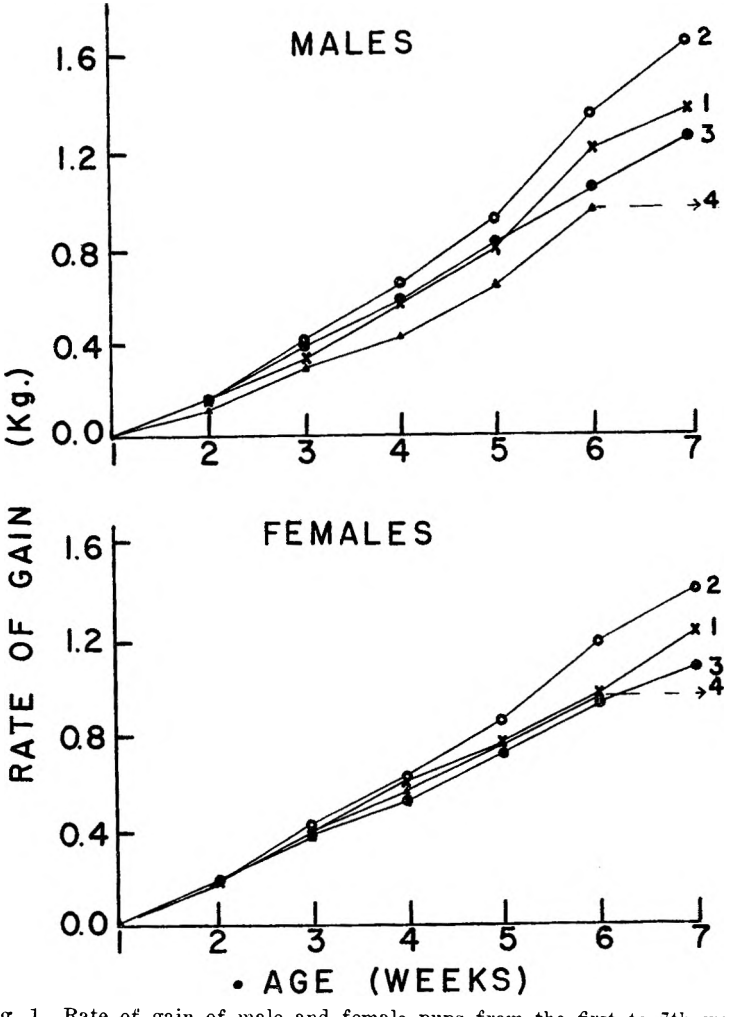

Fig. 1 Rate of gain of male and female pups from the first to 7th week of age. Curve no. 1 — Basal ration; Curve no. 2 — Basal + 4% animal fat; Curve no. 3 — Basal + 8% animal fat; Curve no. 4 — Basal + 18% sucrose.

The average food and caloric efficiency results from the lactating bitches are shown in table 2. These data show excellent calorie utilization of the fat during lactation whereas the calories added as sucrose were not as efficiently utilized as the calories of the basal ration or the rations containing 4 or 8% added fat. These findings indicate that the addition of fat may have enhanced utilization of the crude calories of the basal ration during lactation.

TABLE 3

Rates of gain of male and female pups from 5th–7th week of age

RATION FED BITCH	LITTER NO.	MEAN GAIN ♂	MEAN GAIN ♀	OVER-ALL MEAN [1]	ADJUSTED MEAN [2]
		gm	*gm*	*gm*	*gm*
Basal	I	633	481	504	467
	II	398	360		
Basal 4% fat	I	626	482	586	590
	II	717	538		
P value				less than .15	less than .05

[1] Adjusted for sex variation.

[2] Adjusted for sex and litter variation.

SUMMARY

The maintenance, reproduction and lactation performances of Cocker Spaniel dogs fed since weaning a basal ration either with or without the addition of 4 or 8% fat (antioxidant stabilized choice white grease) or 18% sucrose were observed. The addition of 4 or 8% fat or 18% sucrose increased the efficiency of the ration for maintenance of the females prior to breeding.

The reproduction performance of bitches fed the basal ration plus 4% added fat was somewhat better than the performance of bitches fed the basal ration. The addition of 8% fat to the basal ration appeared to reduce the reproductive capacity of the bitches as judged by the number of pups dead 24 hours after birth, and the weight of the pups at birth, al-

though this result may be due in part to the larger number of pups born per litter for this group. The reproduction capacity of bitches fed 18% added sucrose was poor.

The average rate of gain of pups from bitches fed 4% added fat was higher than any of the other groups tested. This difference was primarily due to greater weight gains in the second litter for this group as compared to those for the basal ration, and was statistically significant. The average rate of gain of pups from bitches fed 8% added fat or 18% added sucrose was slightly less than that for pups from bitches fed the basal ration.

The addition of 4 or 8% fat to the basal ration increased the average food and calorie efficiencies of the lactating bitches when compared to the basal ration.

On the basis of all criteria used, the results obtained were excellent when 4% animal fat was added to the basal diet.

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EXPERIMENTAL RAT CARIES

IV. EFFECT OF A NATURAL SALT MIXTURE ON THE CARIES-CONDUCIVENESS OF AN OTHERWISE PURIFIED DIET [1]

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FIVE FIGURES

(Received for publication January 9, 1954)

Previous observations in this series (Sognnaes, '48, '49) have shown that rats raised on a laboratory stock diet during the period of tooth development were much more caries-resistant than rats raised on a purified diet adequate in known essential nutrients. Since all animals were fed an identical caries-producing diet after tooth eruption, the present study was designed to determine whether the apparent developmental effect could be ascribed to differences in the mineral fractions of the two diets.

EXPERIMENTAL CONDITIONS

The purified caries-producing diet, ration 100, which was used as the control diet in the present study, has been described in detail elsewhere (Shaw, '47). The experimental diet was prepared by replacing the reagent grade salt mixture of the purified ration 100 by an equivalent amount of the mineral ash obtained from the natural stock diet, Purina laboratory chow. A large quantity of this mineral ash was

[1] This investigation, which has extended over several years, has been supported in part by grants from various sources: The Division of Research Grants and Fellowships, U. S. Public Health Service, The Eugene Higgins Trust, The Nutrition Foundation, Inc., New York, and The Sugar Research Foundation, Inc., New York.

prepared from laboratory chow in the following manner: placed in large porcelain trays, the pellets were preheated for 24 hours in an oven at 130°C. whereupon the trays were transferred to a muffle furnace; the temperature was then raised to approximately 550°C. (1,022°F.) and maintained for an additional 24 hours at which time ashing appeared to be complete. By this procedure a recovery of about 7% ash was achieved. The resulting ash was used in the ration directly without any chemical treatment to render it more soluble. Preliminary nutritional experiments had shown the ability of the laboratory rat to use this mixture as a source of the essential minerals.

A comparison between the composition of the purified diet and the laboratory chow has been reported before (Sognnaes, '48). Among other differences it was pointed out that the fluoride content of the purified diet was 2.5 p.p.m. as compared to 21 p.p.m. in the stock diet. Ashing of the stock diet resulted in an appreciable loss of fluorine. Thus the experimental diet into which the natural ash mixture was incorporated at a level of 4% contained 6 p.p.m. of fluoride. From other studies in the literature, it was not possible to determine with any degree of certainty whether these levels of fluoride ingestion during tooth development were of importance in the rat. Experiments to explore the effectiveness of supplements of 6 and of 25 p.p.m. fluoride have been conducted and are reported separately (Shaw and Sognnaes, '54).

The present study covers observations on a total of 239 Norway rats of the Long-Evans strain, divided into two major groups of 110 intact animals and 129 desalivated animals. In the desalivated group all major salivary glands were removed at the time of weaning in order to determine if this additional caries-conducive condition would modify whatever effect was obtained by the ash ration. Within each group of normal and desalivated animals a comparison was made between the caries-susceptibility of rats subsisting throughout on the purified ration 100 (control) and of rats

subsisting on the ash ration (experimental) either during tooth development alone or throughout the experimental period.

The details were as follows: at the beginning of the experiment, an appropriate number of carefully selected adult female rats were maintained on purified ration 100 prior to and during pregnancy and lactation. The first litters raised on this dietary regimen were fed the same ration throughout the entire caries evaluation period (see above). After weaning of the first litter, the above adult females were bred again. The next litters were transferred to the experimental

TABLE 1

Summary table showing the overall response of normal and desalivated rats to the ash-supplemented purified ration

CONDITION OF ANIMALS	NO. OF RATS	DIETARY HISTORY		AVERAGE NUMBER OF CARICUS LESIONS
		Pregnancy and lactation	Post-weaning	
	47	Basic R-100	Basic R-100	6.2
Normal	50	Ash R-100	Basic R-100	4.2
	13	Ash R-100	Ash R-100	1.4
	45	Basic R-100	Basic R-100	21.0
Desalivated	61	Ash R-100	Basic R-100	23.2
	23	Ash R-100	Ash R-100	21.2

ash diet at time of birth and maintained thereon until weaning when they were transferred to the purified ration. Meanwhile, the mothers continued on the ash diet through a third complete reproductive cycle. Thus the third litters were raised on the ash diet both during gestation and lactation after which they were similarly transferred to the purified control diet. The mothers were maintained on the ash diet for a 4th complete reproductive cycle and this time the 4th litters were maintained on the ash diet for the full caries evaluation period.

At the termination of the experimental period the litters were sacrificed for evaluation of caries and histologic examination of their jaws and teeth.

EXPERIMENTAL OBSERVATIONS

Table 1 lists parallel observations made in normal and desalivated rats according to the main experimental grouping of the animals.

In the normal animals, there was observed a consistent trend towards reduction in caries when the ash from the natural stock diet was being used in place of the usual salt mixture in the otherwise purified ration. Within this group

TABLE 2

Effect of a natural salt mixture on experimental rat caries

FAMILY RELATIONSHIP	DIETARY HISTORY			AVER. NO. OF CARIOUS MOLARS	AVER. NO. OF CARIOUS LESIONS
	Pregnancy	Lactation	Post-weaning		
Mother [1]	Natural diet	Natural diet	Basic R-100	0	0
1st litter (#137–142)	Basic R-100	Basic R-100	Basic R-100	4.1	8.3
2nd litter (#160–168)	Basic R-100	Ash R-100	Basic R-100	4.0	5.8
3rd litter (#200–206)	Ash R-100	Ash R-100	Basic R-100	3.0	3.5
4th litter (#231–235)	Ash R-100	Ash R-100	Ash R-100	0.8	1.0

[1] Maintained on Basic R-100 diet for first 10 months, then on ash diet for 10 months (i.e., after birth of second litter).

Each litter was sacrificed 7 months after birth.

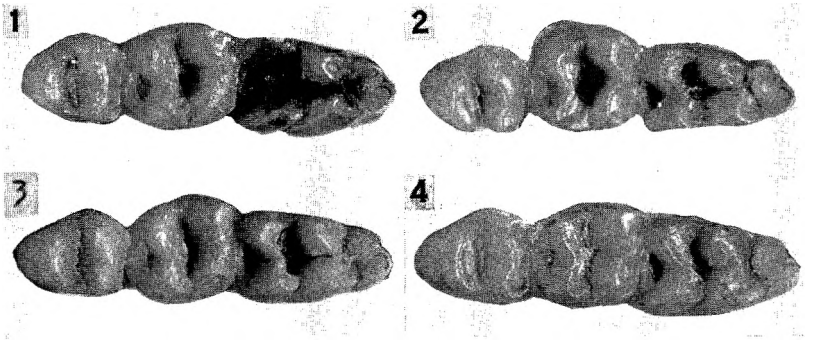
the most comparable series of successive litters from identical parenthood has been selected as those most fully justifying statistical analysis. Thus the data presented in table 2 may be considered the most ideally controlled experiment done to date on this particular problem. The mother of this group of animals belonged to a litter which had been raised on the stock diet and transferred to the purified diet for a period of 10 months after eruption of the teeth. While maintained on the purified diet, she bore her first litter of 6 animals. These rats were maintained on the purified diet until they

were 7 months of age at which time they had an average of 8.3 carious lesions. After the second litter was born, the mother was transferred to the ash diet and maintained thereon for the remaining 10 months of the experiment. The second litter, 9 animals, was transferred to the purified diet at weaning time and kept on that diet for 7 months at which time they showed an average of 5.8 carious lesions. The third litter was conceived, born and reared while the mother was being maintained on the ash diet; but again the offspring, 7 animals, were transferred to the basic purified diet at the time of weaning and, when 7 months old, the third litter had an average of 3.5 carious lesions. The 4th and final litter, 5 animals, had the same prenatal and neonatal history but was continued on the ash diet for the post-weaning period. At 7 months of age this litter had an average of one carious lesion per animal and two of the animals were caries-free. The mother whose teeth developed on the natural stock diet as such remained caries-free throughout.

The critical ratios [2] between the various litters were as follows: between the first and second litter, 3.1; between the first and third litter, 6.9; between the first and 4th litter, 10.4. In another experimental series in which the successive litters were the offspring of closely related but not identical parents the average number of carious lesions was 5.8 in the first litter, 3.3 in the second, 1.6 in the third and 0.8 in the 4th. Subjecting these latter data to statistical analysis, a critical ratio of 2.5 was found between the first and second litter, 4.9 between the first and third and 6.3 between the first and 4th.

[2] The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance, i.e., at a level of 5%; when the ratio is 3.0 or higher, the difference is highly significant, i.e., at a level of 0.3%. (Dunning, J. M., 1950, Variability in dental caries experience and its implication upon sample size. J. Dent. Res., *29:* 541.)

Figures 1 to 4 illustrate jaw segments of animals representing each of the 4 litters described in table 1. Figure 1, representing the first litter, is from a rat with 8 carious lesions, the average for the group being 8.3; figure 2, representing the second litter, is from a rat with 5 carious lesions, the average for the group being 5.8; figure 3, representing the third litter, is from a rat with three carious lesions, the average for the group being 3.5; and figure 4, representing



Figs. 1 to 4 Caries reduction obtained by use of the natural ash mixture in the otherwise purified diet throughout the full experimental cycle (mandibular jaw segments from animals of the litters shown in table 2). × 12.

Fig. 1 From first litter, purified diet.
Fig. 2 From second litter, ash diet — lactation period.
Fig. 3 From third litter, ash diet — prenatal and lactation.
Fig. 4 From 4th litter, ash diet.

the 4th litter, is from a rat with two carious lesions, the average for the group being one.

The rate of growth was compared between animals subsisting on the basic purified diet and those receiving the ash supplement. No significant difference in growth could be observed (fig. 5). Thus the factor or factors in the ash responsible for the difference in caries does not appear to be a growth factor.

The caries-conducive conditions existing in desalivated animals resulted in a frequency of caries which was several times that of intact animals raised on a comparable diet. The

change in the mineral composition of the diet whether introduced before or after tooth eruption was insufficient to influence the caries incidence of the animals under the penalty of desalivation in which the caries-producing factors appear to be overwhelming.

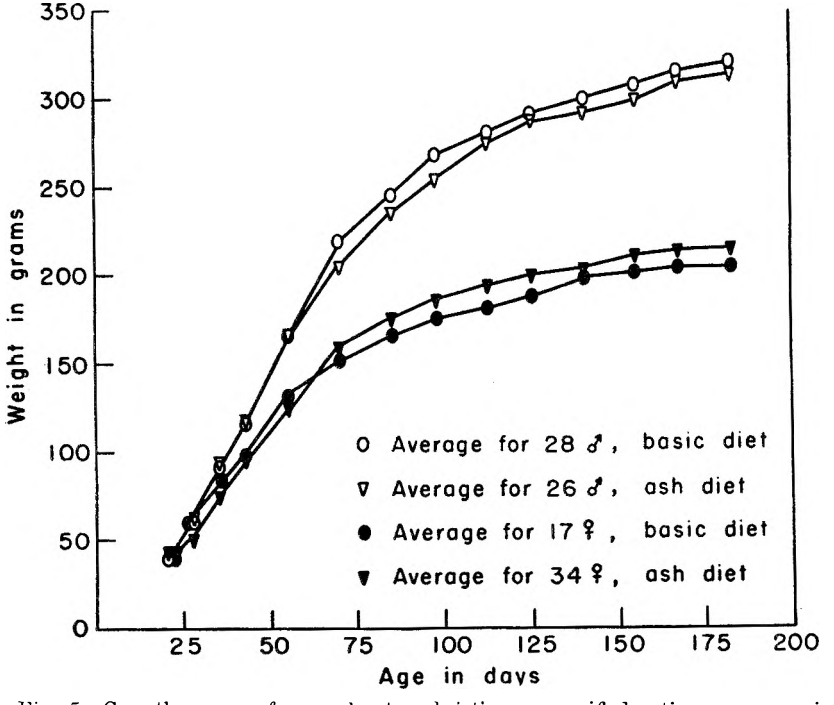


Fig. 5 Growth curves of normal rats subsisting on purified ration versus purified ration containing a natural ash mixture.

HISTOLOGIC EXAMINATION

Representative jaw segments from each experimental group were prepared for microscopic examination. Animals from all groups showed essentially normal development of the calcified structures of the jaws and teeth. In addition, it was observed that in response to attrition and initial caries, there was very favorable tissue response in the form of secondary dentin along the pulpal wall underlying the external injury.

It is apparent, therefore, that the calcifying properties of the two diets, in terms of minerals and vitamins which are essential for development and calcification, were normal.

However, the progress of caries in animals maintained throughout on the purified diet was not halted by the barrier of secondary dentin. The process proceeded into the thick wall of underlying dentin and, in some cases, progressed even into the root canals with bacterial invasion of the dentinal tubules characteristic of dental decay. In the experimental group receiving the ash diet during the period of tooth development, the progress of the lesions appeared to be much slower as though the tooth substance responded in a different manner. Here in some cases the pulp chambers were filled with secondary dentin. Yet there was extensive deposition of bacterial plaques in occlusal fissures of the teeth as well as between the teeth.

Generally speaking, it must be concluded that the microscopic examination of the teeth failed to reveal any significant structural differences attributable to a difference in the development of the teeth formed on the ash diet as compared to those formed on the basic purified diet. Thus there were no microscopically visible landmarks suggesting inadequate or faulty calcification. In explanation of the observed variations in caries susceptibility, it is believed, therefore, that some other structural difference may have been present which, although not visible by ordinary microscopic examination, may have been reflected either in the submicroscopic structure or in the microchemical composition of the tooth substance.

COMMENTS

At the outset, the purpose of the above experiment was to determine whether the total caries protection previously observed in rats raised on a natural stock diet (Sognnaes, '48, '49) might have resulted from factors present in the mineral fraction of that diet. Our observations indicate that *total* caries protection was not provided by the mineral fraction of the stock diet and that, therefore, the "all or nothing"

TABLE 3

Comparative composition of purified and natural stock diet

	PURIFIED RATION [1]	PURINA LAB. CHOW [2]
	%	%
Carbohydrates	67 (sucrose)	48.5
Protein	24 (casein)	26.2
Fat	5 (corn oil)	5.4
Fiber and moisture	0	13.5
Whole liver substance	2	..
Liver concentrate powder (1: 20)	2	..
Total minerals	4 (reagent salts)[3]	6.5 [4]
Individual minerals	*mg/100 gm*	*mg/100 gm*
Calcium	530	1170
Phosphorus	550	870
Sodium	440	(?)
Potassium	450	900
Chloride	760	570
Magnesium	33	196
Sulfur	48	(?)
Iron	15	32.7
Manganese	5.0	10.6
Zinc	3.0	(?)
Copper	1.5	1.7
Iodide	2.0	0.45
Fluoride	0.25	2.1
Cobalt	0.065	0.14
Vitamins	*mg/100 gm*	*mg/100 gm*
Thiamin hydrochloride	.35	1.29
Riboflavin	.35	.75
Pyridoxine hydrochloride	.35	(?)
Nicotinic acid	2.5	6.56
Calcium pantothenate	2.0	5.06
p-amino-benzoic acid	30	(?)
Inositol	1000	(?)
Choline chloride	1000	(?)
Beta-carotene	1.1	0.33
Alpha-tocopherol	5.0	(?)
2-methyl-1,4-naphthoquinone	0.6	(?)
Irradiated ergosterol	300 I.U.	90 I.U.

[1] Values are based upon composition of individual ingredients with the exception of the fluoride analyses conducted by Dr. F. J. McClure, National Institute of Dental Research, Bethesda, Maryland, to whom we are greatly indebted.

[2] Values for Purina Laboratory Chow are based on analyses supplied by Ralston Purina Co. Fluoride value checked by Dr. McClure.

[3] Modification of original Phillips and Hart salt mixture (J. Dent. Res., *28:* 47, 1947).

[4] In addition to the minerals listed, preliminary spectrographic analysis has revealed qualitatively the presence in the stock diet of: Ag, Al, B, Ba, Cd, Cr, Cs, Li, Mo, Ni, Pb, Rb, Si, Sn, St, Ti, V.

effect must in part be dependent upon other fractions of the diet. However, since a significant reduction in the extent of caries did occur, it would appear that certain mineral components were involved in the observed caries reduction.

In a preliminary survey (Sognnaes, '48) the ash of the natural stock diet was found to be high in calcium, phosphorus and other known mineral essentials (see table 3). It is important to note, however, that a 50% increase in the amount of the salt mixture in the purified ration has failed to bring about a reduction in caries (Shaw, '49). It therefore seems more likely that the caries reduction in rats fed the natural ash mixture must have been due to minerals other than those now classified among essential nutrients; however, it has not been ruled out that there may be a more optimal ratio of the essential mineral nutrients than that provided by the purified ration. In addition, a number of trace elements were qualitatively shown to be present in the ash of the stock diet and in teeth developed on that diet (Sognnaes, '50); these and more recent analyses have revealed more than a dozen elements which are not considered essential nutrients (see table 3). Since these data were reported it is interesting to note that one of the observed trace elements, vanadium, has been tested and found to have a measurable caries-inhibiting effect when fed post-developmentally (Geyer, '53) and another, molybdenum, has been shown to be an essential component of the enzyme, xanthine oxidase (Richert and Westerfeld, '53). Any one or several of these may have been involved and need further exploration.

At the moment, there is limited evidence regarding the caries-preventive effects of various trace elements, with the exception of fluorine. When this study was started, it was recognized that in many of the earlier rat experiments the caries-reducing levels of fluorides added to food were extremely high, 125 to 250 p.p.m., and pertained to post-eruptive effects for limited periods after weaning (Hodge and Sognnaes, '46). Since our stock diet contained only a fraction of these amounts, namely, 21 p.p.m., and since the experimental

ash diet used in this study contained only 6 p.p.m., there was no way to predict with any assurance what effect these lower levels might have on the caries incidence. In a separate study which will be reported in detail in the next paper in this series (Shaw and Sognnaes, '54) evidence is presented to indicate that supplements of 6 or 25 p.p.m. of fluoride during tooth development or thereafter were incapable of altering the caries incidence.

Although it might have been tempting to select some other mineral component of the stock diet and test its caries-protecting potentiality, this "hit or miss" approach seemed less rational at this time than a test of the total effect of the whole mineral fraction. From these studies it is evident that the mineral fraction of the stock diet contained elements or ratios of elements besides fluorides which had an appreciable caries-inhibiting effect when consumed during the period of tooth development. In addition, it seems probable that other nutrients besides the minerals must be of significance in the development of teeth fully resistant to dental caries.

The ineffectiveness of the ash in desalivated rats might suggest that the salivary glands and their secretion are the target for the trace element effect. However, this can only explain a part of the observed results, namely those pertaining to the post-weaning period. The increasing protection afforded by the early and continuous use of the ash diet would seem to suggest that the factors involved have no trigger action but only take on full significance when available at an early period of tooth development and continuously thereafter for a prolonged period of time. Perhaps this suggests a pattern of depletion which requires time for complete remedy.

SUMMARY

1. More carious lesions were observed in rats receiving a purified ration complete in known essentials than in those receiving the same ration with the ash of a natural diet as the source of minerals.

2. It appears that the factor or factors involved take on major significance after prolonged ingestion by the mother and offspring during as well as after pregnancy.

3. It is suggested that the observed caries protection may have resulted from the presence of certain trace elements in the ash mixture of the natural stock diet.

ACKNOWLEDGMENT

The skilled technical assistance of Mr. George Pettengill is acknowledged.

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EXPERIMENTAL RAT CARIES

V. EFFECT OF FLUORINE ON THE CARIES-CONDUCIVENESS OF A PURIFIED RATION [1]

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(Received for publication January 9, 1954)

Previous observations in rats have shown that the ingestion of the ash from a natural stock diet as the sole source of minerals during tooth development results in highly caries-resistant teeth (Sognnaes and Shaw, '54). This source of minerals had its greatest significance in the offspring when its ingestion had been maintained by their mothers, during pregnancy and lactation as well as by the offspring throughout the post-developmental caries evaluation period. The above observation indicated the need for further investigation of the element or elements in the ash which were responsible for this degree of caries protection.

The process of evaluating the importance of these added minerals has been begun with the halogens. It has already been shown that the addition of bromine to the purified ration during pregnancy and lactation did not protect the teeth of the offspring from caries (Sognnaes, '49b). In the following studies, the effect of the ingestion of fluorides during the period of tooth development and maintenance has been explored, particularly at the lower levels of fluorine. According to the analyses supplied by the Purina Company, the labora-

[1] This investigation has been supported by grants from The Nutrition Foundation, Inc., New York; The Sugar Research Foundation, Inc., New York; Division of Research Grants and Fellowships, U. S. Public Health Service, and the Eugene Higgins Trust. We wish to express our appreciation to Dr. F. J. McClure of the National Institutes of Health for the Fluoride Analyses.

tory chow used in previous studies (Sognnaes, '48) contained 21 p.p.m. of fluorides; this value has been checked with subsequent analyses which indicated a fluoride content of 23 p.p.m. ($\pm$ 10%). Upon ashing at approximately 550°C. (1,022°F.), an appreciable amount of fluoride is lost, but when this ash is used in place of the synthetic salt mixture in the purified diet, it still contributes 6 p.p.m. of fluoride to the diet (Sognnaes and Shaw, '54). In view of these considerations, it was essential to establish whether the ingestion of dietary levels of 6 or 25 p.p.m. of fluorides by rats during the period of tooth development would provide any detectable degree of caries protection.

EXPERIMENTAL

The purified caries-producing diet (100) used in the previous experiments also served as the control diet throughout the fluoride supplementation studies (Shaw, '47; Sognnaes, '48). The experimental diets were supplemented with either an additional 6 or 25 p.p.m. of fluorine as sodium fluoride. In order to obtain an even distribution of the fluoride throughout the rations, appropriate amounts of a solution of sodium fluoride were sprinkled on aliquots of the vitaminized casein. After thorough mixing, the casein was spread out, dried thoroughly, ground and stored ready for use.

The experimental design used in the fluoride studies was of the same type as that used in the preceding paper in this series (Sognnaes and Shaw, '54) with the exception that one or the other of the fluoride diets was used in place of the ash diet. In the case of the 6 p.p.m. fluoride supplement, female rats of the Long-Evans strain were fed the purified caries-producing ration 100 throughout their early life and up until the weaning of the first litters. On the day of weaning, the offspring were divided into two groups and fed either ration 100 or ration 100 plus 6 p.p.m. of fluoride. After the weaning of the first litter the mothers were transferred to ration 100 plus 6 p.p.m. of fluoride and maintained thereon until the second litter was weaned. The offspring again were divided

into two groups and maintained on either ration 100 or ration 100 plus 6 p.p.m. of fluoride.

In the supplementation with 25 p.p.m. of fluoride, another group of females was maintained on a similar regimen. Furthermore an additional litter was used to test the effect of the administration of the 25 p.p.m. supplement when limited to the lactation period alone.

In all experiments the offspring were maintained on their respective rations for a period of 5 months. At the end of this period they were sacrificed and the skulls evaluated for carious molars and carious lesions. A total of 175 rats was used in these two series of studies.

TABLE 1

Ineffectiveness of a dietary supplement of 6 p.p.m. of fluoride on rat caries during and after the period of tooth development

NO. OF RATS	DIETARY HISTORY		INCIDENCE OF CARIES	
	Pregnancy and lactation	Post-weaning	Average no. of carious molars	Average no. of carious lesions
35	100	100	3.8	8.1
19	100	100 + 6 p.p.m.F	4.2	8.7
54	100 + 6 p.p.m.F	100	4.0	8.5
25	100 + 6 p.p.m.F	100 + 6 p.p.m.F	3.7	7.9

Other experiments with fluoride ingestion increased to 100 p.p.m. during tooth development and thereafter have been conducted to determine where effective levels of fluoride ingestion began. Since these high levels do not pertain to the main problem under investigation here, they will not be described in detail.

RESULTS

The results of the studies with 6 and 25 p.p.m. of fluorides were largely negative and have been presented in terms of average figures for the incidence of carious molars and of carious lesions. The data in table 1 represent a summary of all the offspring used in the first experiment. These data indicate that littermate-distributed rats from two succeeding

pregnancies of the same parents exhibited almost the identical caries incidence whether raised and maintained throughout on the purified ration 100 or whether they received a supplement of 6 p.p.m. of fluoride during tooth development, after tooth development, or both.

The data in table 2 represent results with selected females within the second experiment in which a supplement of 25 p.p.m. of fluoride was studied. In this case, post-developmental supplementation with fluoride for 5 months did not result

TABLE 2

Effect of a supplement of 25 p.p.m. of fluoride during the period of tooth maturation or during post-developmental period

NO. OF RATS	DIETARY HISTORY			INCIDENCE OF CARIES	
	Pregnancy	Lactation	Post-weaning	No. of carious molars	No. of carious lesions
Maturation period					
7 [1]	100	100	100	4.7	9.3
4 [1]	100	100 + 25 p.p.m.F	100	3.8	6.5
4 [1]	100 + 25 p.p.m.F	100 + 25 p.p.m.F	100	4.2	4.8
Post-developmental period					
8 [2]	100	100	100	4.4	10.4
9 [2]	100	100	100 + 25 p.p.m.F	4.0	9.4

[1] Succeeding litters from the same mother and father.

[2] Two litters equally distributed between the two groups.

in any significant decrease in the incidence of carious molars or carious lesions. This is in distinct contrast to the definite effectiveness of post-developmental fluoride ingestion at levels of 125 to 250 p.p.m. of fluoride, the results of which have been reviewed in detail previously (Hodge and Sognnaes, '46). When 25 p.p.m. of fluoride was given during the period of lactation, the rats in the fluoride group had a numerically lower average number of carious molars, average number of carious lesions and average extent of the lesions. The reduction was least for the number of carious molars.

However, these reductions were statistically insignificant. When 25 p.p.m. of fluoride was given throughout pregnancy and lactation, the average number of carious lesions and the average extent of carious lesions was somewhat further reduced, but there was no further reduction in the number of carious molars. The total reduction in number of carious lesions and extent of carious lesions from the values observed in the first litter was of statistical significance. In order to explore the effectiveness of higher levels of fluoride supplementation, other experiments with smaller numbers of rats which are not described herein in detail were conducted at a level of 100 p.p.m. of fluoride in the purified diet. When this level was fed during tooth development, but not thereafter, appreciable reductions in caries incidence were observed for the first 5 months. In those rats from the same litter which were maintained on the experiment for 17 months, no protection could be observed as a result of this brief period of high fluoride ingestion and rampant caries was evident. However, when the 100 p.p.m. fluoride supplement was continued through tooth development and thereafter, as well as before, the caries incidence was maintained at a very low level.

DISCUSSION

It is evident from these two experiments that the caries protection previously observed in rats whose mothers were maintained on the natural stock diet throughout pregnancy and lactation (Sognnaes, '48, '49a) could not be attributed to the presence of approximately 21 p.p.m. of fluorides in that diet. Furthermore, the amount of fluorides (6 p.p.m.) contributed to the purified diet by the incorporation therein of the ash of the natural diet in place of the reagent grade salt mixture was not sufficient to have produced the caries protection observed after the ingestion of the ash diet during tooth development (Sognnaes and Shaw, '54). Therefore, another component or other components present in the natural diet and in the ash of the natural diet, but absent in the

purified diet, must have been responsible for this beneficial influence upon the developing teeth.

It should be emphasized that most of the earlier studies on the effect of fluoride ingestion on the incidence of dental caries in rats were concerned with high levels of fluorides fed *after* weaning when tooth development is essentially complete (Hodge and Sognnaes, '46). Thus Miller ('38) supplemented a caries-producing diet with 250 p.p.m. of fluorides and the drinking water with 4.2 p.p.m. of fluorides for a period of 100 days after weaning. He observed a caries reduction of about 90%. In a subsequent study Arnold and McClure ('41) added 125 p.p.m. of fluorides to the diet for 105 days after weaning and again observed a significant caries-inhibiting effect in this post-developmental period.

The one experimental series where the ingestion of low levels of fluorides was permitted during the period of tooth development gave inconclusive results. Cox and co-workers ('39) studied the effect of the ingestion of 10.3, 20.6 and 41.2 p.p.m. of fluorides during pregnancy and lactation on the caries incidence of the offspring. After the rats were weaned, they were fed a coarse corn meal diet without further fluoride supplementation. In the group of rats where mothers had received the 10.3 p.p.m. fluoride supplement throughout the reproductive cycle, the offspring had less "occlusal caries" than the rats from mothers without any fluoride supplement. However, no reduction in "fissure caries" was noted. The addition of 20.6 p.p.m. of fluoride did not significantly affect the caries susceptibility of the young. The 41.2 p.p.m. fluoride supplement in one part of the experiment caused a 15% reduction and in another part a 41% reduction in occlusal caries and 7 and 30% reductions in fissure caries. These apparent contradictions plus the fact that the caries-producing diet was of the coarse particle variety make comparisons with the current data impossible.

There appear to be no other studies in the rat where the effect of these smaller dosages of fluorides have been studied during the periods of tooth development. In the present stud-

ies, the ingestion of 6 or 25 p.p.m. of fluorides as supplements to the purified diet did not cause any reduction on a post-developmental basis. When fed during tooth development and thereafter, the supplement of 6 p.p.m. of fluoride was completely ineffective in the alleviation of the caries incidence. The supplement of 25 p.p.m. of fluorides during tooth development was responsible for only a partial reduction in caries incidence, whereas this supplement was completely ineffective in alleviating the caries incidence on a post-developmental basis.

These data further illustrate the great difference in the response of rats and of human beings to fluoride ingestion. It is well established that the ingestion by children of 1–2 p.p.m. of fluorides in the drinking water during tooth development and thereafter is sufficient to produce a reduction of 40 to 60% in the dental caries incidence. Two factors may explain the ineffectiveness of the ingestion of 25 p.p.m. of fluoride in the rats. First, it should be emphasized that the caries-producing potentialities of the purified ration used in these studies undoubtedly far exceed those of most human diets. Second, in the rat we are dealing almost exclusively with fissure caries, a type of lesion which is less benefited by fluoride ingestion in human beings, in comparison to lesions on the interproximal and other smooth surfaces. Both these and other points undoubtedly enter into the difference in reaction of the rat and the human being to proportionate levels of fluoride ingestion. However, these differences do not alter the interpretation of our experimental data to the effect that the fluorides ingested from the stock diet or from the ash of the stock diet were insufficient to modify the caries incidence. It is believed, therefore, that some one or more components of the mineral fraction of the natural diet are of importance in the development of caries-resistant teeth.

SUMMARY AND CONCLUSIONS

1. Extensive carious destruction has been produced in Norway rats of the Long-Evans strain which were raised on a

generally adequate purified ration, in spite of the addition of 6 or 25 p.p.m. of fluorine, as sodium fluoride, to the ration during the period before eruption of the teeth.

2. The addition of these amounts of fluoride to the purified ration after the completion of tooth development did not result in any reduction in caries incidence within the experimental periods used in this study.

3. The absence of caries which has been previously reported in rats fed natural stock diets during the period of tooth development cannot be satisfactorily explained by the fluoride content of those diets.

4. While fluorides unquestionably modify the caries picture under certain conditions, these data suggest that there are other minerals or ratios of minerals which are major factors in determining the difference between sound and carious teeth.

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FLUORINE BALANCE STUDIES ON FOUR INFANTS [1]

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(Received for publication July 31, 1953)

A number of experiments dealing with the excretion and retention of fluorine by animals have been made (McClure, '39; Lawrenz and Mitchell, '41a; '41b; Jackson et al., '50) and several fluorine balance studies have been conducted using adult human subjects (Machle et al., '42; Machle and Largent, '43; McClure et al., '45; Largent and Heyroth, '49; Largent, '52). To the authors' knowledge, there has been no fluorine balance study on infants or children recorded in the literature. Yet this group may be ingesting baby foods such as Pablum [2] which contain bonemeal and have a rather high fluorine content (Martin, '48; Ham and Smith, '50).

The present balance studies were undertaken in order to obtain data on the amount of fluorine ingested by infants and its absorption and retention, if any.

METHODS

Procedure used in balance studies

Four normal, healthy male infants [3] served as experimental subjects and a 5th was studied in a preliminary experiment.

[1] Supported in part by the Associate Committee on Dental Research of the National Research Council of Canada.

[2] Pablum is manufactured by Mead Johnson and Company of Canada Limited, Belleville, Ontario, Canada. It is composed of, in per cent: wheatmeal (farina), 52; oatmeal, 18; wheat germ, 15; cornmeal, 10; powdered beef bone (specially prepared for human use), 2; sodium chloride, 1; alfalfa, 1; and dried brewers' yeast, 1; and reduced iron, 11 gm/100 lb. The fluorine content of the Pablum used was in the range of 11–12 p.p.m.

[3] These studies were made possible through the co-operation of the Infants' Homes of Toronto and the assistance of the foster mother, Mrs. F. Mitchell

Two balance periods, each of three days' duration were completed on each infant. In the first period the infants were 6 to 14 weeks old and received a milk formula [4] only. In the second period they were 16 to 18 weeks old and received a milk formula plus 32–50 gm (9–12 tbsp.) Pablum per day. The Pablum had been gradually increased in the diet and the infants had been on the level studied for at least three days before the balance period. The Pablum was moistened with part of the formula before being fed to the infants.

The infants were confined on metabolism beds and were under almost constant supervision during the collection periods. Carmine was used as a marker and the faeces and washings of each subject were pooled for each period and refrigerated. The total amount of fluorine excreted in the faeces over each period was divided by the number of days to give the daily faecal excretion of fluorine. Twenty-four-hour urine samples were collected separately during each balance study. They were preserved with toluene and refrigerated.

Duplicate samples of the milk formulas ingested in a 24-hour period were obtained for analysis. The amount of formula not taken by the infant at each feeding was noted and subtracted from the total. The small amount of fluorine in the carmine marker was added to the fluorine ingestion of the first day of each period.

Analytical method

The method used was, with some modification, essentially that outlined for the determination of fluorine in foods in the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists ('50).

Two-hundred milliliter aliquots of urine or baby formula were made alkaline and used for analysis. The infant faeces and washings were made alkaline, dried at 60°C., ground in a Wiley mill (60 mesh) and mixed by tabling before aliquots

[4] Formulas consisted of evaporated milk, water, dextri-maltose and ADC drops, except in case of subject W who had lactic acid milk plus dextri-maltose and ADC drops.

were taken. Magnesium acetate was used as a fixative (Crutchfield, '42). It was found as effective as low-fluorine lime, gave a low blank and was much simpler to prepare than the lime suspension suggested in the Official and Tentative Methods of Analysis. The samples were dried and charred in platinum dishes, placed in a cold muffle furnace (Godfrey and Shrewsbury, '45) and ashed at 580°C. overnight. The urine samples tended to give a glazed ash which was difficult to remove from the platinum dish. If fluorine-free water was allowed to stand on the ash for some minutes it was more easily transferred. If a considerable amount of carbon remained, fluorine-free water was added, the ash broken up and the sample again evaporated and re-ashed. These instances were rare.

Steam distillation from 60% perchloric acid was begun after two acid-alkali washed beads, and sufficient solid silver sulphate to precipitate the chlorides present, had been added to the still.[5] The amount of silver sulphate used with the faeces and milk samples was approximately 3 gm and that with the urine samples 10 gm. An excess, within limits, seemed permissible. There was considerable bumping with the urine samples as the temperature was being raised, but this was reduced by the addition of steam. It was found by the authors that some fluorine distills over between 125°C. and 135°C. Walker and Finlay ('40) and Hoskins and Ferris ('36) recommended that the collection of distillate begin at 110°C. and this temperature was adopted. The temperature was maintained at 135 to 137°C. until almost 200 ml of distillate had been collected (45–60 minutes). If the acidity of the distillate was greater than could be neutralized by 5.0 ml of 0.05 N potassium hydroxide solution, the distillate was discarded.

Aliquots of the distillate were titrated with thorium nitrate in long-form Nessler tubes (100 ml). To make stirrers for these tubes, soft glass rods were bent at one end at right angles to form a circle to fit just inside the tubes. Standard potassium

[5] The fluorine stills were obtained from the Scientific Glass Apparatus Company, Bloomfield, N. J. (Anal. Chem., *19:* 150, 1947).

fluosilicate [6] solution was used in the back titration and an identical light pink color in both tubes at 100 ml volume was taken as the endpoint. Daylight reflected from a polished white surface beneath the tubes was the source of light when matching colors.

The titration was repeated on a second aliquot of distillate and the average taken. Duplicate titrations usually agreed within 0.10 ml standard potassium fluosilicate solution (1.0 μg of fluorine). Blank determinations were made on each still at frequent intervals. They involved both the reagent blank and "distillation" blank and varied from 6.2 to 11.2 μg of fluorine. The average yield of fluorine per determination of milk formula, Pablum, urine and dried faeces was 31, 64, 21 and 53 μg respectively.

Fluorine-free water (redistilled from alkali) was used throughout the analysis and the reagents were as free of fluorine as possible. All fluorine solutions were stored in waxed bottles.

The precision found using this analytical method varied with the type of material analyzed and the level of fluorine present. With water samples, there was an average difference between duplicate samples of 3 μg in the range of 10 to 150 μg per sample. However, the precision was considerably reduced with biological materials. It was best in the analysis of samples of dried faeces in which the average variation from the mean was 4%. In the urine samples the variations averaged ± 8%. Determinations on aliquots of milk formulas averaged ± 16% from the mean. This was due to the nature of the formulas which made them difficult to sample. When 20 μg of standard fluorine solution were added to samples of milk, faeces and urine, recoveries of 90–95% were obtained.

RESULTS

The results of the infant balance studies in period I, when the infants received a milk formula only, are tabulated in table

[6] Obtained from the Food and Drug Administration, Washington.

1. Those for period II, when they received a milk formula plus 32–50 gm (9–12 tbsp.) Pablum per day are tabulated in table 2. The difference between daily fluorine ingestion and excretion is termed the fluorine balance per day and is expressed in micrograms and as a percentage of that ingested. A positive balance indicates retention of fluorine. If it is presumed that faecal

TABLE 1

Daily ingestion and excretion of fluorine by subjects on milk formula only

	FLUORINE INGESTED			FLUORINE EXCRETED			FLUORINE	
	Formula	Carmine	Total	Urine	Faeces	Total	Balance	Balance
	μg	μg	μg	μg	μg	μg	μg	%
W (14 wk. old)								
Day 1	157	1	158	92	46	132	+ 20	+ 13
Day 2	157		157	67	46	113	+ 44	+ 28
Day 3	157		157	78	46	124	+ 33	+ 21
D (13 wk. old)								
Day 1	146	1	147	46	59	105	+ 42	+ 29
Day 2	150		150	48	59	107	+ 43	+ 29
Day 3	150		150	..	59	...	..	..
E (7 wk. old)								
Day 1	102	1	103	54	55	109	— 6	— 6
Day 2	100		100	46	55	101	— 1	— 1
Day 3	100		100	44	55	99	+ 1	+ 1
R (6 wk. old)								
Day 1	169	2	171	48	84	132	+ 39	+ 23
Day 2	169		169	64	84	148	+ 21	+ 12
Day 3	167		167	52	84	136	+ 31	+ 19

fluorine is unabsorbed fluorine, the fluorine retained plus that excreted in the urine is the amount of fluorine absorbed.

It may be seen from an examination of table 1 that fluorine was retained by three of the infants and one was almost in balance. The fluorine balance for the period, expressed as a percentage, ranged from — 2 to + 29 with an average of + 16. The percentage of ingested fluorine which was excreted in the urine over the period varied from 32 to 50 with an average of 40. The percentage which was excreted in the faeces ranged from 29 to 54 with an average of 43.

The results given in table 2 indicate that there was considerable retention of fluorine by all 4 infants in period II when the diet included Pablum. The per cent retention for the period ranged from 22 to 50 with an average retention of 34. In this period, the percentage of ingested fluorine excreted in the urine

TABLE 2

Daily ingestion and excretion of fluorine by subjects on milk formula plus Pablum

	FLUORINE INGESTED				FLUORINE EXCRETED			FLUORINE	
	For-mula	Pab-lum	Car-mine	Total	Urine	Faeces	Total	Balance	Balance
	μg	μg	μg	μg	μg	μg	μg	μg	%
W (18 wk. old)									
Day 1	180	350	2	532	84	312	396	+ 136	+ 26
Day 2	180	350		530	72	312	384	+ 146	+ 28
Day 3	180	350		530	130	312	442	+ 88	+ 17
D (17 wk. old)									
Day 1	126	350	1	477	54	223	277	+ 200	+ 42
Day 2	126	350		476	78	223	301	+ 175	+ 37
Day 3	126	350		476	56	223	279	+ 197	+ 41
E (17 wk. old)									
Day 1	258	446	2	706	54	291	345	+ 361	+ 51
Day 2	258	446		704	68	291	359	+ 345	+ 49
Day 3	258	446		704	51	291	342	+ 362	+ 51
R (16 wk. old)									
Day 1	100	595	1	696	71	458	529	+ 167	+ 24
Day 2	100	595		695	67	458	525	+ 170	+ 24
Day 3	100	595		695	112	458	570	+ 125	+ 18

was considerably less than that excreted in the faeces. The percentage excreted in the urine varied from 8 to 18 with an average of 13. That in the faeces, however, ranged from 41 to 66 with an average of 53%.

On comparing period I with period II, there appears to be a greater per cent retention in the second period and since the level of fluorine ingested in this period was much higher, the actual amounts of fluorine retained were considerably greater. Certainly, of the fluorine absorbed, a greater percentage was retained in period II than in period I.

DISCUSSION

With the exception of fluorine which may occur in drinking water, bonemeal-containing cereals, such as Pablum, seem to be the chief source of fluorine in the infant diet. Pablum is often fed to the infant at the age of three months or even sooner. The amount is gradually increased as the infant grows and it may form a considerable part of the young child's diet for some time. The level of fluorine in the daily diet of a 4-month-old infant taking a milk formula plus Pablum was found in these studies to range from 476 to 706 μg, an amount as high or higher than that which has been found by the authors and others (Machle, Scott and Largent, '42; McClure, '49) in the much larger amount of food normally consumed by adults.

On the basis of the results from these short balance studies on 4 infants, it appears that there was retention by growing human subjects of the fluorine ingested in milk formulas or in milk formulas plus Pablum. The per cent retention was greater when the subjects were older and received both Pablum and milk formulas and the actual amount of fluorine retained was also considerably higher. It might be noted here that the prenatal history of these infants was not known.

It may be assumed that both in milk formula and Pablum, the fluorine is associated to some degree with calcium. Studies with adult human subjects (Machle and Largent, '43; McClure et al., '45) have shown that fluorine associated with calcium is generally absorbed to a lesser degree than fluorine as sodium fluoride. Particularly was this true of the fluorine in bonemeal.

If there was retention of fluorine, it was also reduced in the presence of calcium. However, the retention of fluorine from the diets used in this study on infants does not seem improbable. Jackson et al. ('50) on feeding bone samples to rats found greater retention of fluorine by the young than by the adult animals. Other evidence suggests that growing subjects retain fluorine. Martin ('48) on analysis of a 9-month human fetus found 9–20 p.p.m. fluorine in the bone and 12.5 p.p.m. in the tooth buds. But Roholm ('37) found that adult human bones averaged 0.09% fluorine. The fluorine in human teeth has been

reported by Martin ('48) to average 0.0202% fluorine. Also, some children who are drinking water containing 1 p.p.m. fluorine may have very mild "mottled" enamel and thus there must be some retention at this level of intake.

SUMMARY

Fluorine balance studies were carried out on 4 male infants. Each infant was studied during two periods which were each three days in length. In period I the infants ingested milk formulas only and in period II, milk formulas plus Pablum which had been gradually increased in the diet to the level studied.

The addition of Pablum to the infants' diets considerably increased the amount of fluorine ingested.

Generally, the studies indicated that the fluorine in infants' diets was retained to some extent. The per cent retention appeared in each case greater in period II than in period I and the absolute amounts retained were also higher.

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FLUORINE BALANCE STUDIES ON THREE WOMEN [1]

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(Received for publication July 31, 1953)

Although numerous investigations have been made concerning the excretion and retention of fluorine by animals, similar work with human subjects has been less extensive. Two groups of workers have reported balance studies in this field. Machle et al. ('42) after a fluorine balance study on a human male subject, stated that "on a normal diet, excretion equals intake if excessive losses by perspiration are avoided and if the study is extended sufficiently." In further experiments at higher levels of intake ranging from 3 to 36 mg fluorine per day, retention of fluorine was found in all instances (Machle and Largent, '43; Largent and Heyroth, '49; Largent, '52). These studies indicated that the absorption of fluorine was dependent on the aqueous solubility of the fluoride salt and its state at the time of ingestion. McClure et al. ('45) in balance studies on 5 young men also found this relationship between absorption and aqueous solubility. However, at the level of fluorine ingested (3 mg supplemental fluorine daily), they found no appreciable retention. These workers included in their determinations the fluorine excreted in perspiration.

To learn more about the absorption of fluorine and its retention, if any, the authors carried out balance experiments on young women. Tea infusion had been found by Ham and Smith ('50) to contain approximately 1 p.p.m. fluorine and was added to the diet in one of the periods as a source of fluorine occur-

[1] Supported in part by the Associate Committee on Dental Research of the National Research Council of Canada.

ring in a soluble form. In another period, the diet was supplemented by Pablum [2] a bonemeal containing baby food which contributed fluorine associated with calcium and in a relatively insoluble form.

METHODS

Three young women aged 23 to 24 years served as experimental subjects. They were studied during three periods. In the first period, the subjects consumed a normal diet, avoiding materials known to be high in fluorine, such as tea and fish. In the second period, they consumed a diet which included approximately 70 gm Pablum per day and in the third period, a diet which included 1,360–1,815 ml of tea infusion per day. Each of the last two periods was preceded by a two-day preperiod.

The subjects lived during this study in an apartment in the same building as the laboratory. All meals were eaten together and they were identical by weight or volume for each item. The amount of water consumed each day by each subject was noted. Samples equal to one quarter of the amount of food ingested each day were collected and refrigerated before analysis. The actual dietary fluorine ingested per day was determined by averaging the results of analysis of aliquots of these quarter portions of the daily diet.

Attempts to dry the meals in a hot air oven at 60°C. under vacuum and then grind in a Wiley mill were unsuccessful. Therefore, each day's food was transferred to the Waring Blendor by means of fluorine-free water and mixed for approximately 10 minutes. It was then weighed in the blendor and aliquots were removed and weighed in platinum dishes. Urine was collected for each day separately during the period, preserved with toluene and refrigerated. Two-hundred milli-

[2] Pablum is manufactured by Mead Johnson and Company of Canada Limited, Belleville, Ontario, Canada. It is composed of, in per cent: wheatmeal (farina), 52; oatmeal, 18; wheat germ, 15; cornmeal, 10; powdered beef bone (specially prepared for human use), 2; sodium chloride, 1; alfalfa, 1; dried brewers' yeast, 1; and reduced iron, 11 gm/100 lb. The fluorine content of the Pablum used was in the range of 11–12 p.p.m. Only 10% of this dissolves in water after soaking 15 minutes at 40°C. (without agitation).

liter aliquots were used for analysis. Norite was used as a marker for faeces in the first period and carmine in succeeding ones. The faeces were dried in a hot air oven at 60°C. in the evaporating dishes in which they were collected. They were ground in a Wiley mill (60 mesh), pooled for each person for each period, and mixed for tabling. Aliquots were then weighed out.

These materials were analyzed for fluorine by the methods outlined by the authors ('54). The precision was such that

TABLE 1

Daily ingestion and excretion of fluorine by subjects on a normal diet

	FLUORINE INGESTED				FLUORINE EXCRETED			FLUORINE	
	Food	Water	Norite	Total	Urine	Faeces	Total	Balance	Balance
	μg	μg	μg	μg	μg	μg	μg	μg	%
B									
Day 1	428	..	1	429	130	141	271	+ 158	+ 37
Day 2	760	32		792	182	141	323	+ 469	+ 59
Day 3	448	16		464	188	141	329	+ 135	+ 29
S									
Day 1	428	8	1	437	154	93	247	+ 190	+ 44
Day 2	760	32		792	144	93	237	+ 555	+ 70
Day 3	448	18		466	198	93	291	+ 175	+ 38
H									
Day 1	428	..	1	429	240	149	389	+ 40	+ 9
Day 2	760	32		792	246	149	395	+ 397	+ 50
Day 3	448	16		464	233	149	382	+ 82	+ 18

determinations on aliquots of meal samples varied by an average of ± 6% from the mean and those on urine and dried faeces samples varied by ± 5% from the mean.

The average yield of fluorine per determination on meals, urine and dried faeces was 38, 47 and 40 μg respectively.

RESULTS

The results of the adult balance studies in period I, when the subjects consumed a normal diet (429–792 μg fluorine per day) are given in table 1. There was retention of fluorine by all three subjects. The per cent retention over the period varied

from 31 to 54, with an average of 43. The percentage of ingested fluorine which was excreted in the urine varied from 29 to 43, with an average of 34. That excreted in the faeces ranged from 16 to 27%, with an average of 23%.

The results for period II when the subjects were on a diet which included approximately 70 gm Pablum are recorded in table 2. The amount of fluorine ingested ranged from 946 to 1,435 μg per day. There was again retention of fluorine by all

TABLE 2

Daily ingestion and excretion of fluorine by subjects on a diet including approximately 70 gm Pablum per day

	FLUORINE INGESTED				FLUORINE EXCRETED			FLUORINE	
	Food	Water	Carmine	Total	Urine	Faeces	Total	Balance	Balance
	μg	μg	μg	μg	μg	μg	μg	μg	%
B									
Day 1	1408	16	8	1432	181	762	943	+ 489	+ 34
Day 2	1184	16		1200	169	762	931	+ 269	+ 22
Day 3	928	18		946	109	762	871	+ 75	+ 8
S									
Day 1	1408	18	9	1435	144	343	492	+ 943	+ 66
Day 2	1184	34		1218	376	343	719	+ 499	+ 41
Day 3	928	18		946	323	343	666	+ 280	+ 30
H									
Day 1	1408	16	9	1433	262	493	755	+ 687	+ 47
Day 2	1184	..		1184	418	493	911	+ 273	+ 23
Day 3	928	18		946	405	493	898	+ 48	+ 5

three subjects. The percentage retained ranged from 23 to 48, with an average of 33. The percentage of ingested fluorine which was excreted in the urine varied from 12 to 30, with an average of 22. That excreted in the faeces averaged 45% ranging from 29 to 64%.

The results for period III, when the diet included 1,360–1,815 ml tea, are found in table 3. The amount of fluorine ingested in this period ranged from 1,200 to 1,368 μg per day. Retention of fluorine by the three subjects was again evident. The per cent retention varied from 34 to 46, with an average of 40. An

average of 44% fluorine ingested was excreted in the urine, ranging from 41 to 51%, and an average of 16% in the faeces, ranging from 13 to 20%. The results from this period seemed the most uniform. It should be noted that in period III, the volume of urine excreted was considerably greater than in the first two periods.

Although the levels of fluorine ingested in the last two periods were very similar, the ratio of urinary to faecal fluorine

TABLE 3

Daily ingestion and excretion of fluorine by subjects on a diet including 1360–1815 ml tea per day

	FLUORINE INGESTED				FLUORINE EXCRETED			FLUORINE	
	Food	Water	Carmine	Total	Urine	Faeces	Total	Balance	Balance
	μg	μg	μg	μg	μg	μg	μg	μg	%
B									
Day 1	1264		11	1275	545	192	737	+ 538	+ 42
Day 2	1200			1200	509	192	701	+ 499	+ 42
Day 3	1368			1368	902	192	1094	+ 274	+ 20
S									
Day 1	1264		10	1274	447	169	616	+ 658	+ 52
Day 2	1200			1200	536	169	705	+ 495	+ 41
Day 3	1368			1368	603	169	772	+ 596	+ 44
H									
Day 1	1264		10	1274	376	250	626	+ 648	+ 51
Day 2	1200			1200	614	250	864	+ 336	+ 28
Day 3	1368			1368	587	250	837	+ 531	+ 39

excretion changed. In period II, more of the fluorine was excreted in the faeces than in the urine, whereas, in period III the reverse was true. The per cent fluorine retained appeared slightly greater in period III than in period II.

If it is presumed that faecal fluorine is unabsorbed fluorine then the percentage of fluorine absorbed was greatest on the diet including tea (84%) and least on that including Pablum (55%). On the other hand, the percentage of absorbed fluorine which was retained was greatest when the diet included bonemeal (60%) and least when tea was ingested (46%).

DISCUSSION

Although these results indicating retention of fluorine on a normal diet are not in agreement with those of Machle and Largent ('42) they do not appear unreasonable. Evidence suggests that there is retention of fluorine throughout life. Glock et al. ('41) have studied the fluorine content of human bone from subjects who had not been exposed to abnormal amounts of fluorine (approximately 0.5 p.p.m. in the drinking water). They found that the per cent fluorine in fat-free rib bone varied from 0.02 to 0.08 in children up to 15 years. It varied from 0.06 to 0.31% in subjects aged 22 to 68 years. Their work indicated that in general there was a rise in the fluorine content of human bones with increase in age. Analyses done in this laboratory (unpublished) confirm this relationship. Samples of foot bones from three individuals aged 78 to 82 had a fluorine content of 0.10 to 0.24%, whereas those from three individuals 17 to 23 years old had a fluorine content of 0.016 to 0.023%. McClure ('46) states that there is a strong probability that a normal increase in the percentage of fluorine in skeletal tissues and perhaps in dental tissue occurs with increasing age. In pregnancy there must be retention of fluorine since the fetus contains some fluorine. There also seems to be a build-up of fluorine in placental tissue depending on the level of ingestion (Gardner et al., '52).

The addition of tea to the diet results in a greater percentage absorption of ingested fluorine than the addition of Pablum. The fluorine in tea seems to be in a form which is easily absorbed, whereas that in Pablum seems less easily removed from the intestinal tract. The fact that tea contains fluorine in solution and probably not in association with calcium to any degree, while Pablum contains fluorine in association with calcium in the form of bonemeal, may explain these results. McClure et al. ('45), in studies on human adults, found relatively poor absorption of the fluorine from bonemeal and in experiments on growing rats, Reid ('36) and Lawrenz and Mitchell ('41) have demonstrated the relative ease of absorption of fluorine from tea.

In considering the results of these studies it must be remembered that the periods were short and the number of subjects small. Also the fluorine excreted in perspiration was not measured. This would not likely be large since the experiments were not conducted during the summer months.

The results reported in this paper and in "Fluorine Balance Studies on Infants" by the authors ('54) have shown that infants and adults tend to retain an appreciable proportion of ingested fluorine. The absolute amount of this retention may be quite considerable if materials high in fluorine, such as tea and cereals containing bonemeal, are consumed. Such studies are particularly essential because of the current interest in the possible prevention of dental caries by supplying fluorine in drinking water, in dentifrices and in other ways.

SUMMARY

Fluorine balance experiments were performed on three young women. They were studied during three periods which were each three days in length. In period I the subjects consumed a normal diet; in period II a diet which included approximately 70 gm of Pablum per day, and in period III a diet which included 1,360–1,815 ml of tea per day. The levels of fluorine ingested during periods II and III were relatively the same and considerably higher than on the normal diet in period I.

There was retention of fluorine by all three subjects in each of these three periods. The fluorine ingested in period II was absorbed to a lesser extent than that ingested in period III. It thus appears that the fluorine in Pablum is not as available for absorption as that in tea.

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THE UTILIZATION BY VITAMIN B_{12}-DEFICIENT CHICKS OF MONOMETHYLAMINOETHANOL, HOMOCYSTINE AND BETAINE AS PRECURSORS OF CHOLINE AND METHIONINE [1]

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(Received for publication December 21, 1953)

INTRODUCTION

The evidence that vitamin B_{12} participates in the actual transfer of a methyl group is conflicting. Gillis and Norris ('51) reported that vitamin B_{12}-depleted chicks fed a diet low in methionine and deficient in choline grew equally as well with homocystine plus betaine in the diet as with methionine. Under their experimental conditions, vitamin B_{12} gave a marked growth response when added to the methyl-deficient diet containing homocystine, indicating that the vitamin was aiding the synthesis of methyl groups which subsequently methylated homocystine to form methionine. Dubnoff ('52) pointed out that an *E. coli* mutant grew on a media containing homocystine and dimethylbetapropiothetin and synthesized methionine in the absence of vitamin B_{12}. Stekol, Weiss, Smith and Weiss ('53) found no evidence for the participation of vitamin B_{12} in the transfer of the methyl groups of methionine, betaine or choline to tissue choline or creatine in the intact rat, as measured by means of isotopic carbon.

[1] This work was supported in part by a grant-in-aid from the International Minerals and Chemical Corporation, Chicago, Illinois, and was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

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On the other hand, Schaefer et al. ('51) reported that chicks fed a high-fat diet were unable to utilize monomethylaminoethanol plus betaine in the absence of vitamin B_{12}. Schaefer and Knowles ('51) reported that vitamin B_{12} and folacin are essential for synthesis by the rat of choline and of methionine from aminoethanol, homocystine and a limited supply of betaine.

Jukes and Stokstad ('52), in an extensive study with vitamin B_{12}-deficient chicks, concluded that vitamin B_{12} is required for the transfer of a methyl group from choline or betaine to homocystine to form methionine.

The present investigation was undertaken to determine the ability of vitamin B_{12}-depleted chicks to utilize monomethylaminoethanol plus betaine, and homocystine plus betaine in place of dietary choline and methionine when supplied in amounts which would just satisfy the chick's requirement.

EXPERIMENTAL

The chicks used in the investigation were hatched from eggs laid by White Plymouth Rock hens mated to New Hampshire roosters. The hens were housed in pens with wire-screen floors and fed a corn-soybean diet deficient in vitamin B_{12}. The hatchability of fertile eggs was reduced to between 50% and 60% by this method. At time of hatching, the sex of the chicks was determined. The males and females were then each divided into weight groups and were distributed so that each lot contained the same number of chicks from each weight group and the same number of males and females. The chicks were kept in battery brooders with wire-screen floors. Feed and water were supplied ad libitum.

The basal diet fed the chicks is given in table 1. The soybean protein used in the diet was assayed microbiologically for the 11 amino acids essential for the chick and for tyrosine and cystine. When supplemented with 0.4% glycine, the diet was made adequate in all of the amino acids except methionine (0.29%) and cystine (0.06%). The choline content of

the diet was determined by the reineckete method of Glick ('44) and was found to be less than 0.005%.

The DL-homocystine used in the investigation was prepared by the method of Butz and du Vigneaud ('32) as modified by Jukes and Stokstad ('52). Microbiological assay showed the presence of less than 0.05% methionine in the homocystine.

TABLE 1

Basal diet

INGREDIENT	AMOUNT	INGREDIENT	AMOUNT
	gm		*mg*
Corn starch	65.8	$ZnCl_2$	1.1
Washed soybean protein [1]	23.5	$CoCl_2 \cdot 6H_2O$	0.14
Cellophane	3.0	Alpha tocopheryl acetate	5.5
Hydrogenated fat	2.5	Niacin	2.64
Dicalcium phosphate	2.0	Calcium pantothenate	1.65
Limestone	1.0	Riboflavin	0.55
Iodized salt	0.6	Pyridoxine HCl	0.55
Glycine	0.4	Thiamine HCl	0.33
KCl	0.42	Menadione	0.22
$MgSO_4$	0.25		
	mg		*µg*
$MnSO_4 \cdot 4H_2O$	22.0	Biotin	15.0
$FeSO_4 \cdot 7H_2O$	10.0	Folic acid	80.0
$CuSO_4 \cdot 7H_2O$	1.1	Vitamins A and D_3 [2]	

[1] Glidden Company "alpha protein."

[2] Vitamin A palmitate and irradiated animal sterols in cottonseed oil were added to the ration to supply 2,000 I.U. of vitamin A and 135 I.C.U. of vitamin D_3 per pound of feed.

Choline and betaine were added to the diet in a water solution as choline chloride and betaine monohydrate, respectively.

Two series of experiments were conducted with the vitamin B_{12}-deficient chicks. The first series covered a study of choline formation from monomethylaminoethanol and betaine as measured by growth and the prevention of perosis. It was shown by Jukes and Welch ('42) and McGinnis, Norris and Heuser ('44) that choline was required *per se* for the prevention of this bone deformity. The second series dealt with

a study of methionine formation from homocystine and betaine as measured by chick growth.

RESULTS

The utilization of monomethylaminoethanol plus betaine in place of dietary choline by vitamin B_{12}-deficent chicks

A preliminary experiment indicated that in the strain of New Hampshire-White Plymouth Rock crossbred chicks used in this investigation 0.08% choline Cl was the border line quantity required for the prevention of perosis. Therefore, this level of choline was used in the following experiments. The monomethylaminoethanol was supplied at a molar concentration equal to 0.08% choline Cl. The formation of choline from monomethylaminoethanol requires the addition of two methyl groups. The betaine was added accordingly at twice the molar concentration of the monomethylaminoethanol. A treatment was also included in the experiment wherein the chicks were fed a diet lacking in vitamin B_{12} and containing monomethylaminoethanol at a molar concentration equivalent to 0.08% choline, and betaine at a concentration twice that necessary to methylate the monomethylaminoethanol. This was done to determine if betaine would supply methyl groups in lieu of those synthesized as a consequence of including vitamin B_{12} in the diet.

The results presented in table 2 are the average 4-week data obtained when the chicks were fed the basal diet which was supplemented with 0.2% cystine giving a total of 0.55% methionine plus cystine. The incidence of perosis reported in this and the following tables was calculated as a percentage of the surviving chicks. The results showed that betaine alone gave a marked growth response either in the presence or absence of vitamin B_{12}, but did not prevent perosis. Monomethylaminoethanol, in the absence of vitamin B_{12}, had no effect on perosis and appeared to have none on growth. The addition of vitamin B_{12} to the methyl-deficient diet con-

TABLE 2

Choline formation from betaine and monomethylaminoethanol in a diet containing 0.55% total methionine plus cystine

EXP. NO.	TREATMENTS	NO VITAMIN B_{12}				PLUS VITAMIN B_{12} (1.5 μG/100 GM)			
		No. chicks	Wt. 4 wks.	Perosis	Mortality	No. chicks	Wt. 4 wks.	Perosis	Mortality
			gm	%	%		*gm*	%	%
4	None	14	67 ± 4.2 [1]	50 [2]	43	14	98 ± 7.7	92	7
4,5,6	0.08% choline	54	88 ± 3.5	18	17	54	163 ± 6.9	24	15
4	0.154% betaine	14	96 ± 5.5	100	7	14	119 ± 5.7	100	14
4	0.043% monomethyl.[3]	14	58 ± 4.9	57	50	14	71 ± 4.8	30	28
4,5,6	0.043% monomethyl. + 0.154% betaine	54	99 ± 3.4	31	22	54	156 ± 6.8	23	4
5,6	0.043% monomethyl. + 0.308% betaine	40	126 ± 5.0	25	10	..	..	..	..

[1] Mean ± standard error of the mean of surviving chicks.
[2] Incidence of perosis in surviving chicks.
[3] Monomethylaminoethanol.

taining monomethylaminoethanol markedly reduced mortality and promoted better chick growth than that of the comparable lot without vitamin B_{12}. The addition of vitamin B_{12} to this diet also caused a reduction in the incidence of perosis. This provided further evidence that vitamin B_{12} functions in methyl synthesis in the chick.

In the absence of vitamin B_{12}, the incidence of perosis was a little higher in the chicks receiving 0.043% monomethylaminoethanol plus 0.154% betaine than in those chicks receiving choline, but approximately the same in the chicks receiving 0.043% monomethylaminoethanol plus 0.308% betaine. The addition of vitamin B_{12} did not correct this condition. It appears, therefore, that vitamin B_{12} is not necessary in the formation of choline from monomethylaminoethanol plus betaine, as measured by the incidence of perosis. In the absence of vitamin B_{12}, monomethylaminoethanol plus betaine promoted greater growth in chicks than supplementary choline. This response was particularly significant for those chicks fed the diet containing 0.308% betaine, twice that necessary to methylate the monomethylaminoethanol. These results indicated that the extra betaine provided methyl groups in place of those synthesized when vitamin B_{12} was present.

In further work, the results of which are presented in table 3, the chicks were fed the basal diet supplemented with 0.2% cystine plus 0.2% DL-methionine to give a total methionine plus cystine content of 0.75%. The choline precursors, monomethylaminoethanol and betaine, were supplied at molar concentrations equivalent to 0.08% choline Cl and 0.16% choline Cl (0.043% monomethylaminoethanol plus 0.154% betaine monohydrate and 0.086% monomethylaminoethanol plus 0.308% betaine monohydrate, respectively). In these experiments, contrary to previous findings, 0.043% monomethylaminoethanol plus 0.154% betaine was not as effective as choline for growth. This may have been due to the better growth resulting from the inclusion of larger quantities of methionine in this basal diet. However, chicks fed 0.043% monomethyl-

TABLE 3

Choline formation from betaine and monomethylaminoethanol in a diet containing 0.75% total methionine plus cystine

EXP. NO.	TREATMENTS	NO VITAMIN B_{12}				PLUS VITAMIN B_{12} (1.5 μG/100 GM)			
		No. chicks	Wt. 3 wks.	Perosis	Mortality	No. chicks	Wt. 3 wks.	Perosis	Mortality
			gm	%	%		*gm*	%	%
7	None	20	92 ± 6.7 [1]	100 [2]	60	20	109 ± 4.8	94	10
7,8	0.08% choline Cl	40	152 ± 5.9	11	32	40	187 ± 6.3	19	10
7	0.154% betaine	20	124 ± 5.5	94	10	20	134 ± 7.1	94	15
7	0.043% monomethyl.[3]	20	104 ± 4.6	33	55	20	149 ± 3.9	75	20
7,8	0.043% monomethyl. + 0.154% betaine	40	127 ± 5.7	23	23	40	181 ± 5.3	33	10
8	0.043% monomethyl. + 0.308% betaine	20	148 ± 6.4	28	10	..	..	..	..
8	0.16% choline Cl	20	155 ± 6.1	0	25	20	218 ± 4.9	10	0
8	0.086% monomethyl. + 0.308% betaine	20	148 ± 5.7	10	0	20	203 ± 5.7	15	0

[1] Mean ± standard error of the mean of surviving chicks.

[2] Incidence of perosis in surviving chicks.

[3] Monomethylaminoethanol.

aminoethanol plus 0.308% betaine and those fed these precursors of choline equivalent to 0.16% choline Cl (0.086% monomethylaminoethanol and 0.308% betaine) grew equally as well as the choline-fed chicks. Again, the incidence of perosis was not reduced by the addition of vitamin B_{12}. Chicks fed choline or its precursors at a level equivalent to 0.16% choline Cl showed essentially no difference in the incidence of perosis, either in the presence or absence of vitamin B_{12}.

Betaine added in excess of that required to methylate the monomethylaminoethanol did not increase growth above that of the chicks supplied choline, when added to a diet adequate in methionine and deficient in vitamin B_{12}. Monomethylaminoethanol, in the absence of vitamin B_{12}, reduced the incidence of perosis and maintained the chick's growth equal to that obtained with the basal diet, indicating that the 0.2% methionine added to this basal diet donated methyl groups to form choline.

The addition of vitamin B_{12} to the diet containing monomethylaminoethanol gave a pronounced growth increase but was only slightly effective in preventing perosis. This can be explained on the basis of competition between the need for choline in the prevention of perosis and the need for choline for growth. The limited amount of choline formed from monomethylaminoethanol and the methyl groups synthesized by vitamin B_{12} was used in part to satisfy the requirement for growth with the result that insufficient choline was available for the prevention of perosis.

The utilization of homocystine plus betaine in place of dietary methionine by vitamin B_{12}-deficient chicks

A preliminary experiment was conducted to determine the methionine requirement of chicks fed the isolated soybean diet. In this experiment, the basal diet (table 1) was supplemented with 0.6 μg of vitamin B_{12} per 100 gm of diet and 0.1% choline Cl. Maximum growth of the chicks fed this

TABLE 4

The formation of methionine from homocystine and betaine under conditions of adequate and deficient levels of vitamin B_{12}

TREATMENTS	NO VITAMIN B_{12}			PLUS VITAMIN B_{12} (0.6 μG/100 GM)		
	Wt. 4 wks.	Perosis	Mortality [1]	Wt. 4 wks.	Perosis	Mortality [1]
	gm	%	%	*gm*	%	%
None	61 ± 2.6 [2]	38 [3]	20	78 ± 3.6	36	30
0.3% methionine	138 ± 6.3	100	35	215 ± 7.9	94	10
0.27% homocystine	71 ± 5.0	57	65	106 ± 4.5	86	30
0.27% betaine	87 ± 5.1	20	0	92 ± 9.6	11	10
0.27% homocystine + 0.27% betaine	136 ± 6.8	89	10	175 ± 11.5	100	5

[1] Twenty chicks per treatment at start.

[2] Mean ± standard error of the mean of surviving chicks.

[3] Incidence of perosis in surviving chicks.

diet was obtained with the addition of 0.3% methionine or a total of 0.65% methionine plus cystine. Maximum feed efficiency was obtained when 0.4% methionine was added to the diet. Since 0.3% supplementary methionine gave maximum growth in chicks fed the isolated soybean diet, this level of methionine or homocystine plus betaine at molar concentrations equivalent to 0.3% methionine was used in the following experiments. The supplements were added to the basal diet (table 1) which was deficient in methionine, choline and vitamin B_{12}.

The 4-week results of the first experiment are presented in table 4. In the absence of vitamin B_{12}, homocystine plus betaine was equally effective as methionine in the promotion of growth and gave a marked growth response over either of these compounds added singly. The diet containing homocystine alone permitted very little growth and was associated with a high mortality. The addition of vitamin B_{12} to this diet resulted in a marked growth response and reduction in mortality indicating that vitamin B_{12} was promoting methyl synthesis with the subsequent formation of methionine. The addition of vitamin B_{12} in each of the diets tested produced a growth response. This response was not as great in the chicks receiving homocystine plus betaine as it was in those receiving methionine.

The next experiments were set up to determine if the chicks could simultaneously methylate both monomethylaminoethanol and homocystine to form choline and methionine, respectively, in the absence of vitamin B_{12}. These precursors were added at a molar concentration equivalent to 0.08% choline Cl and 0.3% methionine. Three levels of betaine were used. The first level of 0.213% betaine was only one-half enough to methylate the monomethylaminoethanol and homocystine. The second level of 0.426% betaine was an amount which would just methylate both compounds and the third level of 0.639% betaine was an amount which would supply an excess of betaine over that necessary to methylate the monomethylaminoethanol and homocystine.

TABLE 5

The ability of various levels of betaine to methylate monomethylaminoethanol and homocystine in the presence and absence of vitamin B_{12}

EXP. NO.	TREATMENTS	NO VITAMIN B_{12}				PLUS VITAMIN B_{12} (1.5 μG/100 GM)			
		No. chicks	Wt. 4 wks.	Perosis	Mortality	No. chicks	Wt. 4 wks.	Perosis	Mortality
			gm	%	%		*gm*	%	%
1	None	20	66 ± 3.5 [1]	18 [2]	10	20	82 ± 4.9	21	5
1,2	0.08% choline Cl + 0.3% methionine	35	206 ± 7.7	18	3	35	268 ± 11.1	9	9
1	0.426% betaine	20	83 ± 7.5	13	20	20	75 ± 4.7	15	0
1	0.043% monomethyl.[3] + 0.27% homocystine	20	54 ± 2.1	0	65	20	106 ± 8.4	22	10
1,2	0.043% monomethyl. + 0.27% homocystine + 0.213% betaine	35	140 ± 8.5	28	29	35	217 ± 8.0	32	3
1,2	0.043% monomethyl. + 0.27% homocystine + 0.426% betaine	35	189 ± 7.6	24	3	35	224 ± 10.2	29	3
1,2	0.043% monomethyl. + 0.27% homocystine + 0.639% betaine	35	198 ± 8.1	21	17	35	243 ± 11.8	17	0

[1] Mean ± standard error of the mean of surviving chicks.

[2] Incidence of perosis in surviving chicks.

[3] Monomethylaminoethanol.

The 4-week results of the experiments were averaged and are presented in table 5. In the chicks receiving homocystine plus monomethylaminoethanol, growth was depressed and there was increased mortality as compared to the basal lot. The addition of 0.213% betaine, half enough to methylate the monomethylaminoethanol plus homocystine, was associated with a marked, although suboptimal, growth response. The addition of adequate betaine to methylate these precursors permitted growth comparable to that of the lot receiving choline and methionine.

In the presence of vitamin B_{12}, the chicks receiving homocystine and monomethylaminoethanol showed a striking improvement in growth and reduction in mortality. Similarly, vitamin B_{12} added to the diet containing half enough betaine gave a growth response which was greater than that of the chicks receiving adequate betaine in the absence of vitamin B_{12} and equal to the growth of chicks receiving adequate betaine in the presence of vitamin B_{12}. In the presence and absence of vitamin B_{12}, the chicks receiving monomethylaminoethanol and homocystine plus adequate betaine grew equally as well as the chicks reared on the comparable diet containing choline and methionine.

The basal diet fed in the experiments reported in tables 4 and 5 was more deficient in methionine and cystine (total of 0.35%) than the diets fed in the previous experiments (tables 2 and 3). Under these conditions, the incidence of perosis was low and was only increased when methionine or homocystine plus betaine was added to diet (table 4). The addition of methionine or its precursors promoted increased growth and an increased demand on the small amount of residual choline in the diet for growth purposes. If choline was added along with the methionine, no increase in perosis was noted (table 5). The results in tables 4 and 5 showed that betaine, when added to the diet very deficient in methionine and choline, did not increase the incidence of perosis and under these conditions betaine may have had a slight antiperotic effect.

DISCUSSION

The results reported here confirm the findings of Gillis and Norris ('51) that chicks severely depleted of their vitaman B_{12} reserves retain their ability to utilize homocystine plus betaine in place of dietary methionine. In addition, it was shown that vitamin B_{12}-depleted chicks were able to utilize monomethylaminoethanol plus betaine in place of dietary choline for growth and the prevention of perosis.

The slightly higher incidence of perosis in all lots of chicks fed the precursors of choline and the small reduction in weight in those chicks fed the precursors of methionine, whether in the presence or absence of vitamin B_{12}, was probably due to an enzymatic equilibrium between the precursors and the enzymatically formed choline or methionine. Thus, the actual amount of choline or methionine formed would be somewhat lower than that theoretically possible if the reaction were entirely complete. Since 0.08% choline Cl and 0.3% methionine are the minimum which will satisfy the chick's requirements, a small decrease in these amounts due to an equilibrium displacement would cause a slight deficiency.

The addition of vitamin B_{12} to the diets containing monomethylaminoethanol plus homocystine and an inadequate supply of methyl groups resulted in a marked increase in growth of chicks (table 5). The fact that the addition of adequate betaine to this diet, in the absence of vitamin B_{12}, gave a growth response comparable to that obtained on the diet containing choline and methionine strongly indicates that the growth response from vitamin B_{12} in a diet deficient in methyl compounds is in a large part due to the *de novo* synthesis of methyl groups. These results are further confirmed in a report by Stekol, Hsu, Weiss and Smith ('53) using chicks. These investigators correlated the results, using the tracer technique, with growth studies and found that vitamin B_{12} is not required for the transfer of the carbon of the methyl group of methionine or betaine to choline or creatine. Their results indicated that vitaimin B_{12} aids in the *de novo* synthesis of methyl groups.

In another investigation, Stekol, Anderson, Weiss and Hsu ('53) reported that in vitamin B_{12}-deficient rats the synthesis of the methyl group of methionine from formate and the alpha carbon of glycine is reduced. However, the transfer of the methyl group of methionine to choline was not reduced. Arnstein and Neuberger ('53), using rats, found that vitamin B_{12} increased the conversion of the beta carbon of serine, the alpha carbon of glycine and the carbon of formate into the methyl groups of methionine and into both the methyl groups and the ethanolamine moiety of choline. The results support the indirect evidence of Stekol and Weiss ('50), Bennett ('50), Gillis and Norris ('51) and the findings presented here that vitamin B_{12} is concerned in methyl synthesis. This function of vitamin B_{12} is particularly evident in chicks fed diets which are inadequate in methyl donor compounds, yet are adequate in methyl acceptors (table 5), for chicks fed the diets containing betaine in amounts adequate or in excess of that necessary to methylate the precursors of choline and methionine grew equally as well in the absence of vitamin B_{12} as those chicks fed choline and methionine. Thus, the responses from vitamin B_{12} obtained by Jukes et al. ('50) where less than one-sixth enough betaine was added to methylate the homocystine and the results of Jukes and Stokstad ('52) where about one-third enough betaine was added to methylate the homocystine can now be explaind as the function of vitamin B_{12} in methyl synthesis and not to the need for vitamin B_{12} in transmethylation as the authors postulated.

SUMMARY

Vitamin B_{12}-depleted chicks were fed a diet deficient in choline and methionine and lacking in vitamin B_{12}. Supplementation with monomethylaminoethanol, homocystine and betaine was compared to choline and methionine at equimolar concentrations, both in the presence and absence of vitamin B_{12}.

The vitamin B_{12}-depleted chicks were able to utilize monomethylaminoethanol plus betaine and homocystine plus be-

taine just as effectively as equimolar concentrations of dietary choline and methionine. These results showed, therefore, that vitamin B_{12} is not required for transmethylation in this species.

In the absence of sufficient betaine to methylate adequately the precursors of choline or methionine, vitamin B_{12} gave a marked growth response indicating it is required in the synthesis of methyl groups in chicks.

Betaine appeared to supply methyl groups for other metabolic functions in lieu of those synthesized by vitamin B_{12} when added to a diet deficient in methionine and vitamin B_{12} in excess of the quantity required to methylate the precursors of choline.

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THE VITAMIN B_6 CONTENT OF MILK PRODUCTS

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ONE FIGURE

(Received for publication February 1, 1954)

The importance of vitamin B_6 in human nutrition, particularly infant nutrition, has only recently been appreciated. Following the reports of vitamin B_6 deficiency in the infant by Snyderman et al. ('53) and Hunt and co-workers ('53), Coursin ('53) found that a convulsive syndrome which occurred in infants fed with one of the commercial sterilized-liquid infant formulas was promptly alleviated by pyridoxine administration. As cow's milk, most frequently used in the form of sterilized-liquids and dried products, is the most important article of diet for nearly all bottle-fed infants, knowledge of the vitamin B_6 content of milk products is of considerable importance.

The only previous publication we have found which reports the vitamin B_6 content of processed milk is that of Hodson ('44), who found that there was no significant difference in the vitamin B_6 content of fresh, pasteurized, evaporated and dried cow's milk; all samples tested containing vitamin B_6 at an average level of 0.65 to 0.73 mg/l (fresh or reconstituted).

In this communication we wish to report (1) a survey of a vitamin B_6 content of processed milks including commercial infant formulas; (2) the effect of heat processing necessary for sterilization of canned liquid milk products on vitamin B_6 content and (3) the heat stability in milk of pyridoxal, pyridoxamine and pyridoxine.

EXPERIMENTAL METHODS

Determinations of the vitamin B_6 content of milk were done by the method of Atkin et al. ('43), as outlined in "Methods of vitamin assay" ('51). This method employs *S. carlsbergensis*, an organism which responds about equally well to pyridoxal, pyridoxamine and pyridoxine. The average absolute error of a single determination by this method, calculated from values obtained in 65 determinations of the vitamin B_6 content of one lot of a powdered infant food, was $\pm$ 11.7% (av. 0.223 mg/l, range [0.160–0.301], $\pm$ 0.026). Unless otherwise stated heat sterilization of milk samples was done under the following conditions: the samples were sealed in enamel-lined tin cans and heated in a production model Anderson-Barngrover continuous sterilizer at a temperature of 245°F. for 15.8 minutes. These conditions of heating are employed in routine production of a sterilized-liquid infant formula. Similar overall heating effects are employed generally in industry to insure sterility in canned milk products.

RESULTS AND DISCUSSION

Survey of milk products

Samples of the various products were purchased without selection from local retailers and it may be assumed that the values obtained, the resultant of both processing and storage losses, represent the vitamin B_6 contents available at the time of use. Inspection of the data of table 1 shows that the vitamin B_6 content of all canned liquid milk products is considerably lower than the amount present in cow's milk (33 to 64%). In contrast, the spray-dried milk products when reconstituted, with one exception (69%), contain only slightly less vitamin B_6 than was originally contained in the milk used in their preparation (79 to 89%). Sweetened condensed milk, a product which is not sterilized by heating, was found to contain 78% as much vitamin B_6 as fresh milk. These results do not agree with those of Hodson ('44) in respect to the vitamin B_6 content of evaporated milk, but are in gen-

TABLE 1

Vitamin B_6 content of milk products reconstituted

TYPE OF PRODUCT AND BRAND	MILK PROTEIN	LOTS TESTED	NO. OF DETERMINATIONS	VITAMIN B_6 CONTENT [1]		
				mg/l		% of original [2]
				Range	Av.	
	%					
Evaporated milk sterilized liquids						
A	3.25	1	5	0.25–0.31	0.28 [3]	51
B	3.25	1	3	0.31–0.41	0.35 [3]	64
Infant formulas sterilized liquids						
C	2.2	4	8	0.13–0.21	0.17	46
D	2.8	4	8	0.15–0.22	0.19	40
E	1.75	4	8	0.80–0.12	0.10	33
F	2.6	2	6	0.16–0.40	0.22	50
G [4]	1.5	10	20	0.51–0.75	0.61	
H	2.2	4	8	0.11–0.15	0.13	35
Sweetened condensed milk						
I	3.25	1	3	0.33–0.49	0.43	78
Infant formulas spray dried						
G [4]	1.5	10	20	0.50–0.78	0.61	
E	1.75	1	2	0.26–	0.26	83
I	2.0	3	7	0.20–0.26	0.24	69
C	2.2	1	2	0.30–0.32	0.31	79
Whole milk spray dried						
N	3.25	2	4	0.46–0.48	0.47	81
Skim milk spray dried						
O	3.25	1	1	0.50–0.55	0.52	89
P	3.25	2	4	0.50–0.56	0.51	88

[1] Vitamin B_6 activity in comparison to the vitamin B_6 activity of pyridoxine hydrochloride. All values listed in this paper, as well as in the references cited, are 1.21 times larger than they would be had pyridoxine itself been used as the standard.

[2] The percentage loss was calculated on the assumption that the fresh milk used contained 0.58 mg/l, the average value obtained for fresh milk in this laboratory. As commercial infant formulas vary in their content of milk solids, the level of vitamin B_6 supplied by milk in these was corrected by multiplying 0.58 by the fraction:

$$\frac{\%\text{ milk protein in formula as fed}}{3.25\ (\%\text{ protein in cow's milk})}$$

[3] As ordinarily used in infant feeding ($\frac{2}{3}$ milk) product A would supply 0.19 mg and product B 0.23 mg/l.

[4] Fortified with pyridoxine hydrochloride.

eral agreement with his finding that dried milk products contain about as much pyridoxine as fresh milks.

Whether the sterilized-liquid-milk products when used as the only food (with or without added carbohydrate) contain adequate amounts of vitamin B_6 for optimum nutrition of all infants is not known. Pertinent findings which bear on the problem are (a) human milk contains an average of 0.04 mg/l (Williams et al., '42) and 0.18 mg/l (Macy, '49). In this laboratory the vitamin B_6 content of mature human milk was found to average 0.13, range (0.035–0.22) mg/l (34 determinations, 30 samples from three donors); (b) the adult requirement for vitamin B_6 is more than 0.5 mg and less than 5 mg per day (Vilter et al., '53); (c) severe deficiency symptoms have occurred in a small percentage of infants fed a formula supplying less than 0.1 mg/l (Coursin, '53); (d) the vitamin B_6 requirement of experimental animals depends upon the kind of dietary carbohydrate, presumably because of synthesis of vitamin B_6 by intestinal flora (Sarma et al., '47). This finding indicates the possibility that the intestinal flora of infants, resulting from the kind of carbohydrate in the diet or undetermined factors, may influence the vitamin B_6 requirement; (e) lower vitamin B_6 contents than expected from microbiological assay values were observed when processed foods were fed to rats (Register et al., '50; Tappan et al., '53).

Effect of heat treatment necessary for sterilization of canned liquid milk products

As shown in table 1, heat-sterilized liquid milk products contain much less vitamin B_6 than fresh milk. A study was made of the effect of the processing steps used in the preparation of a heat-sterilized infant formula on the natural vitamin B_6 content. Losses of vitamin B_6 were not found to occur during compounding, pasteurization, homogenizing and cold storage. A significant loss occurred only during heating at the elevated temperatures necessary for preparation of a sterile product.

Data obtained in experiments designed to show loss of vitamin B_6 after autoclaving are shown in figure 1.

Preliminary investigations in which determinations were made at unspecified times after heating gave confusing results. The reason for the discrepancies turned out to be due to the fact that analyses done immediately after autoclaving indicated only moderate losses in vitamin B_6 content had occurred; analyses done during the next few days indicated further losses in vitamin B_6. In view of these results, to

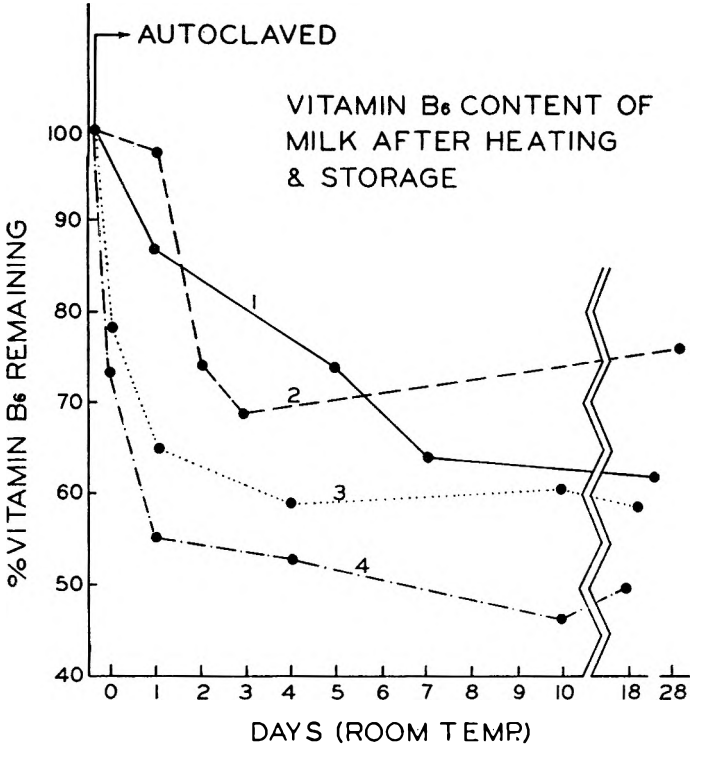

Fig. 1 Decrease in vitamin B_6 activity of milk after autoclaving (see text for conditions) and storage at room temperature. Analyses done on zero days were started within two hours after autoclaving.

Curves: (1) Evaporated milk. (2) Skim milk. (3) Whole milk. (4) Whole milk — autoclaving process done twice. Duplicate determinations, average vitamin B_6 content before autoclaving 0.58 mg/l.

evaluate the total vitamin B_6 losses caused by processing, we routinely wait for 10 days before analyses are made.

The delayed effect of heating on the vitamin B_6 content of milk occurring over a period of several days, as shown in figure 1, is not typical of heat destruction of other vitamins in milk. Possible explanations of the delayed inactivation in vitamin B_6 which occurs after the heating of milk are: (1) the vitamin B_6 of the milk reacts slowly with some compound or compounds produced during autoclaving from other milk constituents or (2) labile substances formed during autoclaving interfere with the assay procedure by causing high values.

After about 10 days of storage further decreases in vitamin B_6 content are slight. For example, storage of evaporated milk (Brands A and B) for 122 days at 35°C. did not cause any definite decrease of the vitamin B_6 content; a sample of evaporated milk (Brand A) after storage of one year at room temperature contained 0.31 mg/l after being reconstituted, as compared to 0.35 mg in a recently purchased sample.

Heat stability of pure compounds with vitamin B_6 activity added to milk

In view of the low vitamin B_6 content of one of the commercial infant formulas, due both to a lowered milk content and losses incurred during autoclaving, studies were done to determine the feasibility of vitamin B_6 fortification. Results of typical experiments are shown in table 2.

The amount of vitamin B_6 activity lost in the milk samples fortified with pyridoxine was no greater than in the unfortified samples. If it is assumed that losses of natural vitamin B_6 and added pyridoxine occur independently, the conclusion may be drawn that pyridoxine was not appreciably destroyed by autoclaving. Pyridoxamine and pyridoxal added to milk were destroyed by autoclaving to roughly the same extent as natural vitamin B_6, a result compatible with the findings of Rabinowitz and Snell ('48). According to these authors

fresh milk contained an average of 0.32 mg pyridoxal, 0.09 mg pyridoxamine and 0 mg pyridoxine/l.

Because of its high degree of heat stability, fortification of sterilized-liquid-milk products with pyridoxine hydrochloride is possible and at the present time is being done with one of the commercial infant formulas.[1]

SUMMARY

1. As estimated by the growth response of *S. carlsbergensis*, sterilized-liquid-milk products were found to contain from 33–64% (0.10–0.35 mg/l) and dried-milk products 69–89%

TABLE 2

Decrease of vitamin B_6 content by heating milk fortified with crystalline compounds

EXPERIMENT NUMBER	SAMPLE	VITAMIN B_6, MG/L		
		Before autoclaving	After autoclaving	Decrease
1[1]	Skim milk	0.54	0.43	0.11
	Skim milk + 0.5 mg pyridoxine hydrochloride/l	1.14	1.02	0.12
	Skim milk + 0.5 mg pyridoxal hydrochloride/l	1.16	0.82	0.34
2[2]	Skim milk	0.50	0.28	0.22
	Skim milk + 0.5 mg pyridoxine hydrochloride/l	0.96	0.75	0.21
	Skim milk + 0.5 mg pyridoxal hydrochloride/l	0.95	0.48	0.47
	Skim milk + 5 mg pyridoxamine dihydrochloride/l	1.00	0.62	0.38
3[1]	Infant formula	0.22	0.09	0.13
	Infant formula + 0.5 mg pyridoxine hydrochloride/l	0.73	0.61	0.12

[1] Conditions of autoclaving described in text.

[2] Samples autoclaved one hour at 15 pounds' pressure (250°F.).

[1] SMA Concentrated Liquid and SMA Powder, Wyeth Laboratories, Inc. The label claim is 0.4 mg vitamin B_6 per quart.

(0.24–0.52 mg/l) of the vitamin B_6 activity of the fresh milk used in their formulation.

2. The heating process commonly used in industry for sterilization of liquid-milk products causes a considerable decrease in vitamin B_6 content.

3. Decreases in vitamin B_6 of sterilized-liquid-milk products occur not only during autoclaving but continue at a relatively rapid rate for as long as 7 days afterwards.

4. Pyridoxine added to sterilized-liquid-milk products is more resistant to heat inactivation than pyridoxal, pyridoxamine or the naturally occurring vitamin B_6 in milk.

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FAILURE OF VITAMIN B_{12} TO INCREASE SURVIVAL OF PROGENY OF RATS FED AN ALL-PLANT DIET [1]

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(Received for publication February 1, 1954)

Halvorson and Schultze ('50) have reported a terminally fatal syndrome in rats 36–48 hour of age which they have characterized as "acute uremia of the newborn." A number of prenatal diets were found to produce this syndrome in the progeny of the females to which the diets were fed. One of these was the diet of Spitzer and Phillips ('46). When the young rats received 0.05 μg of vitamin B_{12}, which was injected subcutaneously shortly after birth, the syndrome was prevented and survival of the young was increased.

These findings bear directly on studies of the reproductive performance of animals fed corn and soybean oil meal type diets which have been done in this laboratory (Spitzer and Phillips, '46; Maruyama and Phillips, '48; Nell and Phillips, '50). For this reason, an investigation of the effect of injections of vitamin B_{12} on survival, and of the concentration of urea [2] in the blood of progeny of female rats which were fed a diet of this type was made. The results indicate that 0.05 μg of vitamin B_{12} per rat did not increase the survival of the progeny of female rats which were fed this type of diet under our conditions. It was also found that the young rats

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[2] The term "urea" is used in the text to refer to urea-ammonia nitrogen.

did not exhibit increased concentrations of urea in their blood except as an accompaniment of starvation and dehydration.

EXPERIMENTAL

Adult female rats of the Sprague-Dawley strain were fed the basal ration (table 1). All of them had been on this ration through one reproductive cycle, and all had been unbred for at least one month since their previous litters had died or were weaned. The management procedure of Spitzer and

TABLE 1

Composition of basal diet

	%
Ground yellow corn	58
Soybean oil meal (expeller)	32
Alfalfa leaf meal	5
Cottonseed oil	3
Salts B [1]	2
Niacin	0.02
Irradiated yeast	0.01

[1] Calcium carbonate, 50; calcium acid phosphate, 24; sodium chloride, 24; magnesium sulfate, 1 and manganese sulfate, 1%.

Phillips ('46) was followed. Within 6 hours following parturition, each litter was reduced to a maximum of 6 rats and was divided into two approximately equal groups. One group was given 0.05 μg of vitamin B_{12} in 0.1 ml of distilled water as described by Schultze ('49). The animals of the other group received 0.1 ml of distilled water.

The procedure for determining urea in blood was adapted from that of Gentzkow ('42) to permit determinations on as little as 0.1 ml of blood from the young rats. Urea recovery determinations were within $\pm$ 5%.

RESULTS AND DISCUSSION

The administration of 0.05 μg of vitamin B_{12} appeared to have no influence on the survival of the young rats under

the conditions of this experiment (table 2). The young which died had no milk in their stomachs. The intraperitoneal injection of 10 international units of pitocin [3] into several mother rats which had litters that appeared to be starving did not result in expression of milk from the mammary glands. For these reasons, it appeared that this suppression of lactation was a factor in the failure of rats fed the basal diet

TABLE 2

Effect of vitamin B_{12} on survival of progeny of rats fed the basal diet

TIME FOLLOWING INJECTION	NUMBER OF YOUNG SURVIVING	
	0.05 μg vitamin B_{12}	Controls, 0.1 ml distilled H_2O
0 hours	44	43
24 hours	35	29
48 hours	23	26
72 hours	15	13
21 days	8	8

TABLE 3

Concentrations of urea-ammonia nitrogen in blood of rats from females fed the basal diet

APPROXIMATE AGE, HOURS	APPEARANCE	UREA-AMMONIA N
		mg %
6	Healthy, milk in stomach	53.0
12	Healthy, milk in stomach	40.3
24	Slightly cyanotic, weak, milk in stomach	37.8
24	Slightly cyanotic, weak, milk in stomach	38.0
48	Emaciated, slightly cyanotic, weak, no milk in stomach	75.2
48	Emaciated, slightly cyanotic, weak, no milk in stomach	94.8
96	Emaciated, weak, no milk in stomach	84.5

[3] Parke, Davis and Co., Detroit, Michigan.

to wean their young. This is in accord with earlier observations (Spitzer and Phillips, '46; Maruyama and Phillips, '48).

Analysis of the blood of some of the young of these rats for urea revealed that some of them did have high concentrations of urea in their blood. Those which had this condition, however, never had milk in their stomachs (table 3). Since an increase in non-protein nitrogen was observed in dehydration (Best and Taylor, '45), it was thought that the increased concentration of urea could be a result of starvation and dehydration consequent to failure of this diet to

TABLE 4

Starvation-dehydration uremia in young rats

HOURS AWAY FROM MOTHER RAT	MG % UREA-AMMONIA N IN BLOOD	
	Newborn rats from basal diet mothers	48-hour-old rats from mothers fed the basal diet + fish solubles [1]
0	45.1 ± 6.5 [2] (4) [3]	40.5 ± 8.42 (6)
12	64.8 ± 15.3 (3)	.....
24	58.3 and 82.2 (2)	82.5 ± 17.5 (5)
36	104 and 94 (2)	.....
48	168 and 107 (2)	71.2 ± 21.5 (5)

[1] 5%, at the expense of the whole diet.
[2] Standard deviation.
[3] Number of observations.

support lactation. In order to investigate this point, young rats were taken from females which were fed the basal diet and from females which received the basal diet supplemented with fish solubles. These rats were sacrificed at intervals and analyses for urea were performed on their blood (table 4). Starvation and dehydration were accompanied by increases in urea concentrations.

The results of these experiments appear to be at variance with those of Halvorson and Schultze ('50). A combination of different laboratory conditions, different strains of rats, and very small differences in the diets employed may account for the divergent results.

The relationship of these findings to observations regarding lactation failure of female rats fed a purified diet containing soybean protein (Schultze, '53) is uncertain. It was reported that soybean oil meal reversed the lactation failure obtained on this diet. Since this material was present in the basal diet employed in this experiment, it appears that the factors responsible for lactation failure in Schultze's experiments and for the apparent lactation failure in the experiments described here, are not the same.

SUMMARY

The survival of the progeny of female rats which were fed an all-plant diet was not increased by the subcutaneous injection of 0.05 μg of vitamin B_{12} within 6 hours after parturition. An increase in the concentration of urea in their blood was not found except when their stomachs contained no milk. Starvation and dehydration were found to result in an increase in the concentration of urea in the blood of young rats from females which were fed either an all-plant diet or this diet supplemented with fish solubles.

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NITROGEN BALANCE STUDIES WITH DOGS ON CASEIN OR METHIONINE-SUPPLEMENTED CASEIN

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(Received for publication February 19, 1954)

Osborne and Mendel showed early ('15) that cystine improved the growth of rats fed diets containing 9 to 12% casein. Subsequently, methionine was found to be effective by Jackson and Block ('32). More recently, Allison et al. ('47), evaluating the biological usefulness of proteins in terms of a "nitrogen balance index" using nitrogen balance studies in normal adult dogs, observed that casein supplemented with 25 mg of methionine N per gram of casein N had a nitrogen balance index about double that of unsupplemented casein. This amount of methionine may be expressed, alternately, as 4.26 gm of methionine per 100 gm of casein (N $\times$ 6.25). A further increase (three-fold) in the methionine supplement to casein did not additionally improve the nitrogen balance index. The nitrogen balance index of casein supplemented with 8 mg of methionine N per gram of casein N [1.36 gm of methionine per 100 gm of casein (N $\times$ 6.25)] was only about one-fifth better than that of unsupplemented casein.

Recently, during the course of investigations on proteins and protein hydrolysates by means of nitrogen balance studies with protein-depleted dogs, we observed that a doubling in the biological value of casein resulted from the addition of 3 gm of methionine per 100 gm of casein (N $\times$ 6.25). This appeared to represent significantly less methionine than had been indicated by Allison et al. as required to effect this degree of improvement in the biological usefulness of casein.

TABLE 1

Weekly nitrogen balance data on protein-depleted dogs fed casein or methionine-supplemented casein as their nitrogen source

NITROGEN SOURCE	DOG NO.	WEIGHT	NITROGEN INTAKE [1]		NITROGEN EXCRETION		NITROGEN BALANCE	CALCULATED [2] NITROGEN INTAKE AT EQUILIBRIUM
			Test	Total	Urinary	Fecal		
		kg	*mg N/kg/day*	*mg N/day*	*mg N/day*		*mg N/kg/day*	*mg N/kg/day*
Casein [3]	32	18.0	225	4050	2311	505	+ 69	156
		18.6	218	4050	3033	453	+ 30	188
	36	10.1	126	1275	966	333	— 2	128
		9.7	131	1275	964	348	— 5	136
		9.6	133	1275	1020	262	— 1	134
		9.6	133	1275	661	313	+ 31	102
		9.5	134	1275	666	284	+ 34	100
		9.6	133	1275	801	307	+ 17	116
	41	11.4	132	1500	1272	354	— 11	143
		11.3	132	1500	1255	284	— 3	135
	56	5.0	180	900	676	275	— 10	190
		5.0	180	900	627	203	+ 14	166
	62	11.7	148	1725	1324	288	+ 10	138
		11.6	149	1725	1316	315	+ 8	141
	66	9.0	167	1500	994	314	+ 21	146
		9.7	155	1500	895	406	+ 20	135
		10.3	146	1500	938	402	+ 16	130
					Weighted mean ± s.e.			147 ± 9
							Median	139
Casein [4] + 1% methionine [5]	66	10.3	117	1274	989	222	+ 6	111
		10.4	117	1274	969	182	+ 12	105
	70	9.0	85	807	515	185	+ 12	73
		9.0	85	807	530	206	+ 8	77
	75	7.4	118	944	580	280	+ 11	107
		7.4	117	944	575	266	+ 14	103
	77	11.8	97	1216	952	265	0	97
		11.8	97	1216	949	326	— 5	102
							Mean ± s.e.	97 ± 7
							Median	102
Casein + 3% methionine	66	10.9	83	957	610	195	+ 14	69
		11.0	82	957	532	197	+ 20	62
		11.0	73	858	516	165	+ 16	57
		11.0	64	760	590	203	— 3	67

TABLE 1 *(continued)*

Weekly nitrogen balance data on protein-depleted dogs fed casein or methionine-supplemented casein as their nitrogen source

NITROGEN SOURCE	DOG NO.	WEIGHT	NITROGEN INTAKE [1]		NITROGEN EXCRETION		NITROGEN BALANCE	CALCULATED [2] NITROGEN INTAKE AT EQUILIBRIUM
			Test	Total	Urinary	Fecal		
		kg	*mg N/ kg/day*	*mg N/ day*	*mg N/ day*		*mg N/ kg/day*	*mg N/ kg/day*
	70	9.0	71	674	492	143	+ 4	67
		9.0	71	674	515	138	+ 2	69
		9.0	71	674	598	166	— 10	81
		9.0	71	674	596	116	— 4	75
	73	7.5	85	705	338	214	+ 20	65
		7.5	85	705	365	200	+ 19	66
		7.6	84	705	385	247	+ 10	74
		7.8	62	549	406	260	— 15	77
	75	7.4	97	798	430	215	+ 21	73
		7.4	97	798	445	210	+ 20	77
		7.4	86	713	440	256	+ 2	84
		7.6	74	643	459	237	— 7	81
	77	11.9	77	979	561	213	+ 17	60
		11.9	77	979	613	250	+ 10	67
		11.9	77	979	607	229	+ 12	65
		11.8	68	866	751	230	— 10	78
	79	6.2	103	711	430	327	— 7	110
		6.2	103	711	412	270	+ 5	98
		6.3	101	711	422	278	+ 2	99
		6.4	99	711	427	290	— 1	100
							Mean ± s.e.	76 ± 6
							Median	72

[1] Each value represents one week's balance.

[2] This value was calculated by increasing or decreasing the known nitrogen intake by the extent of the nitrogen balance (negative or positive) which occurred during the week being studied.

[3] From earlier unpublished data accumulated at this laboratory by Kade, Wyzan and Shepherd, and by Kade, Phillips and Phillips.

[4] The casein sample contained 14.13% N. The 1% and 3% methionine supplemented caseins were prepared by adding 1 gm and 3 gm respectively to 114 gm of casein (16 gm N).

[5] DL-Methionine.

METHOD

The diets and experimental procedures used in this study were the same as those described previously (Wyzan, Shepherd and Arnold, '51; Wyzan, Kade and Shepherd, '51; Kade, Phillips and Phillips, '48; Kade, Houston, Krauel and Sahyun, '46).

Adult mongrel dogs, of moderate size and essentially parasite free, were given adequate calories and about 150 mg casein N per kilogram per day until they came into nitrogen equilibrium. At this time complete balance studies were initiated. The urine was pooled into three samples weekly for analysis: two two-day samples during the week and a three-day sample over the weekend. The completeness of urine collections was verified by creatinine determinations. Carmine markers were used to separate the weekly collection periods of the pooled fecal samples. Nitrogen analyses were done by the macro-Kjeldahl procedure using a copper sulfate catalyst.

The nitrogen source materials were unsupplemented casein [1] and casein to which had been added 1 gm or 3 gm of methionine per 16 gm casein N (100 gm casein at N $\times$ 6.25). The 3% level represented essentially the difference in sulfur amino acids between casein and lactalbumin, a protein with a biological value close to 100.

RESULTS

It may be seen from the data summarized in table 1 that the dogs were maintained in nitrogen equilibrium by median values of 139 mg of casein N, 102 mg of 1% methionine-supplemented casein N, or 72 mg of 3% methionine-supplemented casein N per kilogram per day. The median values are preferred because of the skewed distribution of the results. From the foregoing, it is seen that 73% as much 1% methionine-supplemented casein N was needed as unsupplemented casein to maintain the dogs in nitrogen equilibrium.

[1] Sheffield high-nitrogen casein.

It may also be seen that 3% methionine-supplemented casein N was nearly twice as efficient a nitrogen source as was unsupplemented casein N. Casein with methionine supplements between 1 and 3% was not fed so that no evidence is at hand to determine with this method of test whether casein with a methionine supplement of less than 3% would have equalled that noted with the 3% supplement.

DISCUSSION

The data given in table 1 may be compared with the results of other investigators.

Unsupplemented casein. The amount of casein N required to maintain nitrogen equilibrium in the 6 dogs listed in table 1 is in reasonable agreement with the 140 to 160 mg of casein N per kilogram per day value observed for two dogs similarly protein-depleted by Risser ('46). Kade et al. ('48), on the basis of studies with two dogs each over a two-week period, noted nitrogen equilibrium at intakes closely related to those given in table 1, 130 to 140 mg casein N per kilogram per day. Thus, on the basis of comparisons with related studies on dogs similarly protein-depleted prior to test, the data given on unsupplemented casein in table 1 appear to be reasonably well substantiated so that they may serve as a basis for further discussion.

Methionine-supplemented casein. In comparison with our results on methionine-supplemented casein given in table 1 may be mentioned Risser's findings ('46) that 80 to 100 mg of 4.3% cysteine · HCl · H_2O-supplemented casein N maintained a dog in nitrogen equilibrium. Two additional dogs reported by Risser were maintained in nitrogen equilibrium with 60 to 70 mg of 2% methionine-2.9% cystine · HCl · H_2O-supplemented casein N per kilogram per day. Since these results fall within the range of the data given in table 1, it may be surmised that smaller amounts of sulfur amino acids than those used by Risser might have effectively produced highly efficient nitrogen combinations.

Similarly, Kade, Phillips and Phillips ('48) observed that two dogs were maintained in nitrogen equilibrium over a two- or three-week period with 80 to 90 mg of 6.25% methionine-supplemented casein N per kilogram per day. These investigators, like Risser above, did not claim this level of methionine to be the minimum. With regard to the alternate point of interest, namely, the amount of nitrogen required to maintain the dogs in nitrogen equilibrium, it is evident that their values fall within the range of values given in table 1, so that the data appear to be in essential agreement.

Data on dogs not protein-depleted. Somewhat at variance with the results summarized in table 1 are those submitted by Allison et al. ('47) who used dogs which were not depleted of their protein reserves prior to the test period. These investigators reported that the biological usefulness of casein in dogs was doubled by supplementing with 25 mg of methionine N per gram of casein N. This represents 4.26 mg of methionine per 100 mg of casein (N $\times$ 6.25). The casein N was not further improved by additional methionine (71 mg of methionine N per gram of casein N). A decrease in supplement to 8 mg of methionine N per gram of casein N [1.36 mg of methionine per 100 mg of casein (N $\times$ 6.25)] effected only about a 20% increase over the biological usefulness of the unsupplemented casein. There are several possible reasons for the somewhat greater methionine requirement under the conditions of test of Allison et al.: (1) dogs with normal protein reserves require more methionine, together with casein, than do protein-depleted dogs; (2) less than 4.26 gm of methionine per 16 gm of casein N might have effected improvement equal to that obtained with this level of methionine in the biological usefulness of the casein under the conditions of the Rutgers test had intermediate levels been tested; (3) a difference in the biological value of the casein samples themselves influenced the amount of methionine required to impart maximal biological usefulness to them; (4) some other factor, not apparent to us, exerted an influence. Whether one or more of these reasons serves to explain the difference

in results may best be determined experimentally. While the conditions used in these tests differed from those of the Rutgers studies, the results above clearly show that less methionine than that indicated by the Rutgers group to be needed may be equally effective in imparting maximum utility to casein nitrogen.

Methionine requirement of protein-depleted dogs. The methionine and cystine intakes of the dogs under the described conditions of test may be calculated. Casein may be assumed to supply 3.2% methionine and 0.4% cystine (Bureau of Biological Research, '50). At 139 mg casein N per kilogram per day the dogs received 27.8 mg of methionine and 3.5 mg of cystine per kilogram per day. At 102 mg of casein N supplemented with 1% methionine the dogs received 26.8 mg methionine and 2.6 mg cystine per kilogram per day. At 72 mg of casein N supplemented with 3% methionine, the dogs received 27.9 mg methionine and 1.8 mg cystine per kilogram per day. Under these conditions, with cystine representing 11% or less of the sulfur amino acids, the sulfur amino acid needs of the dogs were about 30 mg per kilogram per day.

Protein requirements. To orient these studies in relation to those of other investigators, the protein content of the diets may be calculated. The dogs given unsupplemented casein were fed diets which contained (medians) about 6.5% casein ($N \times 6.25$). Those fed casein supplemented with 1% methionine received about 5.2% dietary casein ($N \times 6.25$). If the casein was supplemented with 3% methionine, the dogs received about 4.8% dietary casein ($N \times 6.25$). These values are not based on the nitrogen equilibrium figures so that they are not to be taken too literally; however, they do emphasize the low protein requirements of protein-depleted dogs. In dogs not depleted of their protein reserves, Melnick and Cowgill ('37) observed that about 12.1% dietary casein was needed to maintain them in nitrogen equilibrium.

Separately, the point may be raised as to whether low-casein experimental diets might not be supplemented advantageously with methionine. Such diets would thus not be

limiting in sulfur amino acids. The amounts of these required by animals appear to be relatively greater than those required by man (Johnson et al., '47; Cox et al., '47). Alternately, low-protein diet studies could be undertaken with egg proteins, lactalbumin or suitable protein mixtures, wherein sulfur amino acids are not present in limiting amounts.

SUMMARY

For these studies the protein reserves of dogs were brought to a low level by feeding them limiting amounts of casein as their nitrogen source.

Such dogs were maintained in nitrogen equilibrium with (median values) 139 mg of casein N, 102 mg of 1% methionine-supplemented casein N, or 72 mg of 3% methionine-supplemented casein N per kilogram per day.

Since others have demonstrated an equal doubling in the biological usefulness of casein N with somewhat more methionine, it is suggested that a 3% methionine supplement imparts maximal usefulness to casein. That slightly less methionine might be equally effective is not ruled out by these data.

Calculation of the sulfur amino acid intakes of the dogs indicates them to have been maintained in nitrogen equilibrium with about 30 mg of sulfur amino acids per kilogram per day, 11% or less having been contributed by cystine.

ACKNOWLEDGMENT

The technical assistance of D. J. Bradley, Jr., in these studies is gratefully acknowledged.

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STUDIES ON THE INFLUENCE OF ANTIBIOTICS AND METHIONINE ON NITROGEN UTILIZATION AND BASAL METABOLISM OF THE GROWING MALE ALBINO RAT

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ONE FIGURE

(Received for publication January 7, 1954)

Investigations into the mechanisms by which antibiotics may stimulate growth have shown two situations in which a clear-cut function may be cited: disease-level control (Coates et al., '52) and a sparing effect on water-soluble vitamins (Guggenheim et al., '53; Lih et al., '51; and Sauberlich, '52). Studies on utilization of energy and protein as affected by antibiotics fed to rats indicate that storage of energy as fat but not as protein is increased by streptomycin or by a combination of streptomycin, aureomycin and terramycin (Black and Bratzler, '52; Knoebel and Black, '52) or by terramycin (Hartsook and Johnson, '53). The influence of antibiotics on growth produced by chick rations balanced and unbalanced with respect to amino acids seems to vary with the amino acid deficiency in the basal diet. Antibiotic responses were obtained on methionine-low rations (Patrick, '52) but not on lysine-low or tryptophane-low rations (Biely et al., '52).

In the present study, the effects on nitrogen metabolism and basal metabolic rate of supplementing a soybean oil meal diet with methionine or with a mixture of chloromycetin and streptomycin, or both, were investigated, using weanling male albino rats as experimental subjects.

MATERIALS AND METHODS

The experiments described herein were conducted in two sections, one during the winter of 1952 and the other during the following spring. A total of 45 weanling male albino rats of the Sprague-Dawley strain were used. Four groups of 5 animals each were fed in each section of the experiment and an additional group of 5 animals was sacrificed at the start of section 2 to provide basic data from which gains of body constituents could be calculated from carcass analysis. The rats were selected to provide equal average starting weights between groups. Equalized daily feed intake was practiced for all animals in each section of the experiment.

TABLE 1

Percentage composition of experimental diets

INGREDIENT	DIET DESIGNATION				
	A	B	C [1]	D	E
	Section 1			Section 2	
	%	%	%	%	%
Salts 446 [2]	3.0	3.0	3.0	3.0	3.0
NaCl	1.0	1.0	1.0	1.0	1.0
Vitamin mix 691 [3]	5.0	5.0	5.0	5.0	5.0
Cod-liver oil	1.5	1.5	1.5	1.5	1.5
Wheat germ oil	.5	.5	.5	.5	.5
Lard	5.0	5.0	5.0	5.0	5.0
Sucrose	10.0	10.0	10.0	10.0	10.0
Soybean oil meal 112	23.7	23.1	...	...	...
Soybean oil meal 110	...	...	...	24.8	24.4
DL-methionine	...	.3	...	...	.3
Dried egg	...	...	5.3	...	...
Corn oil	2.7	2.7	2.9	2.6	2.6
Woodflock [4]	1.1	1.1	2.0	.8	.8
Cerelose [5]	23.2	23.4	31.9	22.9	23.0
"Modified" cornstarch [6]	23.3	23.4	31.9	22.9	22.9

[1] Diet C was used in both sections.

[2] Spector ('48).

[3] Forbes and Vaughan ('54).

[4] Distributed by Brown Co., Portland, Me.

[5] Corn Products Sales Co., Norfolk, Va.

[6] Amidex. Corn Products Refining Co., New York, N. Y.

The composition of the experimental diets is shown in table 1. All diets were designed to contain 12% crude protein, 10% ether extract and 2% crude fiber, except that the low-nitrogen diet contained 4% protein of whole egg origin. The antibiotic mixture was added to the diet so as to provide 100 p.p.m. streptomycin and 20 p.p.m. chloromycetin.

The treatments given afforded a factorial design and the data were analyzed statistically by the analysis of variance. The experimental design is shown below:

	Section 1	Section 2
TREATMENT	TEST DIET	TEST DIET
ma	A	D
mA	A plus antibiotic	D plus antibiotic
Ma	B	E
MA	B plus antibiotic	E plus antibiotic

Thus there are available, within and between sections, comparisons of the basal treatment "ma" with the basal plus antibiotic "mA," basal plus methionine "Ma," and basal plus methionine and antibiotic "MA." In the above designation, diets A and D are basal and diets B and E contain added methionine as indicated in table 1.

In both sections, the biological value of dietary protein was determined by the Thomas-Mitchell procedure, employing the test diets for a two-week period followed by a two-week period of feeding diet C, containing 4% protein of whole extracted egg. In section 2, rats were returned to their original test diets following the low-nitrogen period (diet C) and were fed for three additional weeks during which time basal metabolic rate determinations were made by the Haldane gravimetric procedure. Following this three-week period the animals were sacrificed and analyses made for total nitrogen, ether extract and water content of the carcasses.

Total nitrogen was determined on feed, feces, urines and carcasses by the Kjeldahl-Wilfarth-Gunning method, using mercury as a catalyst. Ether extract was determined on ground dried carcass samples using petroleum ether in a continuous extraction apparatus. The carcass samples were

TABLE 2

Summary of data

	TREATMENT	BIOLOGICAL VALUE	NITROGEN BALANCE	DIGESTIBILITY OF FEED NITROGEN		DAILY ENDOGENOUS NITROGEN	DAILY METABOLIC FECAL NITROGEN
				Apparent	True		
		%	%	%	%	*mg/gm*$^{3/4}$	*mg/gm food*
Section 1.	ma	62.27	40.91	89.08	93.21	.643	.806
	mA	61.42	43.14	90.12	94.03	.505	.778
	Ma	84.93	61.77	89.03	93.84	.623	.846
	MA	82.20	62.93	90.28	94.47	.512	.797
Section 2.	ma	58.91	32.73	82.21	88.60	.687	1.295
	mA	59.01	37.12	84.68	90.11	.547	1.096
	Ma	80.78	52.91	83.84	89.97	.677	1.239
	MA	80.74	55.88	86.50	91.96	.617	1.105
Pooled variance		3.80	4.81	1.98	1.60	.011	.018

		BASAL METABOLIC RATE (Cal./m²/hr.)	GM CARCASS GAIN				CREATININE N (% of endogenous N)
			Body wt.	Nitrogen	Ether extract	H_2O	
Section 2. (cont'd.)	ma	36.4	85.7	2.78	16.27	49.0	2.79
	mA	34.5	89.6	2.87	17.60	51.6	3.87
	Ma	34.4	104.2	3.43	17.46	62.6	2.66
	MA	33.9	107.7	3.63	16.19	65.0	2.77
Variance		2.58	10.5	.015	10.5	12.8	.41

dried in vacuo at 60°C. prior to ether extraction, and loss of weight resulting from this treatment was taken as a measure of the water content of the sample. Creatinine was determined on the urine by the method of Hare ('50). Basal metabolic rates were determined in 6-hour periods commencing 20 to 22 hours after the animals had finished a day's feed; the Haldane gravimetric procedure was used at 30°C., and the data are expressed as Calories per square meter per hour after calculating the body surface by the formula of Lee ('29).

TABLE 3

True digestibility and biological value of two soybean oil meal preparations

SOYBEAN OIL MEAL	THIS TEST		HAMILTON	
	TDC	BV	TDC	BV
110	88.60	58.91	90.8	63.9
112	93.21	62.27	92.0	65.0

RESULTS

The summarized data are shown in table 2 where each figure shown is the average of 5 observations with exception of the creatinine data. Two urine collections were made from each animal in the course of the biological value determination and creatinine was determined on both samples. Since the calculated percentage of endogenous nitrogen due to creatinine was similar for each treatment in each period, all values were averaged, giving 10 observations per treatment.

The statistical analysis of the data is shown in tables 4 and 5. The analysis of variance of the pooled data (table 4) showed highly significant differences in metabolic fecal nitrogen, apparent and true digestibility of protein, nitrogen balance and biological value between sections of the experiment. Except for the metabolic fecal nitrogen these differences may be ascribed to the different soybean oil meal preparations used. Unpublished data from this laboratory (Hamilton, '53) support this idea as shown in table 3. The difference in

TABLE 4

Analysis of variance of pooled data

SOURCE	DEGREES OF FREEDOM	BIOLOGICAL VALUE		NITROGEN BALANCE		DIGESTIBILITY OF FEED NITROGEN				ENDOGENOUS NITROGEN		METABOLIC FECAL NITROGEN	
						Apparent		True					
		SS	V	SS	V	SS	V	SS	V	SS	V	SS	V
Total	39	4959		4786		394		222		.555		2.144	
Between sections	1	81	81 [2]	559	559 [2]	283	283 [2]	139	139 [2]	.038	.038	1.422	1.422 [2]
Between treatments	3	4745	1582 [2]	4058	1353 [2]	42	14 [2]	27	9 [2]	.135	.045 [1]	.107	.036
Error	35	133	3.8	168	4.81	69	2	55	1.6	.382	.011	.615	.018

Analysis of variance of data from section 2

SOURCE	DEGREES OF FREEDOM	BASAL METABOLIC RATE		GM CARCASS GAIN							
				Body weight		Nitrogen		Ether extract		Water	
		SS	V	SS	V	SS	V	SS	V	SS	V
Total	19	59.80		1911		2.84		175		1142	
Between treatments	3	18.55	6.18	1743	581 [2]	2.60	.867 [2]	8.5	2.8	937	312 [2]
Error	16	41.28	2.58	168	10.5	.24	.015	166	10.5	205	12.8

Analysis of variance of creatinine data

SOURCE	DEGREES OF FREEDOM	CREATININE N (% of endogenous N)	
		SS	V
Total	39	24.11	
Between periods	1	.13	.13
Between treatments	3	9.61	3.20 [2]
Error	35	14.37	.41

[1] $p = .05$.
[2] $p = .01$.

metabolic fecal nitrogen between the sections cannot be explained since conditions known to influence this value did not vary.

TABLE 5

Main effects and interactions of antibiotic mixture and methionine

ITEM	CONTROL GROUP	MEAN MAIN EFFECTS		MEAN INTERACTION
	Mean	Antibiotic	Methionine	
Biological value	60.59	— .88	21.8 [1]	— 1.01
Nitrogen balance	36.82	2.78 [1]	19.9 [1]	— 1.34
Apparent digestibility	85.65	1.86 [1]	.89	.20
True digestibility	90.91	1.24 [1]	1.07 [2]	.14
Endogenous nitrogen	.665	— .11 [1]	.01	.05
Metabolic fecal nitrogen	1.05	— .13 [2]	.003	.02
Basal metabolic rate	36.4	— 1.21	— 1.30	1.40
Body weight gain	85.7	3.7 [2]	18.3 [1]	1.5
Nitrogen gain	2.78	.15 [2]	.70 [1]	.10
Ether extract gain	16.3	— 1.92	.03	— 2.6
Water gain	49.0	2.48	13.5 [1]	.2
Creatinine N (% of endogenous N)	2.79	1.08 [1]	.13 [3]	— .9 [2]

[1] $p = .01$.
[2] $p = .05$.
[3] Mean simple effect.

To localize the source of variance between treatments, analysis of the variance due to interaction between treatments was first made. Since the interactions proved to be small and of no statistical significance (table 5), the variance due to the main effects of the treatments was determined and the probability that such a variance would be obtained by chance alone was calculated. The results of this statistical treatment are shown in table 5. An exception to the above treatment exists in the handling of the creatinine data;

since a significant negative interaction was found, simple effects rather than main effects of the treatments were calculated. Thus, with this exception, the effects of methionine and antibiotic combination closely approximate the sum of their individual effects and there is no evidence of a sparing action between antibiotic and methionine.

The main effects of methionine are in agreement with what might be expected in the light of previous investigations on the nutritive value of soybean oil meal and the technique employed. The greatly increased retention of nitrogen as a result of methionine supplementation is a reflection of the methionine deficiency of the basal ration. In spite of increased gain of protein and water, the rats receiving methionine gained no more fat than did the control animals. This is probably due to the equalization of food intake between groups and the higher total maintenance requirement of the larger animals.

The data on main effects of the antibiotic mixture used provide new information which could not have been predicted. The slightly but consistently higher true digestibility of nitrogen of the antibiotic-containing diets indicates a slightly greater amount of nitrogen available for use by the rats receiving these diets. The effect of antibiotics in lowering the endogenous nitrogen excretion indicates that, of the nitrogen absorbed, less is required for maintaining the integrity of the nitrogen-containing tissues of the body, hence more is available for body gain of nitrogen. Since the biological value (per cent of absorbed nitrogen used for maintenance and for gain of nitrogen) was not changed by antibiotics, the nitrogen balance must have increased. That this situation did exist is readily ascertainable from the data.

As a result of finding, in section 1, that the endogenous nitrogen was lowered by antibiotic treatment, section 2 was planned to include basal metabolism and creatinine determinations. That our data do not show a reduction in basal metabolic rate determination corresponding to that of endogenous nitrogen may be ascribed to one or a combina-

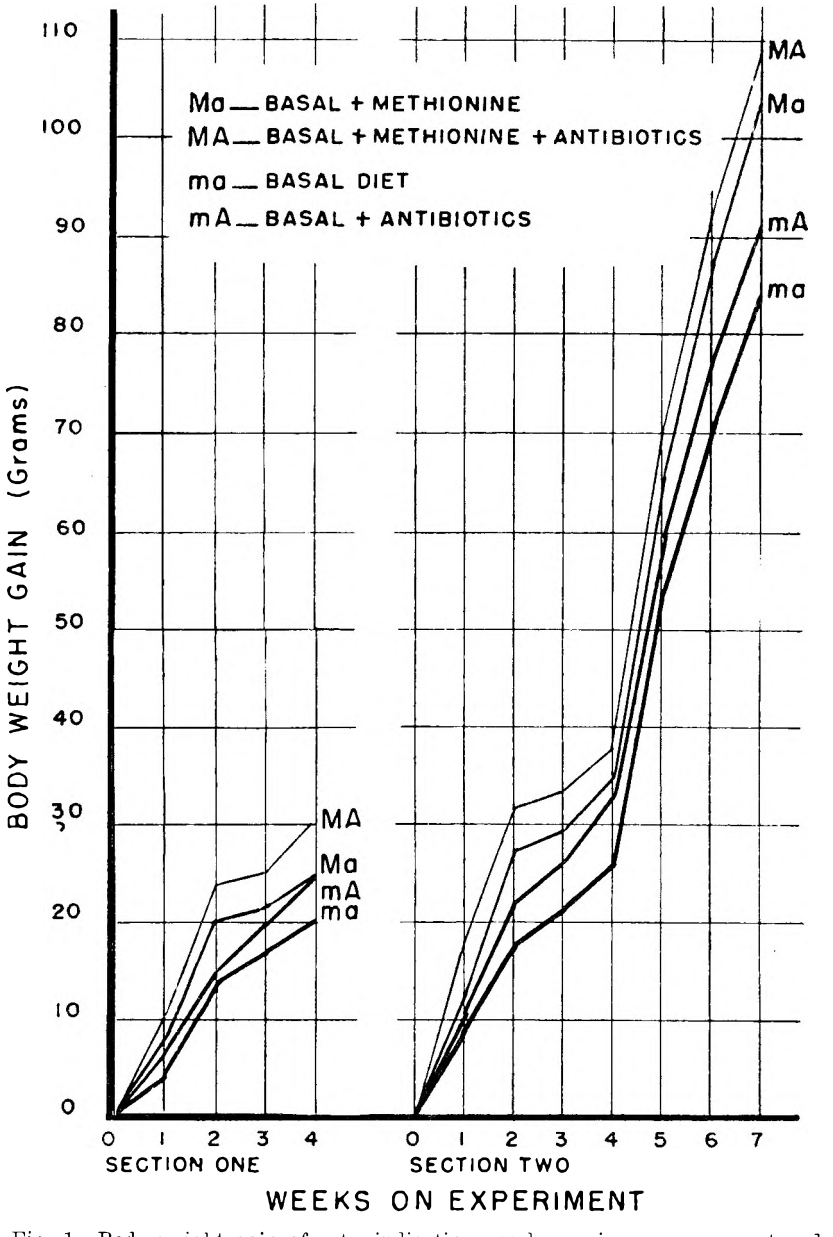

Fig. 1. Body weight gain of rats, indicating nearly maximum response at end of second week. During weeks three and 4 of each section, the 4% egg-protein diet was fed.

tion of the following circumstances. First, there may actually be a shift in the ratio of endogenous nitrogen to basal metabolism. This has been shown to occur in rats as a result of a shift in plane of nutrition (Treichler and Mitchell, '41). Munro ('50) has expressed the view that the association between protein metabolism and basal heat output is a superficial one. Second, the basal metabolism determinations were carried out one to three weeks after the endogenous nitrogen data were obtained. Thus there was opportunity for a previously existing difference in basal metabolic rate to disappear prior to the actual measurements. The weight gain data shown in figure 1 illustrate a phenomenon which has been noted in antibiotic work with chicks and pigs as well as rats (Pecora, '53); there is an initial rapid response to the antibiotic, following which the difference obtained does not increase greatly. The endogenous nitrogen data were obtained during the 4th week of experiment, while the basal metabolism data were obtained during the 6th and 7th weeks. The fact that nitrogen balance by carcass analysis was not so significantly affected by antibiotics as that determined by difference between nitrogen intake and outgo may be logically ascribed to the probability that the absolute difference in nitrogen stored was nearly as great during the second experimental week as at the end of the experiment. Hence, the difference was smaller at the end of the experiment when expressed as a percentage of the basal group.

The data regarding the contribution of creatinine nitrogen to total endogenous nitrogen indicate that different factors may be involved in regulation of metabolism leading to excretion of these products. The creatinine excretion data are considerably lower than expected from published data (Brody, '45). This is a reflection of the method used, chosen for greater specificity gained by separating the creatinine from other Jaffe-positive materials in rat urine. A comparison of the usual method of creatinine determination with that used in this study showed that only 57% of the total Jaffe-positive

substances were adsorbed on Lloyd's reagent and could thus be considered as creatinine.

The stimulus to protein but not to fat deposition obtained by antibiotic supplementation in this experiment is at variance with the findings of Hartsook and Johnson ('53), Black and Bratzler ('52), and Knoebel and Black ('52). This may be a result of difference in combination of antibiotic used or to differences in the level of protein in the diet, or both. Hartsook and Johnson, and Black and Bratzler employed diets containing much higher concentrations of protein. This would tend to obscure small differences which would more easily be detectable if the protein were fed at a level somewhat below the requirement of the rat as was done in the present experiment. The carefully detailed work of Knoebel and Black cannot be criticized on this basis. It seems probable, however, to this writer, that the length of the experimental periods and the time at which measurements are made will affect the results of experiments in the antibiotic field. And it may well be that if the present experiments had continued longer the difference in nitrogen gained as measured by carcass analysis would have lost its significance.

While the data of these experiments do not solve the riddle of the mechanism of antibiotic action they do demonstrate the application of additional criteria of antibiotic effect and thus provide a wider variety of means for investigating this phenomenon.

SUMMARY

The effects of methionine and of a mixture of streptomycin and choloromycetin on nitrogen metabolism, basal metabolic rate, and body composition of growing male albino rats were investigated, using a 2×2 factorial design and equalized food intake. The basal diet to which the above supplements were added was semi-purified and employed soybean oil meal as the sole protein source.

No highly significant interactions between supplements were observed. The highly significant effects of adding methionine with or without antibiotics were to increase biological value

of the dietary protein 36%, nitrogen balance 54%, body weight gain 20%, body nitrogen gain 25%, and body water gain 28%. The highly significant effects of adding antibiotics with or without methionine were to increase nitrogen balance 7.5%, apparent digestibility of protein 2.2%, true digestibility of protein 1.4%, and to decrease endogenous nitrogen excretion 16.8%.

ACKNOWLEDGMENT

The author acknowledges the assistance and advice of M. H. Bert in setting up the statistical analysis of the data in this report.

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STIMULATION AND INHIBITION BY ANTIBIOTICS OF INTESTINAL BACTERIA IN CHICKS[1]

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(Received for publication January 21, 1954)

It has been well established that certain antibiotics stimulate the growth of non-ruminants. Since one property common to all antibiotics is their effect upon microorganisms, the mechanisms proposed to explain acceleration of growth of animals usually involve stimulation or inhibition of microorganisms in the intestinal tract. Branion et al. ('53) have presented a review of this concept in their paper on the effect of antibiotics on the growth of ducks.

The purpose of the study reported here was to determine whether the concentrations of antibiotics which stimulate the growth of chickens receiving rations containing limiting amounts of folic acid have any effect upon the numbers of bacteria in their intestinal contents. This work is a logical extension of previous studies in these laboratories on the intestinal flora of normal chickens (Shapiro and Sarles, '49) and on the nutrition of chickens (Dietrich et al., '50; Monson et al., '50). More recently (Monson et al., '54) we have reported that the growth of chickens fed diets containing limiting amounts of folic acid was increased by supplementing

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds supplied by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

the diets with antibiotics, and that the increase in growth of the chicks fed antibiotics was accompanied by the occurrence in the intestinal tract of coliform bacteria which synthesize increased amounts of extracellular folic acid. We have suggested that this provides evidence for a mechanism by which antibiotics enhance the growth of chickens fed diets deficient in folic acid. This paper presents the results of quantitative bacteriological studies on the intestinal contents of the chicks used in these experiments, and suggests the importance of inhibition as well as stimulation of intestinal bacteria by antibiotics.

EXPERIMENTAL

The procedures used to maintain the chicks and the composition of the diet have been described fully in the previous paper (Monson et al., '54). A synthetic basal ration with three levels of folic acid was used. The high folic acid diet contained 500 μg of folic acid per 100 gm of ration: the low folic acid diets contained 25 μg of folic acid per 100 gm of ration when sucrose was the carbohydrate in the ration, and no folic acid when dextrin was the carbohydrate. Antibiotic supplements were mixed with the dry rations. Aureomycin was fed at the level of 5 mg per 100 gm of ration; a bacitracin + penicillin mixture was fed at the level of 2.5 mg of bacitracin and 10 mg of penicillin per 100 gm of ration. One-day-old chicks were divided into groups according to weight and immediately placed on test; feed and water were supplied ad libitum.

In each sampling during experiment 1, three chicks were taken at random from the appropriate diet group, killed with ether, the gastrointestinal tracts removed, and the intestinal contents immediately collected under aseptic conditions. The intestinal contents of each chick were refrigerated at 4°C. before the next chick of the group was sacrificed. The segments of the intestinal tract which were sampled separately were: duodenum, ascending and descending loops; ileum, the whole length of the ileum from the duodenum to approximately one inch above the cecal junction; and cecal pouches.

The intestinal contents obtained from three chicks in each group were pooled, mixed, and 0.5 gm of the mixed sample was weighed to the nearest milligram on waxed paper. The weighed samples were suspended by shaking with glass beads in 49.5 ml of sterile tap water. Serial decimal dilutions were made in sterile tap water from this 1:100 dilution and immediately used for microbiological work. A similar sampling procedure was followed in experiment 2 except that the individuality of the chicks was maintained. Chicks used in experiment 2 were sacrificed at the termination of the experiment and only the ileum contents were examined.

Plate counts of the numbers of organisms in the intestinal contents were made with the following media: "Difco" eosin methylene blue agar (EMB) for plate counts of coliform bacteria; thioglycollate medium (Baltimore Biological Lab.) with 1.5% agar for plate counts of "total numbers of organisms"; and the Lactobacillus-Selection Medium (LBS medium of Rogosa et al., '51, a "BBL" product) for plate counts of lactobacilli. All plates were incubated at 37°C. for 72 hours before counts were made of the colonies. The LBS medium is highly selective and favors the growth of lactobacilli including those species found in intestinal contents. It does not allow the growth of enterococci, spore forming bacteria, or coliforms.

In experiment 1, the numbers of organisms in the intestinal contents were expressed on the basis of wet weight of the contents. This was necessary because young chicks often had insufficient amounts of intestinal solids to provide material for moisture determination after the sample had been taken for microbiological analysis. Moisture determinations were made of the ileum contents in experiment 2 in which individual chicks were assayed separately; these were 4-week-old chicks and only the ileum contents were sampled. The expression of counts of microorganisms in the intestinal contents of these chicks on a dry weight basis did not alter the relative numbers of the groups of organisms studied. The average amounts of moisture in the intestinal contents of chickens

receiving the different diets were not significantly different. The average moisture content of the ileum material of 4-week-old chickens fed the different diets was as follows: high folic acid diet, 72.7%; low folic acid diet, 73.3%; low folic

TABLE 1

The effect of antibiotics on the intestinal bacteria of chicks fed a low-folic acid-sucrose diet

DAYS	DIET [1]	AV. WT. CHICKS	LEVEL	LOG_{10} NUMBER OF BACTERIA PER GRAM OF WET CONTENTS		
				Coliforms	Total count	Lactobacilli
		gm				
11	Control	93	Duodenum	8.72	8.82	8.60
			Ileum	8.73	9.04	8.55
			Cecum	9.34	10.06	10.38
	Aureomycin	98	Duodenum	6.73	7.12	6.74
			Ileum	8.20	9.00	7.84
			Cecum	10.00	10.00	9.01
18	Control	196	Duodenum	< 5.00	6.11	6.33
			Ileum	5.70	7.22	7.29
			Cecum	8.02	8.29	7.80
	Aureomycin	228	Duodenum	3.00	6.04	5.77
			Ileum	6.98	7.78	7.33
			Cecum	8.92	9.09	6.87
	Bac. + Pen.	249	Duodenum	3.48	3.78	< 3.00
			Ileum	< 4.00	< 4.00	< 4.00
			Cecum	9.70	9.60	5.70
29	Control	284	Duodenum	4.18	5.06	4.98
			Ileum	5.68	7.60	7.76
			Cecum	7.31	9.01	7.94
	Aureomycin	338	Duodenum	6.00	6.57	4.80
			Ileum	6.91	7.70	6.84
			Cecum	8.15	8.63	7.86
	Bac. + Pen.	354	Duodenum	5.11	4.98	2.74
			Ileum	6.89	6.84	2.00
			Cecum	10.81	10.79	4.39

[1] The sucrose basal diet contained 25 μg of folic acid per 100 gm of ration. Antibiotic supplements were added to this basal diet: aureomycin at the rate of 5 mg per 100 gm ration; the bacitracin-penicillin mixture at the rate of 2.5 mg bacitracin + 10 mg penicillin per 100 gm ration.

acid + aureomycin, 77.0%; low folic acid + bacitracin and penicillin, 76.6%.

RESULTS AND DISCUSSION

The data in tables 1–4 show the numbers of organisms in the intestinal contents of the control chicks and of the chicks receiving antibiotics as supplements, as well as average weights of the chicks in these groups.

TABLE 2

The effect of antibiotics on the ileum bacteria of individual chicks fed a low-folic acid-sucrose diet for 4 weeks

DIET [1]	WEIGHT OF CHICKS	LOG_{10} NUMBER OF BACTERIA PER GRAM OF WET CONTENTS		
		Coliforms	Total count	Lactobacilli
	gm			
High folic acid	345	7.25	8.09	8.92
control	405	8.13	8.68	8.13
	346	8.10	8.86	8.37
Average [2]	354 (15)	7.83	8.54	8.47
Low folic acid	262	5.48	6.64	6.00
control	309	5.59	7.85	4.54
	286	6.21	7.75	5.69
Average	289 (15)	5.76	7.41	5.44
Low folic acid	493	8.28	7.67	7.62
+	288	7.87	8.99	5.84
Aureomycin	412	7.95	8.01	5.02
Average	339 (15)	8.03	8.22	6.16
Low folic acid	365	6.76	6.93	2.67
+	361	5.08	5.76	2.00
Bac. — Pen.	372	5.18	6.62	2.00
Average	350 (15)	5.67	6.44	2.22

[1] The high folic acid diet was the sucrose basal diet containing 500 μg of folic acid per 100 gm of ration. The low folic acid diet and the antibiotic supplements to the low folic acid diet as in experiment 1 (table 1).

[2] Average weights are those of all animals in the diet group, the figures in parentheses indicate the number of chicks in these groups. The averages of the numbers of bacteria are obtained from those individual observations recorded in the table.

The efficiency of the antibiotics in sparing dietary folic acid was clearly demonstrated by the weight gains shown by the chicks receiving rations containing antibiotics. Chickens whose diets were supplemented with antibiotics, whether on the low folic acid sucrose diet or the folic acid-free dextrin diet, grew better than those chicks which received diets not supplemented with antibiotics.

The growth differences among the chicks which received these diets were accompanied by changes in the intestinal flora. In experiment 1 (table 1) a slight reduction in the numbers of organisms occurred initially in the duodenum and ileum contents of all chicks fed the sucrose diet containing antibiotics; the reduction in numbers of coliform bacteria was greatest in the duodenum. The inhibitory effect of the mixture of bacitracin + penicillin on the coliform bacteria was greater and more lasting than that of the single antibiotic, aureomycin. A reduction in the numbers of coliform bacteria continued to be apparent in the duodenum and ileum contents of the chicks fed the bacitracin-penicillin mixture after 18 days. By contrast, there were more coliform bacteria present in the ileum contents of the chicks fed aureomycin than were present in the ileum contents of the control birds at this time, although the antibiotic still restricted the numbers of coliform bacteria in the duodenum contents of these chicks. By the end of the experiment, the numbers of coliform bacteria in the intestinal contents of the antibiotic-fed chicks were increased over those found in the control birds. Throughout the experiment the numbers of coliform bacteria in the cecal contents of the chicks fed antibiotic-supplemented sucrose diets were greater than the numbers found in the cecal contents of the control chicks. The bacitracin + penicillin combination caused its greatest increase in numbers of coliform bacteria in the lower portions of the intestinal tract. Assay of the intestinal contents of chicks for antibiotic activity following the administration of bacitracin in the diet has indicated that the antibiotic accumulates in the cecal contents.

Aureomycin had its greatest effect on coliform bacteria in the upper levels of the intestinal tract.

It should be noted, however, that while greater numbers of coliform bacteria occurred after 4 weeks in the intestinal contents of the chicks fed antibiotics than were found in the unsupplemented control chicks, the actual numbers of coliform bacteria were generally less in the intestinal contents of the supplemented chick compared to the numbers in the intestinal tracts of all of the chicks at the beginning of the experiment. The control chicks which received the sucrose diet had a decrease in the numbers of coliform bacteria. This is in agreement with Johansson et al. ('48) who found that chickens fed diets containing sucrose had fewer coliform bacteria in their intestinal contents than those fed dextrin or lactose. Apparently the action of the antibiotics in the current experiment was to maintain large numbers of coliform bacteria in the intestinal tract rather than to cause an increase over the controls in numbers of coliform bacteria. A similar result has been reported previously by Guzman-Garcia et al. ('53) with rats fed a penicillin supplement in a similar basal diet containing sucrose, but deficient in thiamine. Other workers, Anderson et al. ('52) and Cook et al. ('52), have reported that coliform bacteria often increase in numbers in the intestinal flora of chickens and turkeys fed diets containing antibiotics. Still other studies (Rosenberg et al., '52) have not demonstrated any changes in the numbers of coliform bacteria associated with the presence of antibiotics in the diet; or, as in the work of March and Biely ('52), antibiotics have been shown to cause a reduction in numbers of coliform bacteria.

The association of certain numbers of intestinal coliform bacteria with an adequate rate of gain of chicks is of interest because we have found (Monson et al., '54) that coliform bacteria obtained from intestinal contents of the chicks fed antibiotic supplements were able to synthesize greater quantities of extracellular folic acid *in vitro* than coliform bacteria isolated from intestinal contents of the control chicks. An-

derson and co-workers ('53a, b) have shown that the inclusion of antibiotics in the diets of chicks may give rise to the occurrence of "atypical" coliform bacteria in the intestinal tract, and that increased growth may result when cells of these organisms or culture filtrates of them are added to the diet of chicks. Johansson et al. ('53) showed that dietary aureomycin causes a rapid and significant change in the intestinal microflora of the rat characterized by the emergence of aureomycin-resistant bacteria. Johansson further comments on the possibility of an altered nutritional status of bacteria when resistant to antibiotics, as has been demonstrated by Gale and others with penicillin-resistant bacteria. Thus, not only may the presence of more coliform bacteria in the intestinal tract be of significance but also of importance may be the nutritional requirements of these coliform bacteria which occur in the intestinal contents of animals receiving antibiotics.

In experiment 2, the numbers of coliform bacteria in the intestinal contents of the chicks that received the low folic acid control diet were lower than those in the intestinal contents of the chicks that were fed the high folic acid diet. The corrective effect of aureomycin was pronounced: the numbers of coliform bacteria in the intestinal contents of the chicks fed the aureomycin-supplemented low folic acid diet approximated the numbers of coliform bacteria in the intestinal contents of the chicks fed the control diet which contained a high level of folic acid. In both experiments 1 and 2, the growth response of chicks fed the low folic acid sucrose diet supplemented with bacitracin + penicillin was virtually identical with that of chicks fed the sucrose diet which contained an adequate level of dietary folic acid. However, the combination of bacitracin + penicillin was not as effective in experiment 2 in maintaining the numbers of coliform bacteria, although the growth of the chicks which received these antibiotics was equal to that obtained in experiment 1, and superior to the growth of the chicks fed aureomycin in either experiment.

The enhanced growth of the chicks fed the bacitracin + penicillin mixture without the coincident greater numbers of coliform bacteria in the intestinal tract justifies the concept that antibiotics may have effects on the intestinal flora beyond

TABLE 3

Changes in the intestinal bacteria of 4-week-old chicks after the addition of bacitracin + penicillin to the sucrose diet [1]

DAY	LEVEL	LOG_{10} NUMBER OF BACTERIA PER GRAM OF DRY CONTENTS [2]		
		Coliforms	Total count	Lactobacilli
0	Duodenum	4.68	5.15	4.75
	Ileum	5.34	5.74	5.00
	Cecum	8.93	9.06	8.30
1	Duodenum	6.07	6.18	2.00
	Ileum	6.23	6.63	< 2.00
	Cecum	9.28	9.35	< 3.00
2	Duodenum	4.67	4.81	< 2.00
	Ileum	7.61	7.60	2.70
	Cecum	9.40	9.24	< 3.00
3	Duodenum	5.34	5.56	< 2.00
	Ileum	6.80	7.21	< 2.00
	Cecum	9.43	9.30	< 3.00
4	Duodenum	2.60	3.47	3.11
	Ileum	7.34	7.26	3.34
	Cecum	8.92	8.97	< 2.00
5	Duodenum	6.19	6.16	1.78
	Ileum	8.19	8.02	3.50
	Cecum	9.98	9.81	6.10

[1] Control chicks from experiment 1 (table 1) which had received the low folic acid sucrose diet without antibiotic supplements for 4 weeks.

[2] The numbers of bacteria are recorded on the basis of dry weights; the counts were made on the intestinal contents of individual chicks.

their stimulation of "synthesizing" bacteria. It was found that the numbers of lactobacilli in the intestinal contents of all segments of the intestines of the chicks fed antibiotics were reduced throughout the period of the experiment. The reduction in numbers of lactobacilli was not pronounced in the chicks that received the aureomycin supplement, but in

the chicks that received the bacitracin + penicillin supplement there were marked reductions in the numbers of lactobacilli in the intestinal contents. It is significant that few lactobacilli were found in the intestinal contents of chicks that were fed the bacitracin + penicillin mixture in the diets for 4 weeks. No lactobacilli were detected when the intestinal

TABLE 4

The effect of antibiotics on the intestinal bacteria of chicks fed a folic acid-free dextrin diet

DAYS	DIET [1]	AV. WT. CHICKS	LEVEL	LOG_{10} NUMBER OF BACTERIA PER GRAM OF WET CONTENTS		
				Coliforms	Total count	Lactobacilli
		gm				
14	Control	128	Duodenum	5.70	6.18	5.70
			Ileum	7.30	7.78	7.23
			Cecum	9.15	9.49	6.70
	Aureomycin	129	Duodenum	4.00	4.30	< 4.00
			Ileum	6.00	7.34	< 5.00
			Cecum	9.72	9.38	< 5.00
30	Control	210	Duodenum	4.69	6.16	6.18
			Ileum	5.74	6.36	5.96
			Cecum	9.12	9.25	6.74
	Aureomycin	250	Duodenum	2.54	4.34	3.97
			Ileum	5.00	6.36	6.24
			Cecum	8.97	9.02	6.25
	Bac. + Pen.	258	Duodenum	6.69	6.79	< 2.00
			Ileum	6.20	6.22	< 2.00
			Cecum	9.82	9.63	< 2.00

[1] The dextrin basal diet did not contain folic acid. Antibiotic supplements were used as in experiment 1 (table 1).

contents from some of these chicks were added directly to the LBS medium. The reduction in the numbers of lactobacilli in the intestinal contents of chicks and turkeys fed antibiotics has been reported by March and Biely ('52), Cook et al. ('52) and Romoser et al. ('52). It is believed to be significant that the lactobacilli as a group exhibit high requirements for preformed growth factors.

When the control chicks from experiment 1, which had received the low folic acid diet and no antibiotic supplements, were subsequently fed the diet supplemented with the bacitracin + penicillin combination, it was found that the numbers of lactobacilli in the intestinal contents decreased almost immediately after the antibiotics were fed. Table 3 presents the results of this experiment. Six chicks were sampled, one each day, over a 5-day period immediately following the addition of bacitracin + penicillin to the ration. The results show that the numbers of coliform bacteria which occurred in the intestinal contents of the control chicks increased following the addition of the antibiotics to the diet. At the same time, the numbers of lactobacilli were reduced markedly. The use of logarithms tends to obscure the fact that a change of numbers of lactobacilli from 10^6 per gram of contents to 10^2 per gram of contents means that only one ten-thousandth of the original number of these organisms remain.

The effect of these antibiotics on chicks fed the dextrin diet which did not contain folic acid is shown in table 4. Aureomycin was not effective in maintaining or increasing the numbers of coliform bacteria but did result in a reduction in the numbers of lactobacilli in the duodenum. Again, the bacitracin + penicillin combination apparently makes the intestinal tract more suitable for the growth of coliform bacteria and effectively retarded the growth of lactobacilli. The failure of these supplements, especially the bacitracin + penicillin combination, to promote effectively the growth of the chicks in these groups in spite of the effect which they had on the intestinal flora, indicates that a minimum level of folic acid in the diet may be necessary to achieve improved chick growth by feeding antibiotics. If the coliform bacteria are held responsible for the synthesis of the folic acid in these experiments, then they may not have been able to replace completely the requirement of the chicks for folic acid although somewhat improved growth did result. The almost complete removal of lactobacilli from the intestines apparently aided the growth of the chicks; possibly this allowed more of the

folic acid synthesized by the intestinal coliform bacteria to be utilized by the host chicken.

The effect of the folic acid level of the diet is influenced by the kind of carbohydrate in the ration. The carbohydrate in the diet has been shown by Johansson et al. ('48) to influence the intestinal flora of chickens. It was shown that chicks fed diets containing dextrin had a larger proportion of coliform bacteria in their intestinal flora than chicks fed sucrose diets. This finding was corroborated in these experiments. The intestinal contents of chicks fed dextrin contained larger numbers of coliform bacteria than those of chicks fed diets containing sucrose. When dextrin is the carbohydrate in the diet, chicks may develop slowly for a considerable time in the absence of dietary folic acid (Luckey et al., '46; Monson et al., '50).

Some reports on the effects of antibiotics in promoting the growth of animals have stressed that a particular antibiotic may spare only certain growth factors and be ineffective in the prevention or correction of other dietary deficiencies. However, there is disagreement in published reports on specific examples. For example, the work of Coates et al. ('51) demonstrated that penicillin had no effect in relieving deficiencies of thiamine, riboflavin, pyridoxine or pantothenate in the chick but did lessen deficiencies of biotin or folic acid. Waibel et al. ('52) concluded that aureomycin had no sparing effect on sub-optimal levels of pyridoxine in the chick but that either penicillin or aureomycin spared the dietary requirement for thiamine. This may result from the selectivity with which a given antibiotic inhibits or stimulates microorganisms in the intestinal flora, and the synthesizing abilities or nutritional demands of the bacteria which constitute the intestinal flora of the animal maintained on a particular diet.

SUMMARY

Chicks were fed antibiotics in a synthetic ration which contained limiting amounts of folic acid. The numbers of bacteria in the contents of different parts of the intestines of these

chicks were determined at intervals. The feeding of the antibiotics increased the growth of the chicks. The increase in growth of the chicks was accompanied by changes in the numbers of coliform bacteria and lactobacilli in the intestinal tract. The antibiotics generally increased the numbers of coliform bacteria in all levels of the intestinal tract. Aureomycin was most effective in the upper levels of the tract; a combination of bacitracin and penicillin tended to be more effective in lower levels of the tract. In contrast to their effect on coliform bacteria, the antibiotics reduced the numbers of lactobacilli. The bacitracin and penicillin supplement virtually eliminated the lactobacilli in the contents of all levels of the tract. Aureomycin was less effective in depressing the numbers of lactobacilli. Thus, the antibiotics may have acted in these experiments to promote greater synthesis of growth factors by the coliform bacteria and to reduce the competition for folic acid or other essential compounds by the lactobacilli.

ACKNOWLEDGMENTS

We are indebted to Merck and Co., Rahway, N. J., for certain crystalline vitamins and for crystalline penicillin; to Commercial Solvents Corporation, Terre Haute, Ind., for bacitracin; to Wilson and Co., Inc., Chicago, Ill., for gelatin; to Abbott Laboratories, North Chicago, Ill., for haliver oil; and to E. I. DuPont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vitamin D_3.

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THE RELATIONSHIP BETWEEN DIETARY RIBOFLAVIN CONCENTRATION AND THE TISSUE CONCENTRATION OF RIBOFLAVIN-CONTAINING COENZYMES AND ENZYMES

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(Received for publication January 29, 1954)

INTRODUCTION

It has been well established that a metabolic function of riboflavin is its participation as the coenzymes flavin-adenine dinucleotide (FAD) or flavin mononucleotide (FMN) in several oxidative reactions. It would be logical to assume that the concentration of certain enzyme systems requiring the participation of these riboflavin derivatives is diminished in the riboflavin-deficient animal. That this is true has been shown for the xanthine oxidase content of rat liver (Axelrod and Elvehjem, '41) and the D-amino acid oxidase concentration in liver and kidney (Axelrod, Sober and Elvehjem, '40).

In addition, Ochoa and Rossiter ('39) found that the FAD content in the liver and heart muscle of rats maintained on a riboflavin-deficient diet was significantly less than the same organs of normal rats. Bessey, Lowry and Love ('49) have shown that a riboflavin-deficient diet caused a considerable decrease in the tissue concentration of FAD, FMN and free riboflavin especially in liver, kidney and heart. However, no study has been made to compare known dietary intakes of riboflavin with tissue concentrations of the three forms of

[1] Part of the experimental data were taken from a thesis presented by Lucile E. Decker in partial fulfillment of the requirements for the M.S. degree. Presented in part before the Institute of Nutrition at Atlantic City, 1954.

riboflavin or of tissue enzyme activity, nor has a systematic investigation of the amounts of riboflavin in the diet necessary to obtain optimal enzyme or coenzyme concentrations in the tissues been undertaken. Therefore, it seemed of interest to first measure the effect of graded intakes of riboflavin in purified diets on rat tissue FAD, FMN and free riboflavin concentrations, and second to ascertain the effect of variations in intake of riboflavin on D-amino acid oxidase and xanthine oxidase activity of liver.

EXPERIMENTAL

Treatment of animals and diets

Male and female rats of a strain developed in the Michigan State College Chemistry Department were used as experimental animals. The animals, which at the beginning of the feeding period were approximately 21 days old and weighed between 45 and 50 gm, were divided into groups of 8 with each group having an equal number of males and females.

Purified diets which differed only in the riboflavin content were fed to the animals ad libitum for 30 days. The experimental ration, a modification of the Bourquin-Sherman diet ('31) contained the following constituents in per cent: casein [2] (vitamin free) 18, sucrose 68, corn oil [3] 7, salt mixture [4] 4 and roughage [5] 3. A vitamin supplement consisted of the following amounts per 100 gm of diet: thiamine hydrochloride 0.5 mg, nicotinic acid 2.0 mg, pyridoxine hydrochloride 0.25 mg, calcium pantothenate 2.0 mg, folic acid 0.1 mg and choline chloride 100 mg. The riboflavin [6] content of the diets and the intake of the vitamin were varied as shown in tables 1 and 5.

[2] Vitamin-free test casein (14.9% N, 0.14 μg riboflavin per gram) manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio. This casein would supply about 2.5 μg of riboflavin per 100 gm of the experimental diets used. This source of riboflavin would supply about 0.2 μg vitamin B_2 per rat per day.

[3] Mazola.

[4] Hawk and Oser ('31).

[5] Ruffex, a non-nutritive fiber used as a bulk constituent in the diet and obtained from Fisher Scientific Co., Chicago, Ill.

[6] Crystalline riboflavin C. P. grade obtained from Eastman Kodak Company.

Vitamins A and D were fed as three drops of cod liver oil per rat every other day and distilled water was supplied for drinking.

A careful record of food consumption was made. Food cups, especially designed to prevent spilling were weighed every two days and any food spilled was collected on paper towels and also weighed. The animals were kept in individual, raised cages with the room temperature maintained between 75° and 78°F. The experimental diets were fed for a period of 30 days.

TABLE 1

Average dietary intake and average weight gain

DIET NUMBER	DIETARY AVERAGE		AVERAGE DAILY FOOD INTAKE	AVERAGE INITIAL WEIGHT	AVERAGE TOTAL WEIGHT GAINED	AVERAGE DAILY WEIGHT GAINED
	Riboflavin [1]	Daily riboflavin intake [2]				
	μg/100 gm diet	*μg/day*	*gm*	*gm*	*gm/30 days*	*gm/day*
1	0	0.1	4.4	47	3	0.1
2	50	3.0	5.8	47	18	0.6
3	100	6.8	6.6	46	30	1.0
4	200	18.8	9.3	50	75	2.5
5	300	30.3	10.0	47	96	3.2
6	400	43.1	10.7	50	117	3.9

[1] Riboflavin added to diet.

[2] Intake calculated from actual food consumption.

Analytical method for FAD, FMN and free riboflavin

At the end of the feeding period the animals were sacrificed with ether and the kidneys, liver, heart and brain removed as quickly as possible. The tissues were immediately frozen between sheets of solid carbon dioxide and stored at —10°C. until analyzed.

The method used for analysis of FAD, FMN and free riboflavin was the fluorimetric procedure described by Bessey, Lowry and Love ('49). Since riboflavin solutions are sensitive to light, amber glassware was employed and an Eastman safety lamp with Wrattan Filter OA used for illumination

as much as possible. All reagents were prepared with glass redistilled water, and redistilled trichloroacetic acid was used throughout.

An attempt was made to determine the free riboflavin by separation of it from the other components using extraction with benzyl alcohol as described by Bessey et al. ('49). This was unsuccessful since the amount of free riboflavin was too small to be determined accurately with the photofluorometer used. Therefore, the FMN + free riboflavin was calculated as the difference between the total riboflavin and the FAD. In a highly concentrated salt solution the fluorescence of FAD (calculated as riboflavin) is equal to 15% of the fluorescence of free riboflavin. FMN (calculated as riboflavin) and free riboflavin have equal fluorescence. If the apparent riboflavin of the initial sample is R_i and the apparent riboflavin in a sample in which FAD has been hydrolyzed to FMN is R_t, then FAD equals $\frac{R_t - R_i}{0.85}$.

In order to test the validity of using frozen tissue samples, riboflavin analyses were made on fresh tissues, tissues frozen and analyzed immediately and tissues frozen and stored at —10°C. for 6 weeks. No appreciable differences were found in the tissue FAD or FMN + free riboflavin due either to freezing or to storing while frozen.

The diets which were stored in a refrigerator at about 40°C. were analyzed for riboflavin a month after the experiment was concluded. Within the experimental error, the amount of riboflavin found was the same as the amount initially incorporated into the diets.

Measurement of tissue enzymatic activity

For studies of enzyme activity in tissue, diets 7 to 11 were fed. The riboflavin consumption when feeding these diets is shown in table 5.

At the end of the experimental feeding period, each animal was sacrificed with ether and the liver removed. The portion of liver used for D-amino acid oxidase assay was blotted and

weighed immediately, whereas the portion used for xanthine oxidase activity determination was placed on ice for a short time. The length of time between sacrificing the animal and the beginning of the measurement of D-amino acid oxidase activity was about 15 minutes, and between removal of the liver and the beginning of measurement for xanthine oxidase activity no more than 20 minutes.

The method used for assay of D-amino acid oxidase activity was a modification of the methods of Rossiter ('40) and Axelrod, Sober and Elvehjem ('40). First a weighed portion of liver was homogenized with 15 times its weight of ice cold medium (1% KCl plus 0.05 M phosphate buffer pH 7.3) in a Potter-Elvehjem mill. In all cases 1 ml of the homogenate was pipetted into a Warburg flask. The flasks also contained 0.1 ml of 40% KOH in the center well, and 0.2 ml of 4.5% DL-alanine solution, used as the substrate, was placed in the side arm. In the case of the controls, the side arm contained 0.2 ml of redistilled water instead of substrate. The vessels were equilibrated for 10 minutes in a water bath at 37.5°C., closed to the air and the substrate tipped in. The rate of oxygen uptake was measured on the manometer at 10-minute intervals. Oxygen consumption usually remained constant for 20 minutes and thereafter decreased. The D-amino acid oxidase activity is expressed as mm^3 oxygen consumed per gram wet tissue per hour.

FAD [7] was added to the flasks in some cases, and for these studies it was dissolved immediately before use in redistilled water, and 0.1 ml of this solution containing 5 μg of FAD was added to the tissue suspension in the Warburg vessel.

The method used for the determination of xanthine oxidase activity was essentially that of Litwack et al. ('53). A portion of the liver was removed and placed immediately on cracked ice. After chilling a few minutes, the liver was homogenized as described previously with 5 times its weight of ice cold medium. The homogenate was strained through

[7] FAD of 16.8% purity obtained from Sigma Chemical Company, St. Louis, Missouri.

gauze and 5 ml pipetted into each of two 25 ml flasks which were attached to a shaker mechanism (Warburg bath) and immersed in a water bath at 37.5°C. After a 40-minute incubation period, 0.3 ml of buffer and 0.6 ml of water or substrate solution (0.038 M xanthine in 0.038 M NaOH) were added. In some cases in which FAD was added, 0.1 ml of a solution of FAD in water (equivalent to 5 μg of FAD) was introduced into each of the flasks.

Aliquots were removed at convenient time intervals. The protein was precipitated and the xanthine remaining in the supernatant solution determined colorimetrically using the Folin-Ciocalteu reagent.

RESULTS

Growth rate of rats

At the end of the experimental period, the rats on diets 1 and 2 (low in riboflavin) showed typical symptoms of a riboflavin deficiency. Two animals fed diet 1 died before the experimental period was completed and the remaining animals on this diet were small and either had gained very little or had lost weight as shown in table 1. Animals on diet 2 gained more weight but in appearance resembled the animals fed diet 1. Several animals eating diet 3 also showed deficiency symptoms but to a lesser degree.

At the end of the feeding period the animals on diets 4, 5 and 6 were all healthy looking animals. A gain in weight with increased ingestion of riboflavin was observed; the largest gain, 3.9 gm per day, was obtained with diet 6 (43 μg riboflavin consumed per day). There was no noticeable growth difference between the sexes when diets containing lower amounts of riboflavin were fed. However, with diets 4, 5 and 6, the growth of the male rats was greater than that of the females. The average weight gains and the initial and final weights of the animals of each group are given in table 1. In addition to weight gains and dietary concentration of riboflavin, the actual riboflavin intake, which was calculated from the daily food consumption, is presented in tables 1 and 5.

FAD, FMN + free riboflavin and total riboflavin concentration in tissues

The results of the tissue analyses for coenzymes and total riboflavin are given in tables 2, 3 and 4. The coenzyme concentrations were calculated as riboflavin and all were expressed as micrograms of riboflavin per gram of wet tissue since previous experiments of this type have shown that calculations based on either micrograms per gram of dry tissue or micrograms per gram of tissue nitrogen express practically the same relationship as those based on wet tissue weight.

If one examines the effect of dietary riboflavin on the FAD and FMN + free riboflavin concentrations in each tissue it

TABLE 2

Average FAD content of tissues

DIET NUMBER	BRAIN	HEART	KIDNEY	LIVER
	μg FAD/gm fresh tissue [1]	*μg FAD/gm fresh tissue*	*μg FAD/gm fresh tissue*	*μg FAD/gm fresh tissue*
1	1.93 ± 0.06 [2]	9.47 ± 0.65	12.68 ± 0.52	10.61 ± 0.39
2	2.16 ± 0.06	10.43 ± 0.46	16.22 ± 0.74	12.38 ± 0.57
3	2.25 ± 0.06	12.06 ± 0.35	14.46 ± 0.71	11.71 ± 0.42
4	2.39 ± 0.05	13.96 ± 0.39	15.89 ± 2.21	17.14 ± 1.32
5	2.08 ± 0.16	13.19 ± 0.57	15.84 ± 1.03	22.07 ± 0.82
6	2.27 ± 0.06	13.75 ± 0.41	16.94 ± 0.99	19.37 ± 0.35

[1] μg FAD (calculated as riboflavin).

[2] Mean of 6, 7 or 8 analyses and the standard error of the mean.

TABLE 3

Average FMN + free riboflavin content of tissues

DIET NUMBER	BRAIN	HEART	KIDNEY	LIVER
	μg/gm fresh tissue [1]	*μg/gm fresh tissue*	*μg/gm fresh tissue*	*μg/gm fresh tissue*
1	1.01	2.54	5.43	2.23
2	0.94	2.52	7.19	2.99
3	1.08	3.03	11.21	3.77
4	1.24	4.38	14.77	7.49
5	1.12	3.93	15.20	6.05
6	1.09	3.18	14.74	5.57

[1] μg FMN (calculated as riboflavin) + free riboflavin.

is noted that in brain the concentration of these constituents is nearly independent of riboflavin intake. Small differences in brain tissue concentrations of coenzymes are noted but because of the low levels of riboflavin and its phosphorylated derivatives the experimental error of determination is large enough to account for these. In liver, however, the tissue level of FAD increased with increasing amounts of dietary riboflavin up to an intake of about 30 μg per day. Larger intakes of riboflavin did not increase the FAD further, in fact, a small drop of its tissue level was noted. Similar

TABLE 4

Average total riboflavin content of tissues

DIET NUMBER	BRAIN	HEART	KIDNEY	LIVER
	μg/gm fresh tissue [1]	*μg/gm fresh tissue*	*μg/gm fresh tissue*	*μg/gm fresh tissue*
1	2.94 ± 0.05 [2]	12.01 ± 0.36	18.11 ± 0.43	12.84 ± 0.46
2	3.10 ± 0.04	12.95 ± 0.33	23.41 ± 0.73	15.37 ± 0.64
3	3.33 ± 0.08	15.09 ± 0.50	25.67 ± 0.99	15.48 ± 0.52
4	3.63 ± 0.06	18.34 ± 0.41	30.66 ± 0.62	24.53 ± 1.99
5	3.20 ± 0.04	17.12 ± 0.46	31.05 ± 1.01	28.12 ± 0.91
6	3.36 ± 0.02	16.93 ± 0.46	31.68 ± 1.51	24.94 ± 0.36

[1] μg total riboflavin (calculated as riboflavin).

[2] Mean of 6, 7 or 8 analyses and the standard error of the mean.

results were observed for the FMN + free riboflavin concentration in liver.

In heart muscle the pattern was similar to that of liver in that both the FAD and the FMN + free riboflavin concentrations increased up to an intake of 19 μg per day (diet 4), but after feeding diets 5 and 6 the concentration of FAD remained the same whereas the FMN + free riboflavin decreased slightly.

The FAD concentration in kidney increased considerably with a daily intake of approximately 3 μg (diet 2), dropped slightly after feeding diet 3 and then gradually leveled off. The FMN + free riboflavin increased more gradually up to a daily intake of 19 μg riboflavin per day (diet 4). Larger

intakes did not appreciably change the FMN concentration in the kidneys.

It is interesting that a riboflavin deficiency greatly decreased the FMN + free riboflavin concentration in kidney and liver tissue whereas no such extensive decrease was noted in heart muscle. However, the FAD concentration in kidney was not decreased to the same extent as was the FMN concentration in this organ.

TABLE 5

Measurement of liver enzymatic activity

DIET NUMBER	RIBOFLAVIN INTAKE	D-AMINO ACID OXIDASE	XANTHINE OXIDASE
	μg/day	*mm^3 O_2/gm/hr. tissue* [1]	*μM/gm tissue/hr.* [2]
7	0.1	564 ± 37 [3]	4.9 ± 0.26 [3]
8	11.9	648 ± 259	4.9 ± 0.23
9	24.5	994 ± 210	5.2 ± 0.35
10	33.8	879 ± 108	10.1 ± 0.65
11	62.3	1,336 ± 103	11.3 ± 0.84

[1] Net O_2 consumption/gm fresh tissue/hr. resulting from the oxidation of D-alanine.

[2] Micro moles xanthine disappearing/gm fresh tissue/hr.

[3] Mean of 6, 7 or 8 analyses and the standard error of the mean.

D-*Amino acid oxidase and xanthine oxidase activity*

The results of analyses for D-amino acid oxidase activity and xanthine oxidase activity are presented in table 5. The liver xanthine oxidase activity (expressed as μM xanthine oxidized/gm tissue/hr.) after feeding diets 7 and 8 was low, and diet 9 produced only slightly greater activity. After feeding diets 10 and 11 the enzyme concentration appeared to be about twice as great as with the other three diets. The values for the enzymatic activity of D-amino acid oxidase (mm^3 O_2 consumed/gm tissue/hr.) seemed to fall into three rather distinct groups. The values for diets 7 and 8 are low; after feeding diets 9 and 10 the activity appeared to be

greater whereas the enzyme concentration after feeding diet 11 was considerably greater than with the other diets.

There was no significant effect on the enzyme activity with the addition of FAD *in vitro* with either enzyme system. Therefore, these values are not recorded in the table.

The variations in enzymatic activity with variations in riboflavin intake closely parallel the changes in tissue coenzyme concentration under the same conditions. As in the case of the coenzyme concentrations, the enzymatic activities are low on a riboflavin-deficient diet and increase gradually with increased riboflavin intake. At a dietary intake of approximately 30 μg per day the xanthine oxidase activity began to level off although D-amino acid oxidase activity increased up to the highest intake, 62 μg riboflavin per day.

DISCUSSION

In the present study it was found that when the concentration of other dietary constituents was held constant, increasing the quantity of riboflavin ingested by rats from essentially zero to about 20 μg per day resulted in an increase in the concentration of the coenzmyes FAD and FMN in brain, liver, kidney and heart, and in the enzyme xanthine oxidase, a FAD-requiring enzyme, in liver. It appeared that the concentration of coenzymes in all these tissues reached a maximum with an intake of 20 to 30 μg of riboflavin per rat per day. In comparing the coenzyme content in the 4 tissues studied, it was noted that the concentration of brain coenzymes varied least, heart more, and liver and kidney most, following variations in dietary riboflavin. Xanthine oxidase activity in liver also appeared to reach a maximum at a riboflavin intake of about 30 μg per day. However, the activity of D-amino acid oxidase which also requires FAD as a coenzyme increased over the entire range of riboflavin intakes studied, i.e., up to 62 μg per day. It is interesting to observe that the activity of D-amino acid oxidase increased while the concentration of liver FAD remained constant. It was shown previously (Rossiter, '40; Axelrod, Sober and

Elvehjem, '40) that there was a decrease in the activity of D-amino acid oxidase in the livers of riboflavin-deficient rats and our results agree with this finding. The former investigator found that when FAD was added to the enzyme system *in vitro* there was an increased oxygen uptake particularly when the enzyme preparation was from the liver of riboflavin-deficient rats. It was suggested that the protein portion of the enzyme was not altered by a riboflavin deficiency. In our *in vitro* system, however, added FAD had no effect on the rate of oxygen uptake in any instance. In addition, our experiments corroborate the findings of Axelrod and Elvehjem ('41) that there is no apparent effect of added FAD on xanthine oxidase activity *in vitro*. These authors concluded that the liver tissue from riboflavin-deficient rats did not have its normal quantity of the protein component of xanthine oxidase.

It was noted that growth increased beyond the level of riboflavin intake at which the maximum concentration of coenzymes was observed. There appear to be at least two explanations for this finding. One possibility is that whereas the total coenzyme concentration does not increase with increasing ingestion of riboflavin, the activity of some specific riboflavin-requiring enzymes may increase, as was exemplified by liver D-amino acid activity. If such an enzyme were involved in a basic metabolic reaction, raising the concentration of this enzyme might cause the greater growth rate observed in the present study. A second possibility is that the effect on growth of ingestion of increasing quantities of riboflavin is an indirect one. It has been shown (Ham and Scott, '53) that riboflavin in the diet stimulated the production of biotin by intestinal bacteria. Since biotin was not supplied in the diets of the present study, it is possible that part of the growth noted when more than 30 μg of riboflavin was ingested per day was the result of increased intestinal synthesis of biotin and possibly other B-vitamins not supplied in the diet and was not directly the result of increased riboflavin ingestion.

SUMMARY

1. The riboflavin intake necessary to maintain maximum tissue levels of riboflavin coenzymes varies with the organ and the particular coenzyme. In general, an intake of 30 μg per day was required to give maximum FAD and FMN concentrations in all tissues.

2. The FAD and the FMN + free riboflavin concentrations in brain were almost independent of the amount of riboflavin in the diet. However, in kidney and liver, coenzyme concentrations were almost doubled when the intake was increased from no added riboflavin to 30 μg per day. In the heart muscle of riboflavin-deficient animals the FAD concentration and the FMN concentration were about the same whereas in kidney and liver tissues FMN concentration was less than the FAD content.

3. The liver enzymatic activity depended on the riboflavin intake in the same manner as the coenzyme concentration. Both D-amino acid oxidase and xanthine oxidase activity increase with increased intake of riboflavin. Xanthine oxidase activity reached a maximum with an intake of about 30 μg of riboflavin per rat per day. D-Amino acid oxidase activity, on the other hand, increased up to an intake of over 60 μg riboflavin per rat per day.

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