THE EFFECT OF VITAMIN B_{12}, COBALT AND ANTIBIOTIC FEEDING ON THE COMPOSITION OF PORK TISSUE OF 100-POUND PIGS[1,2]

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Many reports have been made pertaining to the growth-promoting effects of vitamin B_{12}, trace minerals, and antibiotics in swine; however, there exists little conclusive information as to their effects on the composition of pork carcasses.

Studies at Iowa State College (Jensen et al., '52; Vohs et al., '51; Catron et al., '53) have consistently yielded data which indicate that the feeding of antibiotics does not result in increased thickness of back fat. Likewise, Wilson et al. ('53) and Robison et al. ('52) observed that antibiotics had no important influence on carcass quality. However, Bowland and McElroy ('52), Vestal ('51) and Perry et al. ('53) concluded that antibiotic supplementation produced carcasses of lower quality. In the foregoing studies 200-pound pigs were the subjects of investigation.

Ling and Chow ('51) found that rats deficient in vitamin B_{12} had lower body fat than their litter mates on the same ration but receiving vitamin B_{12}. Hove and Hardin ('51) and Hartman et al. ('49) concluded from work with rats fed varying amounts of protein that vitamin B_{12} plays an important role in nitrogen utilization. Black and Bratzler ('52)

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observed that while rats receiving vitamin B_{12} made greater gains than their controls, there was no evidence that protein metabolism is more directly associated with vitamin B_{12} than is all-over growth and energy utilization. Their studies did reveal that a vitamin B_{12}-streptomycin supplement brought about changes in body composition which were shown to involve deposition of fat in the carcass. The increase in the fat content of the carcass was attributed to the vitamin B_{12} present in the vitamin B_{12}-streptomycin feed supplement.

EXPERIMENTAL

The experimental designs used and rations fed have been reported by Kline et al. ('54). The animals were slaughtered when they reached 100 pounds live weight. Since preliminary work with young pigs fed high levels of antibiotics indicated a carry-over of antibiotics in the tissues, animals receiving the antibiotics were placed on antibiotic-free rations for three days prior to slaughter. The absence of antibiotics in the tissues of animals not receiving antibiotics three days prior to slaughter was ascertained by employing an aureomycin-sensitive organism (*Bacillus subtilis:* ATCC: 6633). Immediately following the dressing of the carcasses, the livers, kidneys, and spleens were weighed. Samples of these organs, and of the loin (longissimus dorsi at the last rib), and of the ham muscles (gracilis and adductor) were removed and immediately weighed, minced by chopping with a knife and dried under 23 to 28 inches of Hg vacuum at $75 \pm 5°$ C. for 16 to 24 hours to determine moisture content. The water content of the tissues was calculated on a fat-free basis. The water-free tissues, after grinding in a mortar, were subjected to ether extraction for 8 hours to determine the fat content. Samples of fat and water-free tissues were then analyzed for total nitrogen and phosphorus. The total nitrogen and phosphorus contents of the tissues are reported on a fat and water free basis.

The carcass measurements were made after the carcasses were chilled for 24 hours at 34 to 36° F. Specific gravity data

were obtained on these carcasses by weighing in air and in water.

The breaking strength of both femurs was determined, as described by Roberts ('53), after the bones were freed of muscle tissue. Fat, organic matter and ash were determined on the dried ground bones.

The vitamin B_{12} assays were made on liver, spleen, and kidneys which were sharp-frozen at — 30° F. for 24 hours and stored at 0° F. until analyzed. Loin and ham tissues were sharp-frozen after the carcasses were cut (24 hours after slaughtering). After sharp-freezing, these samples were stored at 0° F. All frozen samples were wrapped in aluminum foil to minimize moisture losses. Samples for the vitamin B_{12} assays from the frozen glandular tissues and of the longissimus dorsi, gracilis and adductor muscle tissue were obtained by coring with a motor-driven half-inch drill.

L. leichmannii 313 (ATCC 7830), maintained on yeast, peptonized milk and dextrose medium fortified with added vitamin B_{12} was used for the vitamin B_{12} assays. Transfer of the culture was made every two days. The basal medium finally selected for the vitamin B_{12} analysis was essentially that developed by the U.S.P. collaborative committee. The magnesium content of the media was doubled and cysteine HCl (5 mg per tube) was used as the reducing agent. Crystalline vitamin B_{12} [3] was used as the standard. The assays were carried out with a total volume of 10 ml per tube. The tubes were autoclaved for 5 minutes at 15 pounds pressure and inoculated with one drop of a 12 to 16 hour inoculum (washed three times with 10 ml lots of autoclaved basal medium and suspended in 20 ml of basal medium before use). Assay tubes were incubated for 15 to 18 hours at 37.5° C. Turbidity readings were made in an Evelyn colorimeter using the 660 filter. While considerable work was done on the preparation of the tissue for the vitamin B_{12} assay and on the influence of reducing agents on the assay values for this vitamin, detailed results are not shown since several reports relative to these problems have appeared since this study was made (Scheid and Schweigert, '51; Broquist et al., '51, and Cooperman et al., '51).

A summary of the influence of various sample treatments on the apparent Vitamin B_{12} content of several tissues is

[3] Lederle, lot No. Np 94–73–5.

shown in table 1. Blank corrections for vitamin B_{12} were applied to samples treated with pancreatin and crude trypsin.

Crude preparations of all but one sample of pancreatin gave high blanks. In the case of muscle tissue, where the apparent vitamin B_{12} content is always low, the blank correction often accounted for more than 25% of the total assay value. Crystalline trypsin was therefore used for the "release" of vitamin B_{12} from muscle. The autoclaving of samples with small

TABLE 1

Apparent vitamin B_{12} content of tissues as influenced by pre-assay treatment

SAMPLE [1]	NO. OF TRIALS	TREATMENT	APPARENT VITAMIN B_{12}
			μg/gm
Lamb liver	2	20 mg pancreatin	9.3
Lamb liver	2	20 mg crude trypsin	11.2
Lamb liver	2	Autolysis 24 hours	7.0
Pork liver	6	20 mg pancreatin	12.2 ± 0.6 [2]
Pork liver	8	40 mg pancreatin	12.2 ± 0.3
Pork liver	3	Autolysis 24 hours	8.0 ± 0.5
Pork liver	3	Autoclave 3 min. at 15 lbs. with 20 mg KCN	11.0 ± 0.5
Pork liver	3	Autoclave 3 min. at 15 lbs. with 40 mg KCN	10.0 ± 0.9
Pork liver	2	Autoclave 3 min. at 15 lbs., no KCN	11.0
Pork liver	2	Autoclave 3 min. at 15 lbs. with 10 mg KCN	7.5
Pork liver	2	10 mg crystalline trypsin	7.5
Pork liver	2	Autolysis 24 hours	5.0
Pork loin tissue	4	10 mg crystalline trypsin	0.8 ± 0.2
Pork loin tissue	2	Autoclave 3 min. at 15 lbs. with 10 mg KCN	0.5
Pork loin tissue	2	Autolysis 24 hours	0.5
Pork loin tissue	3	20 mg pancreatin	0.5

[1] Fifty milliliters of homogenate containing 1 gm of tissue and incubated under toluene at 37°C. at pH 7.5 for 24 hours was used for all enzymatic digestions. Samples treated with KCN and samples subjected to autolysis were adjusted to pH 7.0 and incubated for 24 hours at 37°C.

[2] Standard deviation of the means of three to 8 independent assays. The mean value for each assay is the average of duplicate determinations at 4 levels of tissue.

amounts of KCN gave maximum values for apparent vitamin B_{12} in liver, spleen and kidney. The response of the organism to increasing amounts of muscle tissue homogenate was much less marked than that observed when liver tissue was used. This probably indicates that the apparent vitamin B_{12} activity of the muscle was not exclusively due to vitamin B_{12}. For this reason direct comparisons between apparent vitamin B_{12} activity of muscle tissue and that of liver, spleen and kidney tissue are probably not valid. Shive et al. ('48) have reported that desoxyribosides or related substances influence the microbiological values of vitamin B_{12} in tissues low in vitamin B_{12}. Attempts to use the alkali correction method for measuring interfering substances gave highly variable results and therefore it was not employed. The influence of alkali on assay values has been studied by Scheid and Schweigert ('50). The values reported in this study for vitamin B_{12} are therefore more accurately described as apparent vitamin B_{12} activity.

RESULTS

Statistical evaluation of the carcass data is shown in tables 2 and 3. In the animals with no antibiotic supplement it was found that additions of vitamin B_{12}, of cobalt, or the combination of vitamin B_{12} and cobalt did not influence back fat thickness, specific gravity or percentage of lean cuts. In the antibiotic-fed pigs an increase in back fat thickness and a lower specific gravity was found to be associated with both the feeding of vitamin B_{12} and of vitamin B_{12} with cobalt. The weights of the various organs were found to vary within rather narrow limits. However, on the average, the pigs fed the antibiotic-supplemented rations and receiving vitamin B_{12}, cobalt, or a combination of vitamin B_{12} and cobalt had livers lighter in weight than those of the basal group (see the interaction $B_{12} \times Co$ in table 3).

The feeding of vitamin B_{12} in the absence of antibiotics was associated with a significantly lower breaking strength of the femurs. The differences in the bone weight, marrow weight and fat content of the bone were not statistically significant.

In the presence of antibiotics no differences in bone data could be found.

The analytical data for the kidney, liver, spleen, and loin tissues are summarized in table 4.

The only significant difference in the water content, on a fat-free basis, of the various tissues was found in the kidney

TABLE 2

Summary of main treatment comparisons for carcass data, bone data, and organ weights

ITEM	NO ANTIBIOTIC				ANTIBIOTIC			
	No B_{12}	B_{12}	No Co	Co	No B_{12}	B_{12}	No Co	Co
Carcass data								
Back fat, in.	1.1	1.1	1.2	1.0	1.1	1.2	1.1	1.2
Specific gravity	1.042	1.035	1.039	1.038	1.041	1.038	1.041	1.038
Lean cuts, %	55.01	55.08	53.78	56.30	53.86	52.48	53.78	52.56
Organ data								
Liver wt., gm	1088	1054	1081	1060	962	912	949	925
Kidney wt., gm	181	182	183	180	181	178	180	179
Spleen wt., gm	63	58	59	62	67	71	72	71
Bone data								
Bone wt., gm	126	124	123	126	127	134	129	132
Breaking strength, lb./sq. in.	638	543	565	543	703	760	728	735
Marrow wt., gm	9.5	6.2	8.1	7.5	4.8	3.8	4.7	4.0
Bone fat, %	17.9	16.1	17.3	16.7	13.9	13.3	13.5	13.8

tissue from the animals fed vitamin B_{12} and cobalt in the absence of the antibiotics.

There was considerable variation in the fat content of these tissues. Significant differences were found in fat content for loin muscle and for kidney tissue from the animals with no antibiotic supplement. The data show that the dif-

TABLE 3

Analyses of variance of carcass data, bone data, and organ weights

SOURCE OF VARIATION	D.F.	MEAN SQUARES									
		Back fat [1]	Specific gravity [1]	Per cent lean cuts	Bone wt.[2]	Break strength [2]	Bone marrow wt.[2]	Bone fat per cent [2]	Liver wt.	Kidney wt.	Spleen wt.
No antibiotic											
Litters	2	65	0.16	2.56	15.00	450	5.78	14.60	40,096	902	143
Treatments	3	151	0.35	8.81	35.00	8,500	12.00	3.65	2,044	48	27
Vitamin B_{12}	1	70	0.80	0.02	10.58	18,050 [4]	22.44	6.85	3,400	3	65
Cobalt	1	330	.. [3]	19.00	18.60	5,000	0.72	0.72	1,323	21	16
$B_{12} \times Co$	1	52	0.24	7.43	75.64	2,450	10.10	3.38	1,408	120	.. [3]
Exp. error	6	343	0.22	6.51	74.66	1,017	5.43	8.75	956	286	36
Antibiotic											
Litters	2	53	0.11	3.69	72.61	3,612	3.91	0.89	14,896	520	9
Treatments	3	263	0.31	10.27	50.33	3,746	3.96	20.12	14,884	201	83
Vitamin B_{12}	1	341 [4]	0.30 [4]	5.67	85.15	6,613	1.80	0.72	7,500	7	44
Cobalt	1	48	0.18	4.48	18.30	112	0.84	0.18	1,728	24	102
$B_{12} \times Co$	1	320 [4]	0.44 [4]	20.67	47.53	4,512	9.24	59.41	35,425 [4]	574	102
Exp. error	6	49	0.05	4.04	24.46	4,213	1.59	10.08	3,849	119	40

[1] Mean squares multiplied by 10^4.

[2] D.F. for litters 1, for error 3 for these characters.

[3] Less than 0.01.

[4] Significant at $P = 0.05$ or less.

TABLE 4

Summary of average values for certain fractions in pork tissue

DESCRIPTION	TISSUE TREATMENT	KIDNEY			LIVER			SPLEEN			LOIN		
		No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.
No-antibiotic													
Water, %	No Co	80.5	81.8	81.1	70.3	70.5	70.4	79.2	76.6	77.9	75.4	75.3	75.4
	Co	81.6	79.9	80.8	70.9	71.3	71.1	79.0	78.1	78.6	75.6	74.6	75.0
	Av.	81.1	80.9 [1]		70.6	70.9		79.1	77.4		75.5	75.0	
Fat, %	No Co	16.9	20.3	18.6	12.6	14.9	13.8	16.3	20.3	18.3	20.4	30.1	25.3
	Co	15.8	15.1	15.5 [2]	14.0	10.9	12.5	16.8	21.7	19.3	13.7	17.1	15.4 [2]
	Av.	16.4	17.7		13.3	12.9		16.6	21.0		17.1	23.6	
Nitrogen,[3] %	No Co	14.0	13.7	13.9	11.1	11.4	11.3	13.6	13.8	13.7	13.8	14.6	14.2
	Co	13.7	12.9	13.3	11.5	11.3	11.4	13.6	12.9	13.3	14.5	13.7	14.1
	Av.	13.9	13.3		11.3	11.4		13.6	13.4		14.2	14.2	
Phosphorus,[3] mg/gm	No Co	11.6	13.8	12.7	10.5	12.0	11.3	11.9	13.3	12.6	8.9	9.4	9.2
	Co	11.6	10.8	11.2 [2]	11.3	10.5	10.9	12.7	11.2	11.9	9.4	8.6	9.0
	Av.	11.6	12.3	[1]	10.9	11.3	[1]	12.3	12.3	[1]	9.2	9.0	[1]
Vitamin B_{12} μg/100 gm	No Co	1.0	6.0	3.5	2.0	9.0	5.5	0.6	2.0	1.3	0.5	0.8	0.7
	Co	6.0	9.0	7.5 [2]	3.0	8.0	5.5	1.0	2.0	1.5	0.8	0.8	0.8
	Av.	3.5	7.5 [2]		2.5	8.5 [2]	[1]	0.8	2.0 [2]		0.7	0.8	
Cobalt,[3] PPM	No Co				.07	.06	.07				.22	.21	.22
	Co				.32	.38	.35 [2]				.18	.79	.49
	Av.				.20	.22					.20	.50	
Antibiotic													
Water, %	No Co	80.8	80.3	20.6	70.1	71.5	70.8	78.7	78.3	78.5	72.7	72.5	72.6
	Co	80.1	80.6	80.4	70.0	70.1	70.1	77.4	79.0	78.2	73.3	72.1	72.7
	Av.	80.5	80.5		70.1	70.8		78.1	78.7		73.0	72.3	
Fat, %	No Co	13.0	12.7	12.9	9.9	9.5	9.7	10.4	11.0	10.7	16.1	15.6	15.9
	Co	14.9	13.6	14.3	11.3	11.4	11.4	12.4	12.0	12.2	15.9	21.4	18.7
	Av.	14.0	13.2		10.6	10.5		11.4	11.5		16.0	18.5	
Nitrogen,[3] %	No Co	14.2	13.2	13.7	10.2	10.6	10.4	14.2	12.9	13.6	15.3	13.3	14.3
	Co	13.0	13.9	13.5	12.1	10.8	11.5	14.6	14.3	14.5	15.1	15.1	15.1 [2]
	Av.	13.6	13.6		11.2	10.7		14.4	13.7		15.2	14.2 [2]	[1]
Phosphorus,[3] mg/gm	No Co	13.0	12.8	12.9	10.0	11.7	10.9	12.5	12.6	12.6	9.3	8.6	9.0
	Co	13.1	13.4	13.3	12.2	11.5	11.9	13.9	13.2	13.6	10.0	9.2	9.6
	Av.	13.1	13.1		11.1	11.6		13.2	12.9		9.7	8.9	
Vitamin B_{12} μg/100 gm	No Co	2.0	10.0	6.0	3.0	15.0	9.0	0.7	2.0	1.4	1.0	1.0	1.0
	Co	2.0	9.0	5.5	2.0	9.0	5.5	0.7	2.0	1.4	1.0	1.0	1.0
	Av.	2.0	9.5 [2]		2.5	12.0 [2]		0.7	2.0 [2]		1.0	1.0	
Cobalt,[3] PPM	No Co				.06	.08	.07				.11	.17	.14
	Co				.19	.33	.26 [2]				.27	.16	.17
	Av.				.13	.21					.19	.17	

[1] Interaction effect significant at P = 0.05 or less.

[2] Average effect significant at P = 0.05 or less.

[3] Water- and fat-free basis.

ferences were associated with the effect of adding cobalt to the rations. Apart from these considerations there were no consistent relationships between the ration fed and tissue fat content.

The data on nitrogen content failed to show any consistent effect which could be related to the variations in ration treatments.

The relationship between rations fed and total phosphorus content of the tissue was more impressive. The animals fed vitamin B_{12} in the trial without antibiotic had higher levels of tissue phosphorus than their controls on the basal ration. Interaction effects of vitamin B_{12} and cobalt were significant. Only in the kidney tissue could it be shown that the phosphorus content was significantly affected by cobalt feeding. When antibiotic-supplemented rations were fed the differences in the phosphorus content of the tissues disappeared.

The microbiological values for vitamin B_{12} show that assay values for apparent vitamin B_{12} content in the three glandular tissues obtained from animals in both the antibiotic-free and antibiotic-feeding trials were significant. The data for loin muscle tissue revealed no differences.

Antibiotic feeding did not appreciably influence the microbiological assay values for vitamin B_{12} in the tissues since consistent assay values were obtained.

The addition of cobalt to the ration significantly increased the cobalt concentrations in the liver in both the presence and absence of antibiotics but was without effect on loin tissue.

DISCUSSION

The differences in the carcass composition of these animals as determined by dressing per cent, back-fat thickness, specific gravity, or per cent of lean cuts were not consistent when the animals were fed vitamin B_{12}, cobalt, or the combination of vitamin B_{12} and cobalt. However the carcasses from pigs on the ration supplemented with antibiotics and receiving vitamin B_{12} or vitamin B_{12} with cobalt were fatter as measured by back fat thickness and specific gravity.

The large differences in the fat content of the rat carcasses observed by Black and Bratzler ('52) in their studies on the influence of vitamin B_{12} on the carcass composition of the rat were not found in the data obtained from the pigs receiving vitamin B_{12}.

Although some differences were found, the tissue data did not provide conclusive evidence that the feeding of vitamin B_{12} or cobalt either in the absence or presence of antibiotics had any consistent effect on the nitrogen, fat and water content of the kidney, liver, spleen or loin muscle tissue.

There appeared to be no important differences between the levels of apparent vitamin B_{12} in the tissues of animals fed the rations with no antibiotics and the levels of this vitamin in tissues of animals on the corresponding rations with antibiotic supplementation. From these data, one may conclude that antibiotics appear to have no influence on the assay values for vitamin B_{12} in pork tissue.

Differences in the amounts of apparent vitamin B_{12} in the several tissues of the animals on the various rations containing antibiotics could not be correlated with growth performance since the difference in average daily gains and amount of feed required per 100 pounds gain among these animals were statistically non-significant, Kline et al. ('54).

The animals in these studies had lower levels of apparent vitamin B_{12} in the livers than those observed by Richardson et al. ('51) (3.0 μg as compared to the 5.7 μg) in their studies of the vitamin B_{12} requirement of the young pig. They also reported that a vitamin B_{12} deficiency developed on an all plant vitamin B_{12}-deficient basal ration containing a mixture of 10 mg each of aureomycin, streptomycin, terramycin and procaine penicillin G. Since their animals grew poorly because no vitamin B_{12} was supplied in the ration, it was suggested that the antibiotics inhibited vitamin B_{12} synthesis in the intestinal tract. The absence of any observable nutritional deficiency and the excellent growth performance of the animals in the present study (average daily gain was 1.36 pounds) when the much higher level of antibiotics (500 mg per pound of

ration) was fed requires some alternative explanation. Monson et al. ('52) have reported that antibiotics did not affect the levels of pantothenic acid, vitamin B_6, niacin, and vitamin B_{12} in the liver of chickens fed rations supposedly adequate in all the known vitamins. These investigators also observed that the concentrations of several water-soluble vitamins found in the chicken livers could not be correlated with the growth response. Their observations, which indicated that the growth promoting effect of antibiotic-supplemented rations cannot be explained on the basis that the faster-growing animal has larger stores of vitamin in its tissues, are supported by the present findings.

Despite the fact that the basal ration for the pigs contained a nutritionally adequate amount of cobalt in terms of the known requirements for the sheep, increased storage of this element was demonstrated when extra cobalt was supplied. Whether this increase in the amount of liver cobalt is nutritionally beneficial is questionable in view of the observation that the cobalt-supplemented animals made somewhat lower average daily gains. It has been suggested that cobalt supplementation may enhance microbial synthesis of vitamin B_{12} in the intestine. The present investigation failed to demonstrate that this occurs as measured by concentration changes of apparent vitamin B_{12} content of the muscle and organ tissues. Attempts to modify the intestinal synthesis of vitamin B_{12} by feeding as much as 1.5 gm of a mixture of antibiotics and a sulfa drug per day per animal either in the absence or presence of added cobalt failed to demonstrate changes in tissue concentrations of apparent vitamin B_{12}. High-level supplementation of antibiotics to pigs receiving rations containing no vitamin B_{12} did not produce a deficiency syndrome (Kline et al., '54).

The effect of vitamin B_{12}, in the absence of antibiotics, on the phosphorus content of the various tissues appears to be significant. The increase in tissue phosphorus which was

associated with vitamin B_{12} feeding is in harmony with the earlier observations reported by Abbott and James ('50), who found that the injection of vitamin B_{12} enhanced phosphorus retention in the rabbit. It is also noteworthy that this difference disappeared when antibiotics were fed.

SUMMARY

Weanling pigs were individually fed a purified basal ration fortified with vitamins and C.P. salts, including trace mineral elements, from weaning to 100 pounds.

Two 2×2 factorial experiments, one without and one with antibiotics, were used to study the effect of adding vitamin B_{12}, cobalt, or a combination of vitamin B_{12} and cobalt on the tissue composition of the pigs.

No relationships could be found between the vitamin B_{12} content of the tissues and the growth performance when antibiotics were fed.

The carcass quality, as measured by length of body, thickness of back-fat, specific gravity, and percentage of lean cuts, was not appreciably influenced by these treatments. The feeding of a mixture of antibiotics at high levels did not influence the vitamin B_{12} content of tissues.

In the absence of antibiotics larger quantities of phosphorus were found in the tissues when vitamin B_{12} was added to the ration.

The feeding of cobalt had no appreciable effect on tissue composition. Cobalt, when fed in the presence of antibiotics, did not appear to affect the vitamin B_{12} content of the tissue.

The vitamin B_{12} content of liver, spleen, and kidney was increased by feeding vitamin B_{12}.

This investigation and that reported by Kline et al. ('54) failed to provide information which would support adequate explanations for the improved growth performance of the young pig receiving a vitamin B_{12}-deficient, antibiotic supplemented semi-purified ration.

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MICROBIOLOGICAL VERSUS BIOLOGICAL VITAMIN B_{12} ACTIVITY IN BOVINE RUMEN LIQUOR AND FECES[1,2]

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TWO FIGURES

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The presence of a chick-growth factor in cow manure (Rubin and Bird, '46) later shown to be similar in its growth effect to vitamin B_{12} (Lillie et al., '48), suggested that vitamin B_{12} is synthesized in the gastrointestinal tract of ruminant animals. Hale et al. ('50) observed that vitamin B_{12} activity was present in dried sheep-rumen ingesta and the amount of activity was affected by the cobalt content of the ration fed. Subsequently, it has been shown that cobalt supplementation of cattle and sheep rations which were marginal in their cobalt content increased the vitamin B_{12} level in the blood, liver, milk and rumen contents (Collins et al., '51; Harper et al., '51; Hoekstra et al., '52; Moinuddin et al., '53; and Bentley et al., '53).

Vitamin B_{12}-like substances which are active for bacteria but differ structurally from cyanocobalamin and cobalamin have been found in several natural materials, including feces and rumen contents. Such substances are: pseudovitamin B_{12} and B_{12d} isolated by Pfiffner et al. ('52) in a rumen anaerobe; factors A, B and C, discovered by Ford et al. ('53) in calf

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feces; vitamin B_{12m} found by Wijmenga ('51) in pig manure; and vitamin B_{12f} from rat feces (Lewis et al., '52).

Lewis and coworkers ('52) found that vitamin B_{12f} is inactive for the rat but active for *L. leichmannii*. Coates et al. ('51, '53) reported that the chick assay determined less vitamin B_{12} activity in calf feces and rumen ingesta than was estimated by the microbiological assay using either *B. coli* or *L. leichmannii* as the test organism.

The object of the studies to be reported herein was to determine the amount of vitamin B_{12} activity in feces and rumen ingesta from adult animals using both biological and microbiological techniques. It appeared that additional comparative assay results were needed preliminary to further studies on the physiological function of vitamin B_{12} and vitamin B_{12}-like substances, since the latter are synthesized in the rumen of cattle and sheep.

EXPERIMENTAL

Rumen ingesta obtained from a rumen-fistulated steer maintained on alfalfa hay was collected in a double-layered cheesecloth bag and the liquid portion expressed using a large hand press. The liquor thus obtained was either diluted and analyzed microbiologically for its vitamin B_{12} activity or was dried on the corn portion of the rat or chick rations with the aid of fans at room temperature. Efforts to concentrate the rumen liquor *in vacuo* prior to its addition to the rations resulted in excessive losses in activity. However, microbiological analyses of the rations containing dried rumen liquor indicated that 94 to 109% of the original vitamin B_{12} activity was retained.

Microbiological: All microbiological assays were carried out using *L. leichmannii* ATCC No. 7830. The test cultures were incubated for 72 hours at 37°C. and the titrimetric method was used throughout. The assay medium used was a combination of those reported by Skegg et al. ('50), and Thompson et al. ('50). The composition of the assay medium is presented in table 1.

The carrying medium for the organisms consisted of the following ingredients:[4] peptonized milk agar, 2.7 gm; yeast extract, 1.0 gm; glucose, 1.0 gm; vitamin B_{12}, 2 mμg; and Tween 80, 0.2 ml per 100 ml. Stab culture transfers of the test organisms were made every 4 to 6 days.

Liver and kidney samples were homogenized and extracted with water (sodium cyanide added) by autoclaving for three minutes at 15 pounds pressure to release the vitamin B_{12}. Use of a buffer, as suggested by Daniel et al. ('53) or

TABLE 1

Composition of the assay medium (100 ml, double strength)[1]

Casein hydrolysate	1.0 gm	Thiamine	200 μg
Glucose	4.0 gm	Riboflavin	200 μg
Sodium acetate (anhydrous)	2.4 gm	Niacin	200 μg
Tryptophan	40.0 mg	Pyridoxine	400 μg
Asparagine	20.0 mg	Pyridoxal	400 μg
Ribonucleic acid	30.0 mg	Calcium pantothenate	200 μg
Adenine	1.0 mg	Para amino benzoic acid	200 μg
		Biotin	1 μg
Guanine	1.0 mg	Folic acid	200 μg
Uracil	1.0 mg	Tween 80	0.2 ml
Xanthine	1.0 mg	Ascorbic acid[3]	200 mg
Salts A[2]	1.0 ml	Fumaric acid[3]	100 mg
Salts B[2]	1.0 ml	Cysteine[3]	20 mg
		Distilled water to 100 ml	

[1] Potassium hydroxide used to adjust the pH to 6.5

[2] Snell, E. E., and Wright, L. D., J. Biol. Chem., *139:* 675 ('41).

[3] Added in solid form.

autoclaving for more than three minutes did not yield higher results.

Extraction of vitamin B_{12} from dried feces with boiling water (10 parts water and 1 part feces) gave satisfactory results. Autoclaving the water suspension three minutes at 15 pounds pressure gave slightly lower vitamin B_{12} values. Likewise, the use of either sodium cyanide or sodium bisulfite with either procedure did not increase the amount of vitamin B_{12} extracted from the sample (see table 2). When indicated,

[4] Private communication from Dr. J. Kastelic, Iowa State College.

the hot water extracts of feces were lyophilized in the usual manner.

Animal experiments: Weanling male rats (Holtzman) were depleted for two weeks on a ration described by Register et al. ('49) except that vitamin A and D feeding oil was mixed into the ration and iodinated casein [5] was fed at the level of 0.1% of the ration. Depleted rats, weighing between 90 and 107 gm, were divided into 12 groups and fed the depletion ration supplemented as indicated in table 3 for two weeks. The rats were kept in wire bottom cages and allowed water and the rations ad libitum. Weekly weights and total food consumption were recorded.

TABLE 2

Vitamin B_{12} activity of the dried steer feces (microbiological)[1]

SAMPLE TREATMENT	VITAMIN B_{12} ACTIVITY
	μg/gm
Boiling distilled water	1.00
Boiling distilled water + NaCN	0.91
Boiling distilled water + $NaHSO_3$	1.01
Distilled water (autoclaved)	0.90
Distilled water + NaCN (autoclaved)	0.83
Distilled water + $NaHSO_3$ (autoclaved)	0.87

[1] Average of two experiments.

At the termination of the experiments, the animals were sacrificed and livers and kidneys from all of the animals were removed. A composite sample for each dietary group was made which was kept frozen until analyzed for its vitamin B_{12} activity.

Male White Plymouth Rock day-old chicks, obtained from a commercial hatchery, were kept in electrically heated batteries with wire screen bottoms. The chicks were fed a vitamin B_{12}-depletion ration, having the following percentage composition: ground yellow corn, 46.10; soybean oil meal, 50.0; choline chloride, 0.1; DL-methionine, 0.05; vitamin A and D feeding oil (6000 I.U. A and 400 D), 0.25; steamed bone

[5] "Protamone."

TABLE 3

Effect of feces and rumen liquor on rat growth, food consumption, and the vitamin B_{12} activity in liver and kidney [1]

GP. NO.	RATION	AV.[2] GAIN	AV. FOOD CONSUMPTION	LIVER		KIDNEY	
				Vit. B_{12}	Alkali-stable vit. B_{12} activity	Vit. B_{12}	Alkali-stable vit. B_{12} activity
		gm/2 wks.	*gm/rat/2 wks.*	*mμg/gm wet wt.*		*mμg/gm wet wt.*	
1	Basal	18	265	13.9	5.0	61.5	20.1
2	Basal + 2.5 μg cryst. B_{12}/kg	42	240	20.6	8.1	83.5	20.1
3	Basal + 5.0 μg cryst. B_{12}/kg	55	262	26.2	10.0	103.0	20.4
4	Basal + 10.0 μg cryst. B_{12}/kg	68	300	38.0	11.5	160.1	18.3
5	Basal + 2.5 gm dried feces/kg [3]	54	244	23.0	...	81.2	...
6	Basal + 5.0 gm dried feces/kg	59	252	27.0	6.9	93.8	17.1
7	Basal + 2.8 gm lyophilized feces extract/kg [3]	47	304	21.8	...	72.3	...
8	Basal + 5.6 gm lyophilized feces extract/kg	43	237	22.3	7.2	73.5	21.6
9	Basal + 100 ml rumen liquor/kg [4]	36	234	19.7	...	74.4	...
10	Basal + 200 ml rumen liquor/kg	49	230	22.2	6.0	79.1	19.5
11	Basal + 2.5 μg cryst. B_{12} + 2.5 gm lyophilized feces extract/kg	58	259	19.9	6.8	84.9	16.5
12	Basal + 2.5 μg cryst. B_{12} + 100 ml rumen liquor/kg	49	239	18.2	6.4	85.6	12.6

[1] Sixteen rats per treatment (two experiments).

[2] Statistical analysis of growth data: L.S.D. 5% level, 20.82 gm; 1% level, 27.65 gm.

[3] Microbiological activity: dried feces, 1.0 μg/gm; lyophilized feces extract, 0.87 μg/gm.

[4] Microbiological activity: 28 mμg/ml.

meal, 2.0; ground limestone, 1.0; and iodized salt, 0.50. To each 100 pounds of ration the following supplements were added: $MnSO_4 \cdot H_2O$, 6.0 gm; niacin, 1.6 gm; calcium pantothenate, 0.68 gm; and riboflavin, 0.16 gm.

After the depletion period of two weeks, the chicks were divided into groups of 15 chicks each, weighing between 155 and 196 gm and continued on the basal ration supplemented as indicated in table 5. Water troughs were cleaned daily. In addition to the test materials, cobalt (1.0 mg $CoCl_2$/kg ration) was added to all of the rations to compensate for estimated amounts of cobalt that would be contributed by the rumen liquor and feces. At the end of the two-week experimental period, 8 birds from each group were sacrificed and composite liver and kidney samples were analyzed for vitamin B_{12} activity.

RESULTS

The vitamin B_{12} activity of the rumen liquor as determined microbiologically varied between 25 and 30 mμg per milliliter of which 98 to 99% was destroyed when subjected to the alkali treatment suggested by Hoffman et al. ('49).

Various procedures were used to extract the vitamin B_{12} activity from dried steer feces and, as indicated in table 2, comparable values were obtained with boiling-water extraction or with hot water to which sodium cyanide or sodium bisulfite had been added. Because of these observations, hot water extraction was used to prepare samples for microbiological assays for vitamin B_{12}.

The combined results of two rat growth assays for vitamin B_{12} activity are presented in table 3. There was no significant difference in the growth response from comparable amounts of vitamin B_{12} supplied from either crystalline B_{12} or the vitamin B_{12} activity of rumen liquor or feces, although the rats fed dried feces gained slightly more than those receiving comparable amounts of pure vitamin B_{12} (groups 2 and 3 *vs.* 5 and 6, table 3). Contrariwise, the weight gains made by rats receiving rumen liquor were somewhat less than were

expected on the basis of the vitamin B_{12} activity for *L. leichmannii* of the liquor (groups 2 and 3 *vs.* 9 and 10, table 3). In all cases the amount of feces or rumen liquor added to the test rations was based on the vitamin B_{12} content of these materials as determined by the microbiological assay. The rat growth response to the addition of graded amounts of crystalline vitamin B_{12} to the basal ration is shown in figure 1.

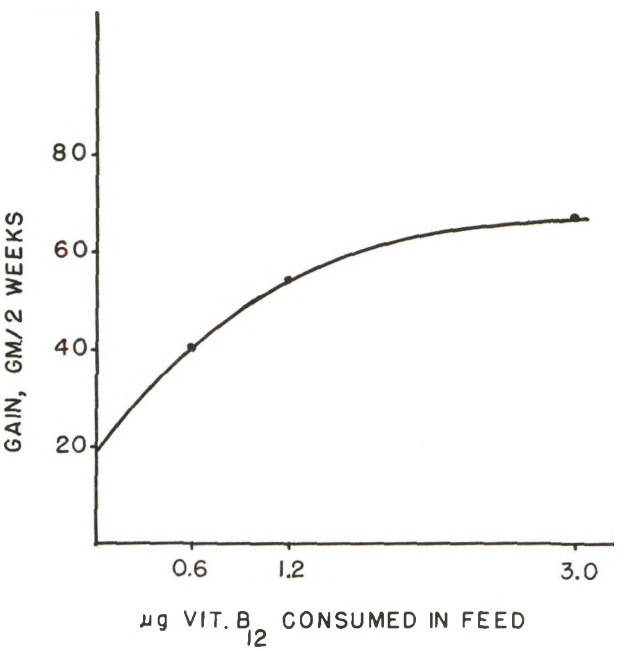

Fig. 1 The average weight gain of rats fed varying amounts of vitamin B_{12}.

As would be expected, the concentration of vitamin B_{12} activity for *L. leichmannii* in the livers and kidneys increased with increasing levels of crystalline vitamin B_{12} in the ration (see table 3). In fact, when these values were plotted against the levels of crystalline vitamin B_{12} in the diet, a linear relationship was observed, as presented in figure 2. This observation afforded an opportunity to compare the microbiological versus the biological activity of the test materials not only on the basis of growth data but also on the basis

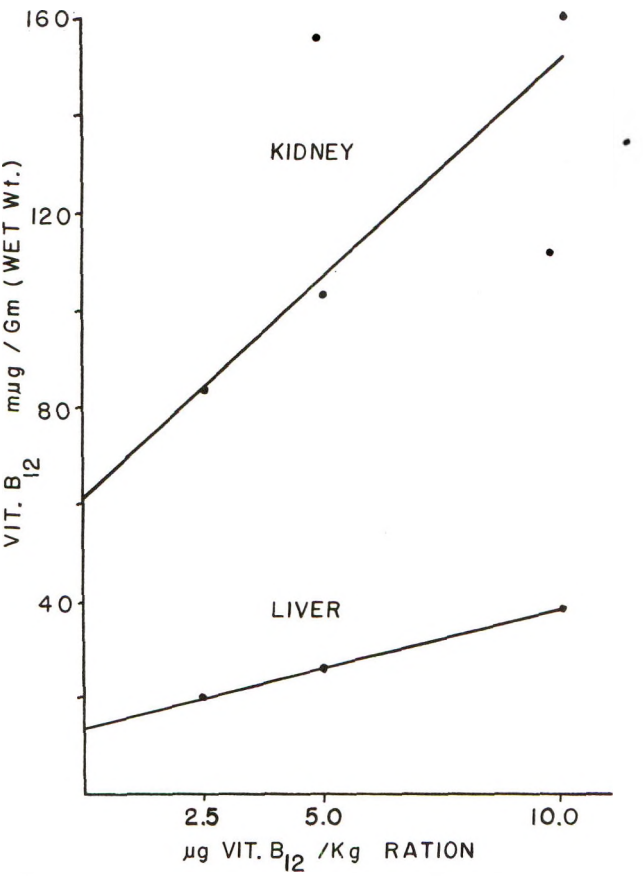

Fig. 2 Vitamin B_{12} content of livers and kidneys from rats fed varying amounts of crystalline vitamin B_{12}.

TABLE 4

Microbiological versus the biological activity [1]

TEST MATERIALS	MICROBIOLOGICAL ACTIVITY	BIOLOGICAL ACTIVITY (RAT ASSAY) EXPRESSED AS PER CENT OF MICROBIOLOGICAL ACTIVITY		
		Growth	Liver	Kidney
Dried feces	1.00 μg/gm	150	124	76
Lyophilized feces extract	0.87 μg/gm	83	100	38
Rumen liquor	0.028 μg/ml	69	71	61

[1] Average of data in table 3.

TABLE 5

Weight gains and liver and kidney vitamin B_{12} content of the chicks

GROUP NO.	RATION	AVERAGE GAIN	LIVER		KIDNEY	
			Vitamin B_{12}	Alkali-stable vitamin B_{12} activity	Vitamin B_{12}	Alkali-stable vitamin B_{12} activity
		gm/2 weeks	*mμg/gm fresh wt.*		*mμg/gm fresh wt.*	
1	Basal	263.4	15.3	11.8	13.9	10.6
2	Basal + 1.0 μg cryst. B_{12}/kg	257.9	25.1	13.4	29.1	8.4
3	Basal + 3.0 μg cryst. B_{12}/kg	265.9	26.2	...	37.1	..
4	Basal + 5.0 μg cryst. B_{12}/kg	285.3	30.1	12.9	39.4	8.0
5	Basal + 10.0 μg cryst. B_{12}/kg	260.6	43.5	9.5	61.4	7.8
6	Basal + 30.0 μg cryst. B_{12}/kg	276.5	180.4	9.5	301.4	8.4
7	Basal + 3.0 gm dried feces/kg [1]	256.3	18.2	..	31.4	..
8	Basal + 10.0 gm dried feces/kg	270.6	27.6	11.3	42.3	8.4
9	Basal + 3.45 gm lyophilized feces extract/kg [1]	272.7	22.9	...	28.2	..
10	Basal + 11.50 gm lyophilized feces extract/kg	283.6	28.2	10.0	39.3	8.2
11	Basal + 107 ml rumen liquor/kg [1]	254.7	21.5	...	38.2	..
12	Basal + 357 ml rumen liquor/kg	287.3	41.0	9.4	49.2	7.6
13	Basal + 3.0 μg vit. B_{12} + 8.0 gm lyophilized feces extract/kg	280.8	32.3	10.2	39.0	7.0
14	Basal + 3.0 μg vit. B_{12} + 250 ml rumen liquor/kg	273.1	22.9	12.2	47.8	7.4

[1] Microbiological activity: dried feces, 1.0 μg/gm; lyophilized feces extract, 0.87 μg/gm; rumen liquor, 28 mμg/ml.

of concentration of vitamin B_{12} activity in the livers and kidneys as well.

The results of the rat growth assay and the vitamin B_{12} liver and kidney analyses are summarized in table 4.

The results obtained in the chick assay are presented in table 5. Since the growth response during the two-week experimental period was of little significance, the weight gains and the vitamin B_{12} content of the livers and kidneys are presented together in the same table.

These data show that the vitamin B_{12} activity in chick livers and kidneys increased as the level of vitamin B_{12} in the diet was raised. However, the total vitamin B_{12} activity in feces and rumen liquor, as measured microbiologically, did not appear to be fully utilized as the level of vitamin B_{12} in the liver and the kidney was somewhat less in the chicks receiving vitamin B_{12} from these materials than from crystalline vitamin B_{12}.

DISCUSSION

The results presented herein indicate that the vitamin B_{12} activity in dried steer feces as determined microbiologically is fully active for the rat. In fact, higher vitamin B_{12} values were obtained with the rat assay than with the microbiological assay using *L. leichmannii* (table 4). However, only about 70% of the microbiologically-determined vitamin B_{12} activity in rumen liquor was used by the rat. Furthermore, a lyophilized hot water extract of the dried feces was less effective as a source of vitamin B_{12} for the rat than would be predicted on the basis of its vitamin B_{12} content, as determined microbiologically.

If dried feces or rumen liquor contained growth factors for the rat other than vitamin B_{12}, increased rat growth might result which would be erroneously considered a vitamin B_{12} response. This possibility seems unlikely since there was an increase in the amount of vitamin B_{12} activity stored in the livers and kidneys of the rats fed either feces or rumen liquor. It is of interest that fairly close agreement was

obtained in the percentage utilization of the *L. leichmannii* assayed vitamin B_{12} activity as calculated on the basis of the liver storage data and the rat growth data (table 4). The livers were assayed microbiologically; however, unpublished data from this laboratory indicate that only vitamin B_{12} and vitamin B_{12b} are found in rat liver. If this is true, then the vitamin B_{12} activity in the liver provides a measure of the biological availability of the vitamin B_{12} ingested in natural materials.

The biological activity of the vitamin B_{12} as measured by *L. leichmannii* in these materials was greater than was obtained by Coates et al. ('53). These investigators found that only 20% of vitamin B_{12} activity (*L. leichmannii*) was utilized by the chick. Unfortunately, in our experiments a growth response to vitamin B_{12} was not obtained when these materials were assayed with chicks, presumably because the chicks were not sufficiently depleted of the vitamin. Nevertheless, the data in table 5 indicate that the storage of vitamin B_{12} in the liver and kidney was less if the chick was forced to obtain its vitamin B_{12} from either dried feces or rumen liquor, suggesting that the vitamin B_{12} activity from these materials was not fully available or utilizable by the growing chick.

Since Ford et al. ('53) had demonstrated the presence in rumen liquor and calf feces of vitamin B_{12}-like substances which were more active for bacteria than for the chick, Coates et al. ('53) assumed that the reason for the low biological activity of the vitamin B_{12} in these materials was due to the presence of the less active compounds. In our laboratory, Johnson et al. ('54) found 6 (or sometimes 7) different compounds in rumen liquor which possess vitamin B_{12}-like activity for bacteria. Thus, there is a possibility that the response of chicks and rats to the various vitamin B_{12}-like compounds present in rumen contents or feces may be a factor in explaining the difference in assay values obtained with the microbiological procedure and the biological assay. Coates et al. ('51) and Dawbarn and Hine ('54) have reported that the vitamin B_{12} activity in rumen liquor can be from

three to 5 times more active for *E. coli* (plate assay) than for *L. leichmannii* which suggests that there is a marked difference in the response of bacteria to these vitamin-like substances.

From these data and the published reports of Ford et al. ('53), Coates et al. ('53), Lewis et al. ('52), Wijmenga ('51), Dawbarn and Hine ('54) and Pfiffner et al. ('52) it is apparent that the relationship of the vitamin-like substances to the true vitamin B_{12} content of materials from the gastrointestinal tract is important. There also appears to be a difference in the response of the rat and the chick to vitamin B_{12}-like substances, as found in feces and rumen liquor. The possibility exists that the chemical constituents in the vitamin B_{12} activity of rumen and fecal samples may vary with the type of ration fed, and the level of rumen microflora activity, e.g. in the calf versus the mature animal. The aforementioned factors appear to provide the only basis for explaining the differences in the results obtained herein and those reported by Coates et al. ('53).

It is also of interest to note that, unlike chick liver and kidney or rat kidney, the amount of alkali-stable vitamin B_{12} activity in rat liver increased as the level of vitamin B_{12} in the ration increased (see tables 3 and 5). In general, rat liver and kidney contained more alkali-stable activity than did similar samples of chick organs.

SUMMARY

Comparative microbiological (*L. leichmannii*) and biological (rat and chick) vitamin B_{12} assays of the dried feces, a lyophilized feces extract, and rumen liquor from a rumen-fistulated steer were carried out. The vitamin B_{12} content of rat and chick liver and kidney was determined microbiologically.

A linear relationship was observed between the vitamin B_{12} activity for *L. leichmannii* found in the liver and kidney and the levels of crystalline vitamin B_{12} in the ration. Although the chicks used in this study did not show a growth response

to the graded levels of vitamin B_{12} in the ration, a definite trend for increased vitamin B_{12} storage in the livers and kidneys was observed as vitamin levels in the ration increased.

On the basis of the criteria used in this study — namely, weight gains and liver vitamin B_{12} content, it was found that the microbiologically active vitamin B_{12} or B_{12}-like substances present in the steer feces were fully active for the rat while only 70% of the activity in rumen liquor was utilized. The activity in dried feces and rumen liquor was less effective, however, for chicks, based on liver and kidney storage of vitamin B_{12}.

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MULTIPLE CONGENITAL ABNORMALITIES RESULTING FROM TRANSITORY DEFICIENCY OF PTEROYLGLUTAMIC ACID DURING GESTATION IN THE RAT

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TWENTY-FIVE FIGURES

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The severity of pteroylglutamic acid (PGA) deficiency as a teratogenic agent for the rat has recently been demonstrated (Nelson, Asling and Evans, '52). Multiple congenital abnormalities in the young resulted in extremely high incidence, 95 to 100%, when a PGA-deficient regimen was instituted 10 or 11 days after breeding. These young were characterized by marked edema and anemia, multiple skeletal abnormalities such as cleft palate and syndactylism, retarded visceral development, and Morgagnian-type cataracts of the eyes. The present communication reports the occurrence of multiple congenital abnormalities, including many anomalies not previously observed, when the mother is given the PGA-deficient diet for only two or three days during the early part of pregnancy.

[1] Presented in part at the Autumn meeting of the National Academy of Sciences, November 1951 (Evans, Nelson and Asling, '51), the 66th meeting of the American Association of Anatomists, March 1953 (Evans et al., '53), and the 120th meeting of the American Association for the Advancement of Science, December 1953 (Nelson, '53). This research was aided by grants from the College of Agriculture of the University of California, the National Vitamin Foundation, the Roche Anniversary Foundation, and the Williams-Waterman Fund.

EXPERIMENTAL

Normal female rats (Long-Evans strain), three to 4 months of age, were bred with normal males and given the PGA-deficient diet for 24, 48, or 72 hours at various periods during the first 14 days of gestation. The animals were then transferred to the PGA-supplemented diet for the remainder of the gestation period. All dietary changes were uniformly made at 10:00 A.M. Food intake was measured for the majority of rats during the period of transitory deficiency. The PGA-deficient diet containing 1% of succinylsulfathiazole (SST) and 0.5% of the "crude" PGA-antagonist (x-methyl-PGA),[2] was the same as that used in previous studies (Nelson, Asling and Evans, '52). The PGA-supplemented diet contained the identical constituents, including the PGA-antagonist, and in addition, 50.5 mg of synthetic PGA per kilogram of diet. Young from mothers maintained on this PGA-supplemented diet throughout gestation showed no abnormalities, either macroscopically or microscopically (Nelson, Asling and Evans, '52).

The young were removed by Cesarean section on the 21st day of gestation and examined carefully for macroscopic abnormalities. This examination included (1) crown-rump measurements and examination of the living or fixed fetus for external abnormalities of body form and (2) examination of the abdominal and thoracic organs[3] of the dissected fetus with a binocular microscope (7 × magnification). In addition, the cerebrum and eyes of some fetuses in each group were examined macroscopically after sectioning with a razor blade. Forty to 60 fetuses from the groups in which marked anomalies were observed were cleared and stained with alizarin red to study the skeletal system; these detailed findings will be reported later.

RESULTS

Table 1 summarizes the reproductive performance of rats placed on the PGA-deficient diet for 24, 48, or 72 hours during

[2] Lot N-125 was used throughout this study.

[3] Details of the cardiovascular anomalies have been reported by Baird et al., '54.

the early part of gestation. All groups averaged 110 to 120 days of age and 200 to 220 gm in body weight on the day of breeding. These animals gained weight and were in good condition throughout the gestation period, regardless of death

TABLE 1

Effect of transitory maternal PGA-deficiency on fetal development

PERIOD OF DEFICIENCY	NO. OF RATS BRED	WT. CHANGE DURING GESTATION	RESORPTIONS	LITTERS	YOUNG		
					Total no.	Av. no. per litter	Abnormal
days of gestation		*gm*	%	%			%
11–14	21	+ 106	0	100	179	8.5	70
10–13	21	+ 85	14	86	130	7.2	100
9–12	10	+ 69	100	0	0	0	
10–12	21	+ 90	0	100	160	7.6	78
9–11	34	+ 77	26	74	128	5.1	99
8–10	10	+ 62	100	0	0	0	
7–9	10	+ 58	100	0	0	0	
7–9 [1]	48	+ 70	62	38	77	4.3	51
9–10	10	+ 103	0	100	93	9.3	0
8–9	12	+ 112	0	100	118	9.8	9
7–8 6–7 5–6	15	+ 112	0	100	143	9.5	0
6–8	10	+ 110	0	100	92	9.2	0
3–6	10	+ 113	0	100	93	9.3	0
0–3	10	+ 114	0	100	97	9.7	0

[1] A lower level of the PGA antagonist was used for this group, i.e., 1.5 to 2.0 gm per kilogram diet instead of 5.0 gm per kilogram diet used for all other groups.

or abnormality of the young. The data show that a two- or three-day period of deficiency instituted after implantation (day 7) markedly affected fetal development. In contrast a one-day period of deficiency during this time had little or no effect, nor did a three-day period of deficiency before implantation.

When the PGA-deficient diet was given for *72 hours* from day 11 [4] to day 14, all rats had living young but 70% of the young were abnormal macroscopically. When the deficient diet was started one day earlier and given from days 10 to 13, living young were present at autopsy in only 86% of the animals and all young were markedly abnormal. When the deficient diet was given still earlier from days 9 to 12, fetal death and resorption invariably occurred in all animals, indicating the greater sensitivity of the younger fetus to the PGA deficiency. The consumption of the PGA-deficient diet during these three-day periods averaged 61.3 gm per rat or 20.4 gm per rat per day.

When the PGA-deficient diet was given for *48 hours* from days 10 to 12, all rats had living young at autopsy with 78% of the young showing macroscopic abnormalities. When the deficient diet was started one day earlier and given from days 9 to 11, only 74% of the animals had living young at autopsy. The number of living young per litter in this group decreased from normal values of 9 to 10 to an average of 5.1, of which 99% were markedly abnormal. Instituting the deficient diet before day 9, i.e. from days 8 to 10 or days 7 to 9, invariably resulted in 100% fetal death and resorption, again demonstrating the greater sensitivity of the younger fetuses to the PGA deficiency. In an attempt to obtain some living fetuses from mothers subjected to the deficiency from days 7 to 9, the level of x-methyl-PGA was lowered from 5.0 gm to 2.0 or 1.5 gm per kilogram of diet.[5] The use of this lower level of the

[4] The day of finding sperm is called the day of breeding and is considered to be day zero. Thus, the PGA-deficiency period from days 11 to 14 is started 11 days after breeding at 10 a.m. and ends 72 hours later, 14 days after breeding at 10 a.m. Implantation sites can be observed macroscopically as minute swellings in the uterus 7 days after breeding in the Long-Evans strain.

[5] One-half of these animals received the PGA-supplemented diet with the antagonist omitted after the deficiency period and the remainder received a stock diet of natual foodstuffs as the control diet. The stock diet, a modification of McCollum's Diet I, is composed of 67.5% ground whole wheat, 15.0% technical casein, 7.5% skimmilk powder, 6.75% hydrogenated vegetable oil, 1.5% calcium carbonate, 0.75% iodized sodium chloride, and 1.0% fish oil (vitamin A-D concentrate). This diet was also used for all animals from the day of breeding to the beginning of the PGA-deficiency period.

PGA antagonist from days 7 to 9 resulted in a milder deficiency and 38% of the rats had living young at autopsy. The average number of living young per litter was only 4.3 and half of these young were abnormal. For all two-day deficiency periods, the average food intake was 37.7 gm per rat or 18.9 gm per rat per day.

In contrast to the deleterious effects observed with the 48- and 72-hour periods, practically no effects on fetal development were observed with a *24-hour* period of deficiency from days 5 to 6, 6 to 7, 7 to 8, 8 to 9, or 9 to 10. All rats in these groups had living young at autopsy and the average number of living young per litter was normal. No macroscopic abnormalities were observed except in the offspring from mothers given the PGA-deficient diet from days 8 to 9, in which 9% of the young showed some abnormality. As it is difficult to ensure consumption of the PGA-supplemented diet immediately after transferring the animals, it is possible that these few abnormal young (11 of 118 young) resulted from delayed consumption of the vitamin-supplemented diet. The food intake during the 24-hour periods of deficiency averaged 20.5 gm per rat.

Although a 72-hour deficiency period instituted after implantation (day 7) was markedly injurious to fetal development, the same period instituted before implantation had no observable effects, i.e. from days 0 to 3, or from days 3 to 6. All rats in these groups had living young at autopsy with a normal number of young per litter. No macroscopic abnormalities were observed. The group with a 48-hour deficiency period during implantation, from days 6 to 8, also showed normal fetal development. This deficiency period included one day before implantation and one day after implantation, so was apparently equivalent to the group with the 24-hour period of deficiency from days 7 to 8. The quantitative data on weight gain, number of young per litter, etc., were practically identical for the two groups.

It may also be noted that all groups with 24-hour deficiency periods and those groups with 72-hour deficiency periods be-

fore implantation averaged a maternal weight gain per rat of over 100 gm with 9.5 living young per litter. This is equivalent or slightly superior to the reproductive performance of stock rats maintained on the stock diet of natural foodstuffs throughout gestation.

DESCRIPTION OF ABNORMAL YOUNG

Over 1300 young were examined macroscopically for abnormalities. Table 2 shows the incidence and types of macroscopic anomalies encountered in the abnormal young of the deficient groups and reveals the pattern of abnormalities in these groups. The abnormal young of mothers given the PGA-deficient diet from *days 11 to 14* were characterized by a moderate to high incidence of skeletal anomalies; no anomalies of other systems were observed. Cleft palates, which were found in half of the young, were usually narrow in width; these defects were occasionally accompanied by a short mandible or protruding tongue. Syndactylism was more frequent in the hindpaws than in the forepaws and usually affected only two toes. Tails were slightly short or crooked in practically all young and clubfoot was observed occasionally. These young were of normal size and length or only slightly smaller, varying from 3.5 to 4.0 cm in length.

The abnormal young from mothers given the PGA-deficient diet on *days 10 to 13* were characterized by multiple anomalies of greater severity. There was a high incidence of skeletal anomalies and a moderate number of urogenital and cardiovascular anomalies. Cleft palates were present in all young and varied from moderate to wide extensive clefts (figs. 1–6). These clefts were invariably accompanied by a short mandible. Harelip (figs. 7–9) was observed in a few young. Digital deformities occurred in practically all young and affected forepaws and hindpaws equally. These deformities consisted of syndactylism and malformed, stubby or splayed toes; in a few cases fusion of all digits was noted. Clubfoot occurred in more than 90% of the young and forelimb deformities, short

TABLE 2

Incidence of macroscopic abnormalities encountered in abnormal young from mothers submitted to a transitory PGA-deficiency during gestation

PERIOD OF DEFICIENCY	YOUNG ABNORMAL	TYPES OF ABNORMALITIES										
		Cleft palate	Harelip	Forepaws	Hindpaws	Club foot	Kidneys	Gonads	Cardio-vascular	Hernias		Cerebral
										Abd.	Diaph.	
days of gestation	*no.*	%	%	%	%	%	%	%	%	%	%	%
11–14	124	48	..	60	92	17	..	..	..	..	..	..
10–13	126	100	19	90	88	94	66	51	29	..	..	..
10–12	115	61	21	..	61 [1]	54	11	7	32	..	..	..
9–11	125	86	93	5	48	62	49	24	58	22	13	..
7–9 [2]	39	..	..	..	..	13	8	..	53	21	..	49

[1] Thirty-eight per cent syndactylism and 23% polydactylism.

[2] A lower level of the PGA antagonist was used for this group, i.e., 1.5 to 2.0 gm per kilogram diet instead of 5.0 gm per kilogram diet used for all other groups.

stubby limbs bowed backwards, in half of the young. In all young the tails were markedly short or stubby and either crooked or curled.

Two-thirds of the fetuses in this group showed marked anomalies of the urinary system, including abnormalities of kidney position (in the pelvis, midline, or partially embedded in muscle, figs. 18–21), hydronephrosis or hydro-ureter (figs. 17, 19, 21), renal hypoplasia (fig. 20), and apparent absence of one or both kidneys (figs. 19–20). Gonadal abnormalities occurred in half the young and included abnormalities of position, namely failure of descent or abnormal relation to the kidneys (figs. 16–19, 21) or markedly retarded development so that sex could not be determined by macroscopic examination. Occasionally the adrenals exhibited abnormalities in position. Cardiovascular anomalies were present in approximately one-third of the young examined. The lungs were either unexpanded or only partially expanded in more than 80% of the young. All fetuses were smaller than normal and varied from 2.5 to 3.5 cm in length.

The abnormal young from mothers given the PGA-deficient diet on *days 10 to 12* were similar to those of the preceding group (days 10 to 13) but in general showed a lower incidence and less severe anomalies. A few cases of additional defects such as oblique facial fissures, mandibular clefts or umbilical hernia were observed occasionally. The most striking difference was the occurrence of polydactylism, 6 to 7 toes, in one-quarter of the young in this group. Occasionally both polydactylism and syndactylism occurred in the same animal on different paws. All young were smaller than normal and varied from 2.5 to 3.5 cm in length.

The abnormal young of mothers maintained on the PGA-deficient diet from *days 9 to 11* showed more types of abnormalities than those of any other group studied. These young were characterized by a high incidence of skeletal anomalies, a moderate incidence of urogenital and cardiovascular anomalies, and a low incidence of abdominal and diaphragmatic herniations. Cleft palates were usually wide extensive clefts

(figs. 10–12) and always involved the median palatine as well as the lateral palatine processes. Harelip occurred in more than 90% of the young and was usually bilateral (figs. 10–12); only 10 of 115 cases were unilateral. An occasional malar or oblique facial cleft (figs. 13–15) was noted as well as marked atrophy or absence of the nose. Digital deformities occurred in the hindpaws of half the young and consisted principally of the absence of one toe. Clubfoot and crooked or curly "pig" tails occurred frequently.

Urogenital anomalies were similar to those observed in young from mothers given the PGA-deficient diet one day later, from days 10 to 13, but occurred less frequently. Of interest was the occurrence of umbilical herniation or gastroschisis with partial or complete ectopia of the abdominal viscera in more than one-fifth of the young. Diaphragmatic abnormalities were less frequent. These defects included right diaphragmatic hernia, left diaphragmatic hernia through an enlarged esophageal hiatus, incomplete diaphragm with double or triple defects, apparent absence of the diaphragm, and enlargement of the esophageal or aortic hiatus without herniation. Cardiac or vascular anomalies were observed in 58% of the young examined, the highest incidence of any of the groups studied. The lungs were only partially expanded in more than half of the young. These young were retarded in size and length to a variable extent, i.e. 2.3 to 3.8 cm in length.

The abnormal young from mothers given the PGA-deficient diet containing a low level (1.5 to 2.0 gm per kilogram of diet) of x-methyl-PGA during *days 7 to 9* exhibited cerebral anomalies, defects which had not been observed in any other group with transitory PGA-deficiency. These anomalies included hydrocephalus, exencephaly (figs. 22–23), and acephalus (fig. 24). Cardiovascular anomalies, principally interventricular septal defects, occurred in more than half the young and gastroschisis or umbilical herniation (figs. 22–24) less frequently. Urogenital abnormalities of size or position, skeletal anomalies such as clubfoot or slightly deformed tails,

and diaphragmatic hernia were only rarely observed. The few abnormal young from mothers given the PGA-deficient diet with the usual level of x-methyl-PGA (5 gm per kilogram of diet) on *days 8 to 9* were similar to the days 7 to 9 group in that all young exhibited hydrocephalus; one fetus, in addition, showed microphthalmia.

Skeletal anomalies, including retardation or absence of ossification, interference with the normal sequence of ossification, and deformed or misshapen bones, were revealed in great variety in virtually all young when cleared and stained with alizarin red. Practically all of the skeletal defects observed in young from mothers given the PGA-deficient diet from days 11 to 21 (Asling et al., '55) were found, together with additional and more severe types of deformities. Malformations of the vertebral column and of the thoracic cage, disproportionate shortening of curvature of the long bones of the extremities, and even aplasia of certain bones occurred frequently in young from mothers given the PGA-deficient diet starting on day 9 or 10. There was no specific pattern of skeletal abnormalities such as that reported for riboflavin-deficient young (Warkany and Nelson, '41) as the skeletal changes varied in type, severity and incidence according to the timing and duration of the PGA-deficiency.

Various types of *eye* abnormalities were observed in all groups. The most frequently encountered defect was the Morgagnian-type cataract previously reported (Nelson, Asling and Evans, '52) as being present in all young from mothers given the PGA-deficient diet during days 11 to 21. Other anomalies such as coloboma and eversion of the retina with retinal cysts, "open" eyes (figs. 13–15), lenticular cysts microphthalmia and anophthalmia also occurred, particularly in the young from mothers receiving the PGA-deficient diet during days 7 to 9 or days 9 to 11. The highest incidence and most severe anomalies were observed in young from the group receiving the deficient diet during the earliest period, days 7 to 9.

Occasionally encountered was the unusual anomaly of strophosomus, a type of celosomus, in which the extremities are reflected on to the back with the distal ends resting on the head (fig. 25). Macroscopic abnormalities of the ear were observed more frequently. Elongation of the esophagus resulting from ectopia of the stomach occurred occasionally and one instance of aplasia of the stomach was found.

DISCUSSION

The data presented show the severity of even a transitory deficiency of pteroylglutamic acid as a teratogenic agent for the rat. Providing the deficiency was instituted at a critical time during gestation, a very high incidence and wide range of congenital malformations resulted from as brief a period as 48 hours on the PGA-deficient diet. The abnormalities observed have included practically all congenital anomalies hitherto produced in the rat or mouse by any experimental procedure (Hogan, '53) and the majority of anomalies encountered in human teratology.

In this study no typical pattern of anomalies was observed for PGA-deficiency, as the type and frequency of deformity varied with the duration and the time of instituting the deficiency. In studies on PGA-deficiency by other investigators, various types of abnormalities have likewise been observed, usually in low incidence. Hydrocephalus has been produced in the rat by omission of PGA from the diet for long periods prior to pregnancy (Richardson and Hogan, '46). Hydrocephalus together with the occasional occurrence of spina bifida, cranium bifidum, anophthalmia, microphthalmia, harelip, cleft palate and edema resulted when the diet used contained low levels of x-methyl-PGA and vegetable protein (O'Dell, Whitley and Hogan, '51). Defects of the eyes, cerebrum, and body walls and in addition an occasional harelip or facial fissure occurred when a PGA-deficient diet containing 5% SST was used (Giroud et al., '52). Injection or oral administration of the PGA antimetabolite, aminopterin, early in pregnancy resulted in hydrocephalus, harelip, micromelia,

arthrygryposis and clubfoot in rat fetuses (Sansone and Zunin, '54), and in hydrocephalus, meningo-encephalocele, cleft palate, and harelip in human fetuses (Thiersch, '52).

In contrast to the varying types of anomalies resulting from PGA-deficiency are those studies on other dietary deficiencies in which a pattern of abnormalities or "syndrome" has been reported, e.g., the skeletal anomalies resulting from chronic riboflavin deficiency (Warkany, '45), the visceral and ocular anomalies produced by chronic vitamin A deficiency (Warkany and Roth, '48), and anomalies of the nervous tissues reported for pantothenic acid deficiency (Lefebvres-Boisselot, '51). The possibility that such a pattern may be due to the timing and the severity of a deficiency is suggested by other studies with the same vitamins. In addition to the skeletal anomalies found by Warkany, multiple types of anomalies (cardiovascular, urogenital, cerebral, ocular, epidermal and body wall defects) have resulted from an acute riboflavin deficiency produced by addition of the antimetabolite, galactoflavin, to the vitamin-deficient diet (Nelson, '53, '55; Baird et al., '55). Cerebral and ocular anomalies in addition to skeletal defects have recently been reported by Grainger et al. ('54) for chronic riboflavin deficiency; in their studies these additional anomalies were prevented by vitamin B_{12}. Wilson et al. ('53) found that vitamin A supplementation given at progressively earlier times during gestation reduced the incidence of dead young and also modified to some extent the typical "syndrome" of malformation. In the pig, chronic vitamin A deficiency combined with vitamin A supplementation after the first month of pregnancy resulted in a pattern of anomalies differing from that observed in the rat (Hale, '37). The similarity of the congenital abnormalities produced in mammals by different dietary deficiencies and by other teratogenic agents such as hormones, nitrogen mustrads, trypan blue, hypoxia, and irradiation, emphasizes the non-specific results of teratogenic agents.

The failure of the PGA-deficient diet to affect embryonic development when instituted before implantation indicates

that the teratogenic effect results only from imposing the deficiency at a precise and later time in development. The decrease in sensitivity to PGA-deficiency with increasing embryonic age after the 9th day is similar to the findings of Wilson ('54) with irradiation of implantation sites in the rat.

Determination of the mechanism by which this vitamin deficiency interferes with development must await further studies. For some types of anomalies, e.g., those of the cerebrum, eyes, and heart, the PGA-deficient period included part or all of the period of organogenesis. In other cases, particularly for skeletal defects, the deficient period preceded organogenesis. Some of the anomalies observed, notably in the urogenital system and in the skeleton, represented developmental arrests, i.e., persistence of the relationships characteristic of an earlier stage of fetal development. In many other instances an abnormal course of development apparently resulted from retardation.

SUMMARY

Multiple congenital abnormalities have been produced in the rat by an extremely short and transitory deficiency of pteroylglutamic acid instituted during the early part of pregnancy, the "critical" period of differentiation and organogenesis. A deficiency period of only 48 hours during this period, after implantation on day 7 and before day 12, resulted in 70 to 100% abnormal young or fetal death. In contrast a 24-hour period of deficiency after implantation had practically no effect on fetal development nor did a 72-hour deficiency period before implantation. The earlier phases of embryonic development were more severely affected by the same length of deficiency than the later phases. Throughout the experiments the mothers receiving the PGA-deficient diet gained weight and were in good condition, regardless of death or abnormality of the fetuses.

The abnormalities produced included multiple types of defects of the nervous, ocular, skeletal, respiratory, cardiovascular, and urogenital systems; the diaphragm and body walls were also affected. The type of anomaly and the incidence

varied both with the duration and with the time of instituting the deficiency.

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PLATE 1

EXPLANATION OF FIGURES

Lateral and frontal views of the head and ventral views of the palate in 21-day-old fetuses of mothers maintained on the PGA-control or deficient diet, × 2.7. × 3.6.

1, 2, 3 Control.

4, 5, 6 PGA-deficient, days 10 to 13, showing a wide cleft palate and short mandible.

7, 8, 9 PGA-deficient, days 10 to 13, showing cleft palate, unilateral harelip, and extremely short mandible.

10, 11, 12 PGA-deficient, days 9 to 11, with cleft palate, bilateral harelip, right oblique facial cleft, and extremely short mandible.

13, 14, 15 PGA-deficient, days 9 to 11. Note extremely short mandible (virtual agnathia), cleft palate with bilateral harelip, malar or oblique facial clefts extending into the orbital fossae on both sides, and bilateral "open" eyes due to failure of eyelid formation.

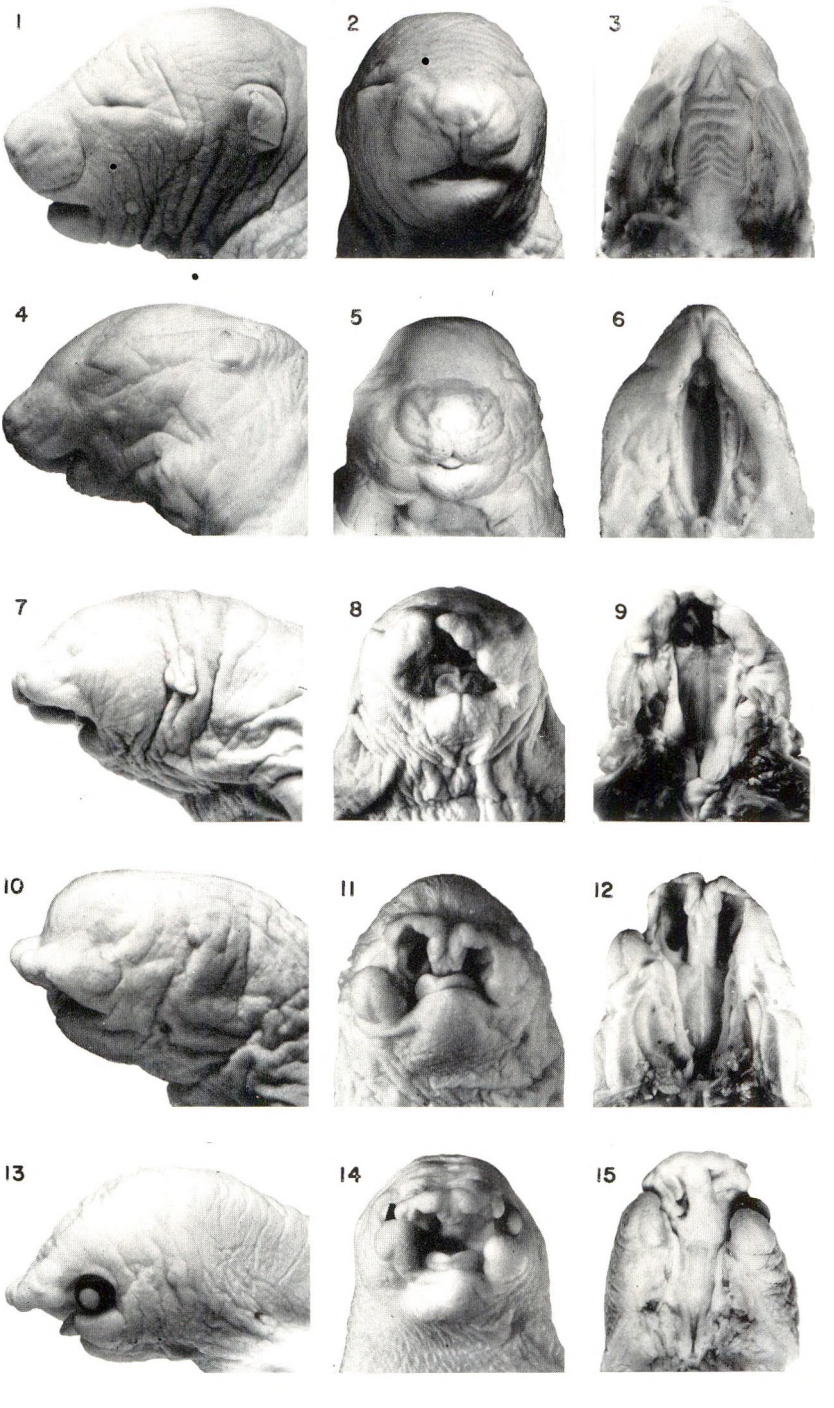
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PLATE 2

EXPLANATION OF FIGURES

Dissections showing the urogenital system of 21-day-old fetuses of mothers maintained on the PGA-control or deficient diet from days 10 to 13 of gestation, × 3.6.

16 Control, showing kidneys, ureters, bladder and uteri.

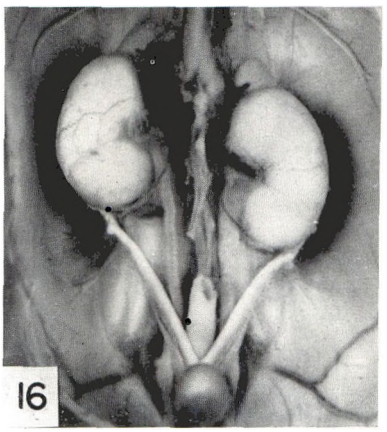
17 PGA-deficient. Hydronephrosis and hydro-ureter with marked distention of the bladder. The ovaries are lateral to the kidney instead of in the normal posterior position.

18 PGA-deficient. Retention of the early embryologic relationships with both kidneys in the pelvis and the gonads cephalad.

19 PGA-deficient. Marked hydronephrosis of right kidney, apparent absence of left kidney (which is deeply imbedded in the posterior abdominal musculature) and incomplete descent of the left testis.

20 PGA-deficient. Apparent absence of right kidney with left kidney small but in normal position.

21 PGA-deficient. Right "pelvic" kidney, incomplete descent of the right testis, and left hydro-ureter.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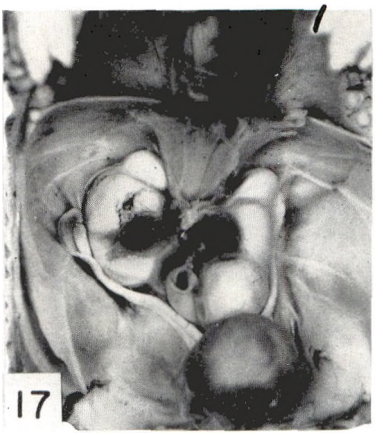
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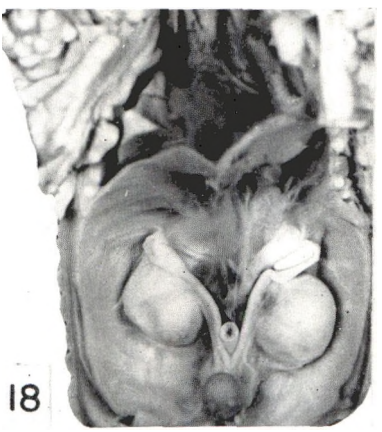
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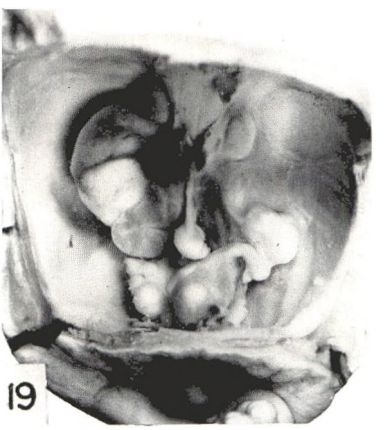
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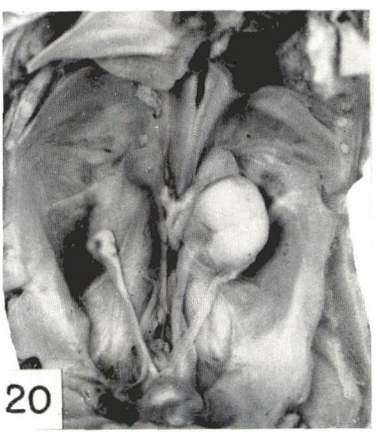
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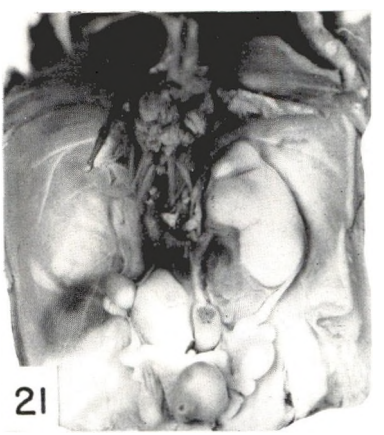
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PLATE 3

EXPLANATION OF FIGURES

Lateral or ventral views of 21-day-old fetuses of mothers maintained on the PGA-deficient diet, × 2.7.

22, 23 PGA-deficient, days 7 to 9, showing exencephaly (encephalocele in the occipital region) and gastroschisis with ectopia of abdominal viscera.

24 PGA-deficient, days 7 to 9, showing acephalus, the lack of all cranial and facial structures above the tongue, and gastroschisis with ectopia of abdominal viscera.

25 PGA-deficient, days 9 to 11. Strophosomus, a type of celosomus in which the extremities are reflexed on to the back with the distal ends resting on the head.

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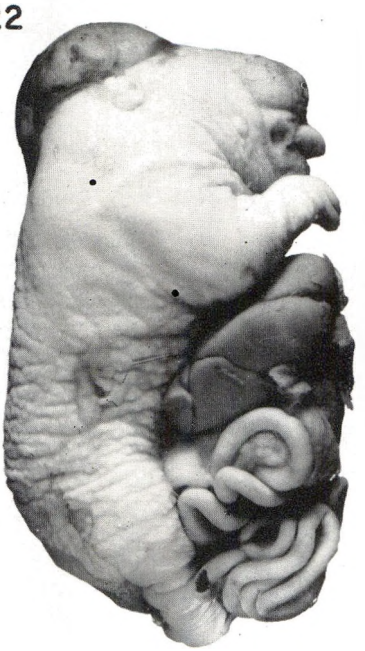

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STUDIES OF NIACIN REQUIREMENT IN MAN

II. REQUIREMENT ON WHEAT AND CORN DIETS LOW IN TRYPTOPHAN [1]

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ONE FIGURE

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The niacin requirement of humans can be determined only in relation to the tryptophan content of the diet since it has been shown that the amino acid tryptophan is converted to niacin compounds in man as well as in many other species (Sarett and Goldsmith, '47, '49; Perlzweig et al., '47). Two basic diets have been devised for investigating niacin requirement, one high in corn, the other high in wheat. Each diet furnishes approximately 200 mg of tryptophan which should supply little more than the minimum requirement for this amino acid (Rose, '49). In a previous study (Goldsmith et al., '52) each of three subjects who received the "corn" diet, which furnished approximately 4.7 mg of niacin and 190 mg of tryptophan for more than 50 days developed pellagra. On the other hand, no signs of niacin deficiency were observed in one subject who received the "corn" diet supplemented

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with 2 mg of niacinamide daily for 122 days, nor in a second subject who received a "wheat" diet, which furnished approximately 5.7 mg of niacin and 230 mg of tryptophan, for 95 days.

The induction of pellagra with the unsupplemented "corn" diet and the failure of signs of deficiency to appear with the "wheat" regimen, may have been due to the slightly lower niacin and tryptophan contents of the "corn" diet but there are other possibilities. In a further investigation of this problem, three subjects have been maintained on a "wheat" diet, which furnished approximately the same amounts of niacin and tryptophan as did the "corn" diet with which pellagra was induced. In this report, clinical findings and the urinary excretion of niacin and tryptophan metabolites during the "wheat" regimen will be discussed, and compared with those observed previously when the "corn" diet was administered. In addition, data obtained in 6 subjects who received the "corn" diet supplemented with several levels of niacinamide will be presented.

METHODS

The three subjects who received the "wheat" diet were white females, R. L., L. M. G. and P. C., aged 26, 51 and 60 years respectively. One subject (L. M. G.) had participated in the earlier study with the "corn" diet. A 4th subject (L. M.), a negro female 46 years of age, received the "wheat" and "corn" diets, alternately, for periods of 20 days each. Six additional subjects, two white females, three colored females and one white male, aged 31 to 57 years, received the "corn" diet supplemented with varying doses of niacinamide. The subjects were essentially free of organic disease except for the white male, who had a post-encephalic tremor of 30-years duration.

All subjects were maintained in a metabolism ward and were ambulatory throughout the period of study. Clinical examinations for signs suggestive of nutritional deficiency were made several times weekly.

Basal diets were weighed and administered under strict supervision. The complete diet for two days was analyzed at intervals for niacin, tryptophan and nitrogen by methods previously described (Goldsmith et al., '52). All urine was collected for 24-hour periods in dark bottles containing 5 ml of glacial acetic acid and kept in the refrigerator until poooled for analysis in two- or 4-day periods. A portion of each pooled specimen was adjusted to pH 6.9 and stored in the frozen state until analyzed. Procedures for determination of total nitrogen, creatinine, niacin, quinolinic acid, tryptophan, N^1-methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Me (pyridone) were those described in a previous paper (Goldsmith et al., '52). The method for estimating pyridone is relatively insensitive when excretion is less than 2 mg daily, and reproducibility of values is poor. At these low levels, differences of 1 mg or less do not appear to be significant. Other laboratory tests which were carried out at intervals included: gastric analysis, complete blood count, concentration of urea nitrogen and sugar in blood and of albumin and globulin in serum, glucose tolerance test, basal metabolic rate, electro-cardiogram, and liver function tests (cephalin flocculation, serum bilirubin, thymol turbidity, and bromsulfalein excretion).

The "wheat" diet used in the present study (table 1) included 50 gm of farina and 90 gm of white flour, both unenriched. The "corn" diet was similar except that the cereal content was 89 gm of unenriched corn products, grits and corn meal, and 35 gm of unenriched white flour. There were minor differences in the quantities of vegetables and fruit in the two diets. The energy values were adjusted to fit individual need by modifying the sugar and oleomargarine content and varied from 1600 to 2100 calories. The "wheat" diet was found by analysis to contain approximately 5 mg of niacin and 200 mg of tryptophan, the "corn" diet 4.7 mg of niacin and 190 mg of tryptophan.

The subjects consumed most of the food presented to them except subject L. M. G. who developed anorexia in the

latter part of the experimental period. When adjustments were made for actual food consumption, the average daily intakes were 4.8, 4.9 and 5 mg of niacin and 169, 195 and 200 mg of tryptophan in the three subjects on the "wheat" diet.

TABLE 1

Wheat diet low in niacin and tryptophan

BREAKFAST	gm	SUPPER	gm
Orange juice or		Potato	50
Grapefruit juice	200	Beets or carrots [3]	100
Farina	50 (dry weight)	Biscuit [1]	30
White bread [1]	30	Pears, pineapple	
Oleomargarine	15	or peaches [3]	125
Sugar	10	Oleomargarine	15
		Grape juice	100
		Gelatine	20
		Sugar	30
		Lemon juice	20
DINNER		BETWEEN MEALS 3:00 P.M.	
Wilson beef [2]	20		
Rice	25 (dry weight)	Lemon juice	30
Green beans or	100	Sugar	20
Mustard greens [3]	50	8:00 P.M.	
Biscuit [1]	30	Prune juice	100
Fruit cocktail or	100		
Applesauce [3]	150		
Oleomargarine	15		
Apple juice	200		

Analysis of diet: Nitrogen 6.2 gm, niacin 5.0 mg, tryptophan 200 mg.

[1] Recipes are given in previous report (Goldsmith et al., '52). The unenriched white flour (Gold Medal) was generously furnished by General Mills, Inc., through the courtesy of Dr. H. S. Faulkner.

[2] Same product as Mor Beef used in the corn diet.

[3] Canned, drained weight, given alternately.

Similar data for the three subjects previously maintained on the "corn" diet were: 4.4, 4.6 and 4.6 mg of niacin and 177, 186 and 195 mg of tryptophan. The nitrogen content was 7.0 gm for the "corn" diet and 6.2 gm for the "wheat" diet. The content of other nutrients in these diets, as calculated from standard tables, was as follows: 11,000 I.U. vitamin A,

0.5 mg of thiamine, 0.5 mg of riboflavin, 35 mg of ascorbic acid, 0.2 gm of calcium, and 8.0 mg of iron.

To avoid complicating deficiencies, vitamin supplements were given in the following amounts daily: 1 mg of thiamine, 1.5 mg of riboflavin, 5 mg of pantothenic acid, 5 mg of pyridoxine, 2.5 mg of folic acid and 5 μg of vitamin B_{12}.

In studies in which the corn diet was supplemented with niacinamide, the daily supplement ranged from 1.5 to 15 mg and was divided into three portions, one being given with each meal. Each supplement was administered for a period of 12 to 20 days (usually 16 days) and each subject was studied at 4 to 6 levels of niacin intake. In half the subjects supplements were given in increasing and in the other half in decreasing amounts.

RESULTS

A — Experiments with the "wheat" diet

Of the three subjects who received the "wheat" diet, one (R. L.) developed amenorrhea after the second month and showed slight redness of the tongue papillae and herpes of the lip at the end of the third month. The experiment was terminated at this time as the subject was unwilling to remain on the diet. The second subject (P. C.) showed nothing which could be definitely attributed to niacin deficiency, although lassitude and depression were noted prior to termination of the study on the 95th day. At this time, illness developed in the patient's family and it was necessary for her to return to her home. The third subject (L. M. G.) developed characteristic signs of niacin deficiency, which were first observed after 80 days on the "wheat" diet. This subject had been studied previously on the "corn" diet and had developed the first evidence of niacin deficiency about the 50th day (Goldsmith et al., '52). The signs which occurred during the two regimens were very similar but appeared earlier and were more severe on the "corn" regimen (table 2). It was necessary to terminate the "corn" experiment on the 82nd day due to the extent of the deficiency. In the present

TABLE 2

Clinical findings in subject L.M.G. on the "Corn" and "Wheat" diets

CORN DIET (Previous study)		WHEAT DIET (Present study)	
Days after institution of diet	Symptoms and signs of niacin deficiency	Days after institution of diet	Symptoms and signs of niacin deficiency
50 to 59	Heartburn; weakness; atrophy tongue papillae.	80 to 95	Atrophy tongue papillae. Dry, scaly skin — elbows.
60 to 69	Decreased activity; apathy; white patch under tongue; dermatitis — vulval, perirectal.	95 to 104	Erythema — oral, vaginal, rectal mucosa. Dysphagia. Dermatitis — vulval, perirectal. Moderate glossitis.
70 to 79	Weakness — confined to bed. Mouth sore; unable to use false teeth; anorexia; indigestion; cheilosis; angular stomatitis.	105	Bloody diarrhea.
80 to 81	Depression; dysphagia. "Raw" sensation, mouth to rectum. Severe glossitis. Erythema — oral, vaginal, rectal mucosa. Diarrhea. Small ulcers vulva. Bleeding — nose, intestine.		

study with the "wheat" diet, the patient left the hospital, against advice, after 105 days. At this time, the deficiency was less acute than at the end of the corn experiment.

Urinary excretion findings in the three subjects who received the "wheat" diet are shown in table 3. Nitrogen excretion remained relatively constant except in subject L. M. G. whose food intake decreased toward the end of the experimental period. The excretion of niacin and of quinolinic

TABLE 3

Urinary excretion data — subjects on the "Wheat" diet

SUBJECT	DAY	NITROGEN	NIACIN	QUINOLINIC ACID	N^1-Me	PYRIDONE	TRYPTOPHAN
		gm	*mg*	*mg*	*mg*	*mg*	*mg*
R.L. Age 26	0 to 19	5.0	0.3	2.4	1.5	1.1	8
wt.: 40.9 kg	20 to 39	5.3	0.3	2.5	1.0	0.3	9
	54 to 83	4.8	0.3	2.6	0.8	0.3	9
P.C. Age 60	3 to 22	6.1	0.4	4.9	1.5	1.6	8
wt.: 48.2 kg	23 to 46	5.9	0.3	4.7	1.0	0.7	7
	60 to 71	6.0	0.3	4.9	0.8	0.6	6
	82 to 95	5.8	0.3	4.8	0.7	0.7	6
L.M.G. Age 51	5 to 21	5.7	0.4	4.0	1.8	2.0	6
wt.: 65.5 kg	22 to 41	5.2	0.4	3.9	1.1	1.0	5
	42 to 61	5.8	0.4	4.2	1.0	0.7	5
	62 to 81	4.8	0.3	3.9	0.7	0.5	6
	82 to 104	4.4	0.3	3.4	0.6	0.6	4

acid remained constant during the period of study in all subjects except L. M. G. in whom a slight decrease in quinolinic acid excretion occurred after the 82nd day. In all subjects, the excretion of N^1-methylnicotinamide (N^1-Me) decreased gradually throughout the experiment, reaching levels of 0.6 to 0.8 mg by about the 80th day. Excretion of the 6-pyridone of N^1-Me decreased rapidly reaching less than 1 mg by about the 40th day.

B — Comparison of excretion data from subjects on "wheat" and "corn" diets

It is of interest to compare urinary excretion data obtained in the three subjects on the "wheat" diet with those

previously reported in three subjects on the "corn" diet. Excretion of niacin was essentially the same during the two regimens: 0.3 to 0.4 mg daily. With the "corn" diet, quinolinic acid excretion was about 2.5 mg in two subjects, 4.3 mg in the third; with the "wheat" diet, excretion was about 4.5 mg in two subjects, 2.5 mg in the third. Excretion of tryptophan was slightly greater on the "wheat" than on the "corn" diet, averaging 5, 7 and 9 mg daily in the three subjects on the "wheat" regimen and 3, 4 and 6 mg on the "corn" regimen.

The excretion of N^1-Me decreased to minimum levels of 0.5 to 0.6 mg daily within 50 to 80 days in subjects receiving

TABLE 4

Urinary excretion of N^1-Me in subjects on "Corn" and "Wheat" diets

	CORN DIET [1]			WHEAT DIET			CORN DIET [1]
	Niacin 4.7 mg Tryptophan 190 mg			Niacin 5.0 mg Tryptophan 200 mg			Niacin 6.7 mg Tryptophan 190 mg
days	*L.M.G.*	*H.K.*	*R.C.*	*P.C.*	*R.L.*	*L.M.G.*	*D.H.*
0 to 20	1.2	0.9	0.7	1.5	1.5	1.8	1.4
21 to 50	0.8	0.7	0.5	1.0	1.0	1.1	1.0
51 to 80	0.6	0.5	0.5	0.8	0.8	0.7	0.9
81 to 120	..	0.5	0.5	0.7 [2]		0.6 [3]	0.9
121 to 135	..	0.5					

[1] Data reported in previous study (Goldsmith et al., '52).
[2] Experiment discontinued on 95th day.
[3] Experiment discontinued on 104th day.

the "corn" diet. The excretion decreased to similar levels in only one subject receiving the "wheat" diet and this occurred after a longer period on the diet. (table 4). In the subject who received the "corn" diet supplemented with 2 mg of niacinamide (niacin intake 6.7 mg), excretion of N^1-Me remained at slightly higher levels after 50 days than in subjects maintained on the "wheat" diet.

Pyridone excretion may have fallen more slowly in subjects who received the "wheat" diet (table 3) than in those given the diet containing corn. During the latter regimen, pyridone excretion became nondetectable after the first three weeks. The small differences observed are probably not significant

due to insensitivity of the pyridone method at low levels of excretion.

In subject L. M. G., who was observed on both the "corn" and "wheat" regimens, the excretions of tryptophan, quinolinic acid, N^1-Me and pyridone were lower when the "corn" diet was administered than when the "wheat" diet was prescribed.

One subject (L. M.) received the "wheat" and "corn" diets for alternate periods of 20 days each. Urinary excretion of niacin metabolites is given in table 5. Excretion of N^1-Me and pyridone was essentially the same during the second 20-day period on each diet. This subject developed typical skin lesions of pellagra after working all day in the sun at

TABLE 5

Urinary excretion of niacin metabolites on "Wheat" and "Corn" diets

(Subject L.M.)

DIET	DAYS	EXCRETION		
		N^1-Me	Pyridone	Total
		mg	*mg*	*mg*
Wheat	1 to 20	1.5	1.2	2.7
Corn	21 to 40	1.0	0.6	1.6
Wheat	41 to 60	0.8	0.4	1.2
Corn	61 to 80	0.7	0.5	1.2

home after the experiment was terminated. Thus, in this individual, a difference between the two diets was not apparent as far as excretion findings were concerned and consumption of the "wheat" diet for half the experimental period did not prevent the development of pellagra. However, this patient was subjected to stress factors which were not applied in other subjects, namely physical exertion and sunlight.

In subjects who received the "wheat" diet, there were no significant changes in findings obtained with the following laboratory tests: basal metabolic rate, electrocardiogram, serum protein concentration, blood urea nitrogen, glucose tolerance, liver function, gastric analysis after histamine stimulation or complete blood counts.

In the previous study with the "corn" diet, a decrease in gastric acidity following histamine stimulation was observed in two subjects; in one instance, no free acid was obtained. In addition, one subject developed a mild anemia which did not improve with niacin but responded to folic acid. This subject, and one other, had not received folic acid as a dietary supplement. Findings in the other laboratory tests listed were essentially normal.

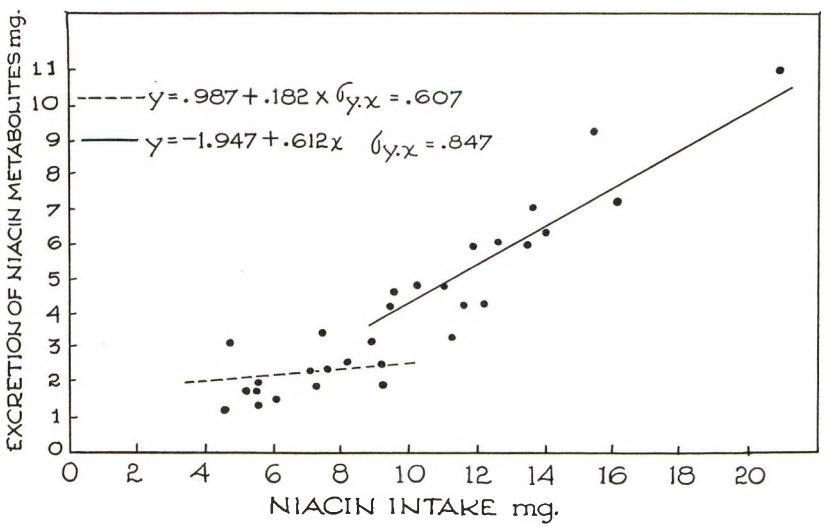

Fig. 1 Urinary excretion of niacin metabolites in relation to niacin intake.

C — Studies with the "corn" diet supplemented with niacinamide

Six subjects were maintained on the "corn" diet to which supplements of niacinamide were added, as described above. The total niacin furnished by the diet plus the supplements ranged from 4.6 to 21.2 mg daily. In 5 of 6 subjects, there appeared to be a change in the proportion of the niacin intake excreted as metabolites when the diet furnished 8 to 10 mg of niacin daily. The significance of this pattern of excretion was tested by fitting straight lines. The best fit was obtained by the two lines shown in figure 1 rather than by a single line. It was

found that about 0.2 mg of niacin metabolites were excreted for each milligram ingested up to the level of 8 to 10 mg daily; above this level, the excretion rate was 0.6 mg of metabolites for each milligram of niacin intake. This difference is statistically significant, the P value being < 0.01. These data suggest that an intake of 8 to 10 mg of niacin is adequate to prevent depletion of body stores when the diet contains corn and furnishes 200 mg of tryptophan.

DISCUSSION

The association of corn diets with pellagra has been recognized for centuries. Possible explanations for this association include (1) the low tryptophan content of corn, (2) the presence of "bound" niacin in corn which is unavailable to the body, (3) imbalance of amino acids in diets which contain large quantities of corn products and (4) a "toxic" or inhibitory factor in corn.

Comparison of findings obtained with the "wheat" diet of the present study with those obtained previously with the "corn" diet suggests that niacin deficiency was induced less readily and was less severe during the wheat regimen. Also, the excretion of N^1-Me decreased to lower levels in a given period of time with the "corn" than with the "wheat" diet. As noted above, the two diets furnished approximately the same total quantity of niacin and tryptophan after adjustment was made for actual food consumption of the several subjects. One explanation of the differences observed might be that body niacin stores were greater in subjects who received "wheat" than in those given "corn" diets. In an attempt to determine the adequacy of niacin stores the subjects, with one exception, were given a test dose of 50 mg of niacinamide prior to institution of the test diet. In each instance, more than 70% of the dose was excreted in the next 24 hours, a finding consistent with normal niacin nutrition. However, this test is not sufficiently sensitive to detect small differences in body stores which might have influenced findings.

When the intake of niacin and tryptophan was computed in relation to body weight and surface area, a relationship was noted between the intake per unit of body size and the development of pellagra on both "corn" and "wheat" diets. These data are shown in table 6 which includes computations in subject R.L. (1) who received a "wheat" diet furnishing 5.7 mg of niacin and 230 mg of tryptophan, and in subject D.H. who received the "corn" diet supplemented with 2 mg niacinamide daily (Goldsmith et al., '52). With the unsupplemented "corn" diet, signs of niacin deficiency appeared earliest in subject L.M.G. who had the lowest niacin and tryptophan intake. Deficiency occurred somewhat later in subject H.K. and still later in subject R.C. Likewise, the severity of the lesions could be rated in the same order. Subject D.H., who received the "corn" diet supplemented with niacinamide, did not develop pellagra. With the "wheat" diet, only one subject (L.M.G) showed signs typical of niacin deficiency although findings in the other subjects might have represented early changes. Data in these 8 subjects suggest that niacin requirement is greater than 0.1 mg per kilogram of body weight daily if the tryptophan intake is less than 4.0 to 4.5 mg per kilogram.

The intake of niacin and tryptophan per unit of body size may not completely explain the differences observed between the "wheat" and "corn" diets. The intake of subject L.M.G. was almost identical during the two regimens, yet pellagra developed more slowly when the "wheat" diet was consumed. As noted above, it is possible that body niacin stores were greater when the "wheat" diet was initiated than when the "corn" diet was prescribed. Subject R.C. ("corn" diet) received amounts of niacin and tryptophan in relation to body size equivalent to those received by subject P.C. ("wheat" diet), yet definite evidence of deficiency did not develop in the latter subject. Variations in requirement among individuals may account for this difference.

Since the pellagragenic effect of the "corn" diet may not be explicable solely on the basis of niacin and tryptophan

TABLE 6

Average daily intake of niacin and tryptophan in relation to body size by subjects on "Corn" and "Wheat" diets

SUBJECT	DIET	INTAKE		INTAKE		INDUCTION OF PELLAGRA
		Niacin	Tryptophan	Niacin	Tryptophan	
		mg/kg body weight		*mg/M² surface area*		
L.M.G.[1]	Corn	.07	2.7	2.7	109	+
H.K.[1]	Corn	.08	3.3	2.8	115	+
R.C.[1]	Corn	.10	4.3	3.4	145	+
L.M.G.	Wheat	.07	2.8	2.9	114	+
R.L. (2)	Wheat	.12	4.8	3.7	146	—
P.C.	Wheat	.10	4.2	3.4	135	—
R.L. (1) [1]	Wheat	.11	4.9	3.2	146	—
D.H.[1]	Corn	.11	3.1	4.0	120	—

[1] Previous studies (Goldsmith et al., '52)

Subject D.H. received the corn diet supplemented with 2 mg niacinamide daily.

Subject R.L. (1) received a wheat diet which furnished 5.7 mg niacin and 230 mg tryptophan daily.

content, alternative explanations should be considered. The presence of "bound" niacin in corn, unavailable to the organism unless hydrolyzed by alkali (Kodicek, '51; Laguna and Carpenter, '51) may explain the corn-pellagra relationship. The incidence of pellagra is relatively low in some areas of the world, such as Central America, where diets high in corn are common but where corn is treated with lime prior to ingestion. The effect of lime treatment on the pellagragenic effect of corn is currently under investigation. Preliminary findings suggest that such treatment fails to modify, appreciably, the development of pellagra in subjects receiving corn diets (Goldsmith et al., '54).

Amino acid imbalance is another factor which may explain the pellagragenic effect of corn diets. In animals, such imbalance can result in niacin-tryptophan deficiency (anonymous, '52). In Frazier and Friedemann's ('46) extensive analysis of diets which produced or prevented pellagra, it is evident that not only the intake of niacin and animal protein influence the development of pellagra in humans or corn diets, but also other factors which make up the total composition of the diet. Perhaps amino acid imbalance will account for these factors. The possibility that corn contains an inhibitory factor which influences niacin requirement or utilization, as suggested by Wooley's ('46) experiments with mice, has not been ruled out.

When the "corn" diet, which furnished about 200 mg of tryptophan, was supplemented with varying amounts of niacinamide, a significant increase in excretion of niacin metabolites was noted when the intake approximated 8 to 10 mg daily. These findings suggest that under the dietary conditions of this experiment, a daily supply of 8 to 10 mg of niacin is sufficient to maintain body niacin stores.

SUMMARY

Three subjects were maintained for 90 to 105 days on a "wheat" diet which furnished approximately 5 mg of niacin and 200 mg of tryptophan daily. One subject developed typical

niacin deficiency beginning 80 days after the diet was instituted, a second developed amenorrhea, herpes of the lip and slight redness of the tongue papillae, and a third showed lassitude and depression as the only clinical findings. In contrast to this, each of three subjects previously maintained on a "corn" diet of comparable niacin and tryptophan content showed the characteristic clinical picture of niacin deficiency after about 50 days of the experimental period. Excretion of N^1-methylnicotinamide decreased to lower levels within a shorter period of time, and tryptophan excretion was slightly lower during the corn than during the wheat regimen.

The time at which pellagra developed, and the severity of the deficiency, seemed to be related to the intake of niacin and tryptophan per unit of body size with both the "wheat" and "corn" diets. However, this relationship may not completely explain differences in clinical and laboratory findings between the two regimens. The low tryptophan content of corn may not be the sole explanation of the pellagragenic effect of this cereal.

When the "corn" diet was supplemented with varying amounts of niacinamide a significant increase in excretion of niacin metabolites occurred when the intake approximated 8 to 10 mg daily. These data suggest that with the "corn" diet, which furnishes 200 mg of tryptophan daily, body niacin stores approach adequacy when the diet supplies 8 to 10 mg of niacin.

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STUDIES ON VITAMIN E IN POULTRY NUTRITION [1]

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In most of the studies which have been conducted on the role of vitamin E in animal nutrition, the basal diets employed have contained appreciable amounts of vitamin E, thereby requiring treatment with ferric chloride or the addition of a vitamin E-oxidizing fat such as lard or cod liver oil in order to produce E-deficiency symptoms. Under these conditions, as Dam ('44) reports, the symptoms of vitamin E deficiency in chicks can be enhanced or suppressed by dietary changes which are unrelated to the vitamin E content of the diet. For example, Dam showed that when vitamin E-low diets were used no symptoms occurred unless highly unsaturated fats were added to the diets. He found that the fatty acids from cod liver oil, lard and linseed oil produced exudative diathesis as the main symptom, whereas fatty acids from hog liver favored the production of encephalomalacia. As curative agents in addition to vitamin E, Dam found that inositol (1.5%) counteracted both symptoms while lipocaic (2%) prevented only exudative diathesis. Cholesterol (1%) hastened exudative diathesis when the diet contained 5% of cod liver oil and a low salt content, and prevented encephalomalacia when the diet contained 30% of lard. Such wide variability in results, brought about by relatively minor changes in the diet, has made the study of vitamin E difficult and the results obtained open to a variety of interpretations.

[1] This work was aided by a grant, samples of stripped lard, vitamin E and other assistance from Distillation Products Industries, Rochester, New York.

In view of this, investigations have been undertaken in this laboratory to develop a basal diet severely deficient in vitamin E, by the use of which the production of vitamin E-deficiency symptoms in the chick is neither enhanced nor suppressed by the addition of pro-oxidants or antioxidants to the diet.

In the course of studies on dietary necrotic liver degeneration in rats, Schwarz ('51) found that the feeding of a vitamin E-deficient purified diet, containing American Torula yeast as the source of protein, produced the liver necrosis in 100% incidence in repeated experiments. In confirmation of earlier experiments, using European yeasts, it was found that the addition of alpha-tocopheryl acetate to the Torula yeast diet prevented the disease in all instances.

It was considered possible that, by modifying the basal rat diet used by Schwarz in order to make it adequate in all nutritional essentials required by the chick with the exception of vitamin E, this diet might produce uncomplicated vitamin E deficiency symptoms in chicks and poults. This would allow a straightforward investigation into the role of vitamin E in poultry nutrition.

Interest in this subject has arisen in this laboratory from the finding reported by Scott ('53) that vitamin E is concerned in the prevention of the enlarged hock disorder in turkeys. Since dried brewers' yeast appeared to spare the vitamin E requirement for this function, it was considered desirable to determine the relationship between vitamin E and the unknown vitamin E-sparing principle in dried brewers' yeast using a diet severely deficient in vitamin E. The development of such a diet and some of the results obtained with it are the subjects of this report.

EXPERIMENTAL

The basal diet employed by Schwarz ('51) contained Torula yeast, 30%; sucrose, 59%; salts, 5%; lard (vitamin E-free), 5%; and vitamin powder, 1%. Since the requirement of the chick for protein exceeds that of the rat, it was considered necessary to modify Schwarz's diet by the use of an increased

level of Torula yeast. In calculating the amount of yeast necessary to meet the protein and amino acid requirements of the chick, the crude protein content (N × 6.25) was assumed to consist of 80% amino acid nitrogen and 20% purine and pyrimidine nitrogen. These values were obtained by von Soden and Dirr ('42) from studies of amino nitrogen in enzymatic hydrolysates of yeast. Later work by Dirr and Decker ('44) indicated that the "pure" protein content of yeast is only 64 to 76% of the crude protein content. However, Roine

TABLE 1

Composition of basal diets

INGREDIENTS	DIET A	DIET B
	%	%
Torula yeast	58.50	40.00
Glucose (Cerelose)	28.26	—
Sucrose	—	46.64
Lard, vitamin E-free (stripped)	5.00	5.00
Cellophane, ground	3.00	3.00
Glycine	0.50	1.00
L-Arginine HCl	0.24	0.36
DL-Methionine	0.30	—
Minerals [1]	+	+
Vitamins [2]	+	+

[1] Dicalcium phosphate, 2.55; $CaCO_3$, 0.95; NaCl, 0.6; $FeSO_4 \cdot 7H_2O$, 0.054; $MnSO_4 \cdot H_2O$, 0.036; KI, 0.003; $CoCl_2 \cdot 6H_2O$, 0.0002%.

[2] Thiamine HCl, 0.2; riboflavin, 0.4; niacin, 2.7; calcium pantothenate, 1.5; pyridoxine HCl, 0.45; biotin, 0.015; folic acid, 0.08; vitamin K (menadione), 0.08; vitamin B_{12}, 0.0005 mg and vitamin A, 459 I.U.; vitamin D_3, 30 I.C.U. per 100 gm of diet.

('46) showed that of the soluble nitrogenous compounds in yeast, which represent about 13 to 16% of the total nitrogen (extremes for Torula yeast were 9 and 22%) approximately one-half is present as free amino acids, largely glutamic and aspartic acids, alanine, glutamine and asparagine.

The diet was further modified by the use of dextrose instead of sucrose. The composition of the basal diet is shown in table 1 as Diet A. Since a nitrogen determination conducted with the sample of Torula yeast used indicated a crude protein level of 46%, the amount of yeast used in the basal diet

supplied approximately 21.5% true protein, assuming that 80% of the total nitrogen represented amino acid nitrogen. Calculations of the amounts of essential amino acids present in this amount of yeast according to figures supplied by Singruen ('53) indicated that the diet might be slightly deficient in arginine, methionine and glycine. These amino acids, therefore, were added as supplements to the basal diet.

White Plymouth Rock male chicks were used in the first three and White Leghorn male chicks in the last two experiments. The chicks were housed in groups of 10 to 20 in thermostatically controlled, electrically heated pens with wire-mesh floors. Feed and water were supplied ad libitum.

Experiment 1. For the first experiment, the basal diet was fed alone and supplemented with vitamin E (d,alpha-tocopheryl acetate) and with a variety of different antioxidants. Since the basal diet contained 5% of vitamin E-free lard, a known pro-oxidant, two lots of chicks were fed diets having the same composition except that in one lot the lard was replaced by an equal amount of dextrose and in the other with completely hydrogenated vitamin E-free lard. An additional lot received gamma-tocopherol in order to determine whether or not this form of tocopherol possessed vitamin E activity for the chicks under the conditions of the experiment.

The results of the experiment, presented in table 2, show that all of the chicks receiving the basal diet developed severe symptoms of exudative diathesis by the third week and succumbed before the end of the 4th week. In contrast to the results obtained with rats by Schwarz ('51), none of the chicks showed symptoms of liver necrosis even when examined in the last stages before death. No evidence was found of gizzard erosion, which has been reported to occur on diets which were rendered deficient in vitamin E by means of the addition of cod liver oil or other forms of highly unsaturated fat. Many of the chicks showed slight symptoms of encephalomalacia and edema of the cerebellum, but none showed gross hemorrhages of the cerebellum or severe symptoms of encephalomalacia.

TABLE 2

Specificity of vitamin E for the prevention of exudative diathesis

TREATMENT	AVERAGE WEIGHT			INCIDENCE OF EX. DIATH.
	3 weeks	4 weeks	5 weeks	
	gm	*gm*	*gm*	%
Basal diet (5% vitamin E-free lard)	188 (10) [1]	— (0)	— (0)	100
Alpha-tocopheryl acetate, 5 mg/lb.	259 (14)	382 (13)	531 (12)	0
Gamma-tocopherol, 20 mg/lb.	235 (15)	311 (15)	385 (6)	100
Diphenyl-p-phenylene-diamine (DPPD), 100 mg/lb.	223 (12)	217 (3)	— (0)	100
Butylated hydroxyanisole (BHA), 100 mg/lb.	194 (13)	— (0)	— (0)	100
Nordihydroguaiaretic acid (NDGA), 100 mg/lb.	195 (12)	— (0)	— (0)	100
2,5-Ditertiary-amyl-hydro-quinone (DAH), 100 mg/lb.	209 (10)	— (0)	— (0)	100
6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinone (Santoquin,[2] 100 mg/lb.	230 (12)	242 (2)	— (0)	100
Crude lecithin (Alcolec S),[3] 0.1%	175 (14)	189 (2)	— (0)	100
Purified phosphatides, 0.1%	200 (12)	194 (4)	— (0)	100
Basal diet (lard omitted) [4]	184 (12)	— (0)	— (0)	100
Basal diet (5% vitamin E-free lard, hydrogenated) [5]	161 (13)	193 (5)	— (0)	100

[1] Figures in parentheses indicate number of survivors. Each lot contained 15 male White Plymouth Rock chicks at start.

[2] Monsanto Chemical Company, St. Louis, Missouri.

[3] American Lecithin Company, Inc., Woodside, New York.

[4] Dextrose substituted for lard.

[5] Substituted for lard in basal diet.

All symptoms were prevented and a good rate of growth was obtained in the lot receiving d,alpha-tocopheryl acetate. On the other hand, none of the other treatments had a beneficial effect upon the vitamin E deficiency, with the exception of gamma-tocopherol which, at the level used, caused some delay in the onset of symptoms in some of the chicks and a reduction in mortality during the experimental period of 4 weeks. Between the 4th and 5th week, however, all of the chicks receiving gamma-tocopherol developed severe symptoms of exudative diathesis. Many of these died by the end of the 5th week, when the experiment was discontinued.

Experiment 2. One of the purposes in conducting these studies was to determine if dried brewers' yeast contains an unknown factor capable of sparing the vitamin E requirement of the chick, and if so, whether or not this activity is due to the antioxidant properties of the yeast. Accordingly, a second experiment was conducted using the same basal diet employed in experiment 1, alone and supplemented with d,alpha-tocopheryl acetate, dried brewers' yeast and di-phenyl-para-phenylene diamine (DPPD). Dried brewers' yeast was supplied at a level of 10% in place of an equal amount of Torula yeast. Additional lots received dried brewers' yeast in combination with alpha-tocopheryl acetate or DPPD. The results of the experiment are presented in table 3.

These results show that dried brewers' yeast was as effective as vitamin E in preventing symptoms of E-deficiency, while DPPD again had no effect upon the chicks.

Experiment 3. In view of the fact that dried brewers' yeast produced results equivalent in every respect to those obtained with vitamin E, it was considered desirable to determine whether the vitamin E-sparing factor in yeast is fat soluble. A sample of the dried brewers' yeast was extracted with hexane [2] for 72 hours. The hexane extract and unextractable residue were then added as supplements to the basal diet and compared to vitamin E and whole dried brewers' yeast. The

[2] Skellysolve B.

experimental plan and results of the experiment are presented in table 4.

These results show that the vitamin E-sparing activity of dried brewers' yeast is present in the hexane-insoluble fraction of the yeast, thereby clearly differentiating it from free vitamin E, a fat-soluble vitamin.

Hematologic studies. In view of the fact that the main deficiency symptom occurring in chicks receiving the basal diet was exudative diathesis, which is a form of edema accompanied by extensive capillary hemorrhaging, an examination

TABLE 3

Effectiveness of dried brewers' yeast in preventing symptoms of vitamin E deficiency in chicks

TREATMENT	AVERAGE WT. 4 WEEKS	INCIDENCE OF EX. DIATH.
	gm	%
Basal diet	244 (3)	82
Alpha-tocopheryl acetate (E), 5 mg/lb.	335 (20)	0
Dried brewers' yeast (DBY), 10% [1]	331 (19)	0
DPPD, 100 mg/lb.	274 (3)	78
DBY + E	334 (20)	0
DBY + DPPD	343 (18)	0

[1] Substituted for 10% of Torula yeast in basal diet.

TABLE 4

Presence of vitamin E-like activity of dried brewers' yeast in fat-soluble fraction

TREATMENT	AVERAGE WT. 4 WEEKS	INCIDENCE OF EX. DIATH.
	gm	%
Basal diet	224 (5)	100
Alpha-tocopheryl acetate, 5 mg/lb.	314 (10)	0
Dried brewers' yeast, 10%	323 (8)	0
Hexane (Skellysolve B) extract of dried brewers' yeast ≏ 10% yeast	250 (3)	90
Hexane insoluble residue of dried brewers' yeast ≏ 10% yeast	310 (10)	0

was made of the blood constituents in order to determine whether or not the chicks suffering from vitamin E deficiency were anemic. Hemoglobin, hematocrit, and red blood cell determinations were conducted on blood from at least 5 chicks from each lot in experiment 3. The results of these studies are presented as experiment 3a in table 5. These results

TABLE 5

Microcytic anemia and low reticulocyte counts in vitamin E-deficient chicks

TREATMENT	HEMOGLOBIN	RED BLOOD CELLS	HEMATOCRIT	MEAN CORPUSCULAR VOLUME	RETICULOCYTES
	gm/100 ml	*millions/mm³*	*ml/100 ml*	*μ³*	*%*
		Experiment 3a			
Basal diet	7.7	1.85	18	99	..
D-Alpha-tocopheryl acetate	10.4	2.11	23	109	..
Dried brewers' yeast (DBY)	11.0	2.24	27	120	..
Hexane-extract of DBY	8.3	1.86	20	112	..
Hexane-insoluble residue of DBY	10.5	1.98	24	124	..
		Experiment 3b			
Basal diet	7.5	1.69	19.8	112	15
d,Alpha-tocopheryl acetate	10.2	2.25	29.2	131	85

showed that the chicks receiving the basal diet or this diet supplemented with the hexane extract of dried brewers' yeast were anemic, whereas the blood pictures of the chicks receiving vitamin E, dried brewers' yeast, or the hexane-insoluble fraction of dried brewers' yeast were approximately normal.

In order to determine if the anemia was normocytic, as would be expected if it were due simply to loss of blood brought about by the hemorrhaging, calculations were made

(according to Wintrobe, '42) to determine the mean corpuscular volume for the chicks from each lot. The results showed that the vitamin E-deficient chicks were suffering from a *microcytic* anemia. This was a surprising finding in view of the fact that if the anemia was due to hemorrhaging, the loss of blood should result in a stimulation of production of new erythrocytes which, as reticulocytes, ordinarily have a *larger* mean cell volume than mature erythrocytes.

In order to obtain information concerning the percentage of immature red blood cells in chicks receiving the basal diet as compared to those receiving vitamin E, a further experiment was conducted using these two treatments. When the chicks were three weeks of age, reticulocyte counts were made, in addition to hemoglobin, hematocrit and red blood cell determinations. The results of this study, presented as experiment 3b in table 5, show that reticulated red blood cells were present in much lower concentration in the vitamin E-deficient chicks than in those receiving vitamin E. These results indicate, therefore, that vitamin E is concerned in erythropoiesis. Apparently, the unknown vitamin E-like activity in dried brewers' yeast is as effective in preventing anemia as it is in the prevention of other E-deficiency symptoms.

Studies with a lower protein diet. If one considers that only 80% of the crude protein in yeast is true protein, then the diet used by Schwarz in studies on liver necrosis in rats contained only approximately 12% of true protein. György et al. ('50) used an even lower protein level (18% British bakers' yeast) in their studies of hepatic necrosis in rats. McLean and Beveridge ('52) showed that rats receiving yeast [3] at levels under 30% developed symptoms of liver necrosis, whereas those receiving diets containing 30%, 40% or 60% yeast did not show the disorder.

Experiment 4. In view of the fact that no liver necrosis occurred in the chicks receiving the basal diet in the present studies, it was decided to reduce the amount of Torula yeast to a level of 40% and to omit methionine from the basal diet.

[3] Nutritional Biochemicals Co., U. S. P. grade yeast.

The composition of the modified basal diet is shown in table 1 as diet B. This diet was fed alone and supplemented with methionine and d,alpha-tocopheryl acetate, singly and in combination.

The results of experiment 4, presented in table 6, show that very poor growth, a high severity of exudative diathesis, severe leg weakness, and gizzard erosion occurred in the chicks receiving the basal diet, but no abnormal livers were noted. The addition of methionine, alone, to the basal diet improved

TABLE 6

Results using the low-protein basal diet

TREATMENT	AV. WT. 4 WEEKS	SYMPTOMS			
		Ex. diath.	Severe leg weakness [1]	Liver necrosis	Gizzard erosion
	gm	%	%	%	*average score* [2]
Basal diet (low-protein)	92.5	27	18	0	2.0
Alpha-tocopheryl acetate (vitamin E), 20 mg/lb.	129	0	0	0	1.5
DL-Methionine, 0.5%	169	82	75	0	0.1
Vitamin E + methionine	293	0	0	0	0.9

[1] Chicks unable to stand erect.

[2] Scores made on a basis of 0 for no erosion to 5 for severe erosion.

growth and alleviated gizzard erosion but did not affect exudative diathesis or leg weakness. Addition of both methionine and vitamin E promoted a marked increase in growth and prevented all symptoms.

Experiment 5. Since Abell and Beveridge ('49, '51) reported that the inclusion of cod liver oil in the basal diet for rats increased the incidence and severity of liver necrosis, a further experiment was conducted with chicks using a basal diet similar to that used in the previous experiment except that 5% of cod liver oil was substituted for the 5% of vita-

min E-free lard, and DL-methionine was added to the basal diet at a level of 0.5%. The diet was fed alone and supplemented with D-alpha-tocopheryl acetate. In order to determine whether or not dried brewers' yeast would prevent symptoms on this low-protein diet, a further lot of chicks received a diet in which 10% of the Torula yeast was replaced by dried brewers' yeast. Since cystine has been implicated in some of the work on liver necrosis in rats, an additional lot was included in this experiment in which cystine was fed at a level of 0.4%.

TABLE 7

Results using the low-protein basal diet containing cod liver oil

TREATMENT	AV. WT. 4 WEEKS	SYMPTOMS			
		Ex. diath.	Severe leg weakness	Liver necrosis	Gizzard erosion
	gm	%	%	%	*average score*
Basal diet [1]	134	100	75	0	0.9
Alpha-tocopheryl acetate, 20 mg/lb.	246	0	0	0	1.4
Dried brewers' yeast, 10% [2]	277	0	0	0	0.8
Cystine, 0.4%	193	91	83	0	0.3

[1] Basal diet contained 0.5% methionine and 5% cod liver oil.

[2] Substituted for an equal amount of Torula yeast.

The results of this experiment, presented in table 7, confirmed the previous experiments and showed that dried brewers' yeast is as effective as alpha-tocopheryl acetate in preventing the symptoms of vitamin E deficiency which occur on this type of basal diet. Supplementing the diet with cystine improved growth and gizzard erosion, but did not affect any of the symptoms of vitamin E deficiency. Even in the presence of cod liver oil, no symptoms of liver necrosis appeared, indicating that necrosis of the liver is not a symptom of vitamin E deficiency in the chick.

DISCUSSION

A basal diet has been developed which appears to be suitable for use in the study of uncomplicated vitamin E deficiency

in the chick. The occurrence of vitamin E deficiency symptoms in chicks receiving the basal diet was not influenced to a measurable degree by the presence or absence of lard in the diet. Since the Torula yeast used in these experiments contained only 1.3% of ether-extractable material, the diet from which the lard was omitted contained approximately 0.66% fat. It seems unlikely that this small amount of fat could have a marked effect upon the vitamin E requirements of the chicks. The failure of DPPD to prevent symptoms of vitamin E deficiency appears to be contrary to the results reported by Singsen et al. ('53) and Bunnell et al. ('54). However, the basal diet used by these workers was known to contain small amounts of natural vitamin E and the addition of 2% of fish oil was necessary in order to produce symptoms.

Exudative diathesis, the most prominent symptom exhibited by the chicks receiving the basal diet, was first shown to be due to vitamin E deficiency by Dam and Glavind ('39). In the present studies, chicks suffering from exudative diathesis and showing gross puffy edema under the skin recovered within 24 to 48 hours following a single oral administration of 10 mg of d,alpha-tocopheryl acetate. In view of these findings, vitamin E appears to be concerned in the maintenance of proper osmotic relationships between the intra-vascular and extra-vascular fluids within the body of the chick.

In a recent report from this laboratory (Miller, Small and Norris, '55) results were presented which indicated that when vitamin E deficiency is produced in chicks by actively oxidizing fats, the most acute symptom which occurs is encephalomalacia. This symptom of vitamin E deficiency was first described in chicks by Pappenheimer and Goettsch ('31). No obvious explanation exists for the fact that the chicks in the present experiments died with symptoms of severe exudative diathesis without showing marked symptoms of encephalomalacia.

Jungherr and Pappenheimer ('37) reported that in turkeys receiving a vitamin E-deficient diet the primary symptom observed was necrosis of the smooth muscle of the gizzard

wall, unaccompanied by significant lesions in other organs or tissues. Many of the chicks receiving the basal Torula yeast diet in the present experiments also showed a marked necrosis of the gizzard musculature characterized by a thin, white, hyaline appearance instead of the normal thick, red smooth muscle.

Jungherr and Pappenheimer ('37) also reported that the majority of the poults receiving their basal diet developed severe perosis within 18 days, whereas the controls remained free from this deformity. A large number of the chicks receiving the basal, vitamin E-deficient diet suffered from severe leg weakness and displayed an enlargement of the hock joint which closely resembled that studied in turkeys by Scott ('50; '51a, b; '53). Since this disorder in the chicks was prevented by the addition of either vitamin E or dried brewers' yeast, it is possible that the condition is the counterpart in chicks of the enlarged hock disorder in turkeys.

Since the substitution of 10% of dried brewers' yeast for an equal amount of Torula yeast in the basal diet was as effective as vitamin E in preventing all of the symptoms referred to above, it appears that the sample of dried brewers' yeast used in these experiments contained a factor having biological activity similar to that of vitamin E. In view of the failure to extract this factor from dried brewers' yeast with hot hexane,[4] it appears that this material, unlike free or esterified vitamin E, is not fat-soluble. Since antioxidants, lecithins and gamma-tocopherol were ineffective for prevention of exudative diathesis and the other disorders, these dysfunctions appear to be due to a specific deficiency of vitamin E. This indicates the possibility that brewers' yeast contains an active derivative of vitamin E. On the other hand Schwarz ('51b) has reported the presence of a factor (Factor 3) in brewers' yeast believed to be distinct from vitamin E. Whether or not Factor 3 is responsible for the vitamin E-sparing activity of brewers' yeast for the chick remains to be determined.

[4] See footnote 2, page 392.

SUMMARY

A basal diet has been developed which appears to be suitable for use in the study of uncomplicated vitamin E deficiency in the chick. The occurrence of vitamin E-deficiency symptoms in chicks receiving the basal diet was not influenced to a measurable extent by the presence or absence of pro-oxidants or antioxidants in the diet.

The symptoms which occurred in chicks receiving the basal diet were, for the most part, similar to those which have been described previously for vitamin E-deficient chicks. In addition, the chicks receiving the basal diet displayed a microcytic anemia and a low reticulocyte count, indicating that vitamin E may be concerned in erythropoiesis. Necrosis of the liver does not appear to be a symptom of vitamin E deficiency in the chick. Erosion of the gizzard lining was not influenced by the level of vitamin E in the diet, but appeared instead to be due to a deficiency of essential amino acids, particularly methionine and cystine.

The brewers' yeast used in these studies contained biological activity similar to that of vitamin E.

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EFFECT OF GERMINATION ON VITAMIN B_{12} VALUES OF PULSES (LEGUMINOUS SEEDS)

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A study of the vitamin content of germinated pulses has been carried out in this laboratory. It was observed that the germinated pulses contained significantly increased amounts of carotene (Chattopadhyay and Banerjee, '51a), tocopherol (Chattopadhyay and Banerjee, '52), ascorbic acid (Nandi and Banerjee, '50a), thiamine (Chattopadhyay, Nandi and Banerjee, '50), riboflavin (Nandi and Banerjee, '50b), pantothenic acid, biotin and niacin (Banerjee, Rohatgi and Lahiri, '54) and choline (Chattopadhyay and Banerjee, '51b) than the ungerminated pulses. Germinated pulses, however, contained less folic acid (Banerjee et al., '54). As vitamin B_{12} plays an important role in normal hemopoiesis in humans it was of interest to estimate vitamin B_{12} in ungerminated and germinated pulses because pulses form an important item in the daily diet of Indians.

A number of different extraction procedures have been suggested which would produce maximum release of vitamin B_{12} from natural materials. Coates, Ford, Harrison, Kon, Porter, Cuthbertson and Regler ('51) observed that the extraction of calf and chick feces and chick cecal contents in the presence of cyanide resulted in higher vitamin B_{12} values. Thompson, Dietrich and Elvehjem ('50) extracted vitamin B_{12} from various animal proteins by digesting the homogenized samples with trypsin in 0.8% sodium bicarbonate solution for

24 hours at pH 7. Denton and Kellog ('50) reported that extraction of egg yolk and other materials in the presence of sodium cyanide or sodium bisulphite acetate buffer of pH 4.5 gave an increased vitamin B_{12} activity.

In the present investigation several of the extraction procedures were carried out in order to find a method which would release maximum vitamin B_{12} from the pulses. The vitamin B_{12} contents of 7 varieties of pulses, germinated for two or 4 days or ungerminated, were determined microbiologically.

EXPERIMENTAL AND RESULTS

Extraction of vitamin B_{12} from pulses. In order to find out which method of extraction would give the maximum vitamin B_{12} value, samples of *Phaseolus mungo* were extracted by the following different procedures:

1. Enzymatic digestion with trypsin at pH 7 in 0.8% sodium bicarbonate solution for 24 hours according to the method of Thompson et al. ('50).
2. Autoclaving the samples in acetate buffer at pH 4.5 for 30 minutes with sodium cyanide according to the method of Denton and Kellog ('50).
3. Autoclaving the samples in acetate buffer at pH 4.5 for 30 minutes with sodium bisulphite.
4. Enzymatic digestion with trypsin at pH 7 in 0.8% sodium bicarbonate for 24 hours followed by autoclaving with sodium cyanide in acetate buffer of pH 4.5.

After extraction with the above methods, vitamin B_{12} activity was estimated microbiologically and the results are given in table 1. It was observed that samples which were first digested with trypsin and then treated with sodium cyanide and autoclaved for 30 minutes gave highest vitamin B_{12} values. This procedure was, therefore, adopted for the extraction of vitamin B_{12}.

In order to verify the accuracy of this method of extraction a known amount of vitamin B_{12} was added to 1 gm of crushed *Phaseolus mungo* and the pulse was extracted as described above. Vitamin B_{12} was estimated microbiologically. The

results are given in table 2. A good recovery of the added vitamin B_{12} was obtained by this method.

Germination of the seedling. About 1 gm portions of clean, dry and healthy seeds were weighed and kept in clean and sterilized petri dishes. Two milliliters of sterile redistilled water were added on the first day and 1 ml on subsequent

TABLE 1

Effect of different methods of extraction on the vitamin B_{12} contents of Phaseolus mungo (μg per 100 gm)

SAMPLE NO.	AFTER DIGESTION WITH TRYPSIN	AFTER AUTOCLAVING WITH SODIUM CYANIDE	AFTER AUTOCLAVING WITH SODIUM BISULFITE	AFTER DIGESTION WITH TRYPSIN FOLLOWED BY TREATMENT WITH SODIUM CYANIDE
1	0.275	0.275	0.310	0.425
2	0.275	0.275	0.300	0.410
3	0.280	0.270	0.300	0.430
4	0.275	0.275	0.300	0.437
5	0.250	0.285	0.290	0.437
6	0.260	0.270	0.280	0.400

TABLE 2

Recovery of vitamin B_{12} added to the pulse Phaseolus mungo

SAMPLE NO.	VITAMIN B_{12} ADDED	VITAMIN B_{12} ESTIMATED	VITAMIN B_{12} RECOVERED
	$m\mu g$	$m\mu g/gm$	%
1	0	4.0	...
2	10	13.8	98
3	10	14.0	100
4	12	16.0	100
5	12	15.8	98
6	12	16.1	100.8

days to each petri dish. The dishes were kept away from direct sunlight at room temperature (30°C.). The seeds germinated well and the seedlings were healthy.

Extraction and estimation of vitamin B_{12}. Powdered ungerminated seeds and seeds germinated for 48 and 96 hours were crushed in a glass mortar with 0.8% sodium bicarbonate solution and transferred quantitatively into a 250 ml Erlen-

myer flask with the bicarbonate solution, the total quantity of which was 18 ml in each case. Five milligrams of trypsin dissolved in 2 ml of 0.8% bicarbonate solution and 1 ml toluene were added to each flask which was then incubated for 24 hours at 37°C. After the incubation 100 ml of acetate buffer of pH 4.5 were added to the flask and the contents were mixed by shaking. After the addition of 10 mg of sodium cyanide in aqueous solution the flask with the contents was autoclaved at

TABLE 3

Vitamin B_{12} contents of pulses. (Average of 10 determinations)

LOCAL NAME	BOTANICAL NAME	UNGERMINATED	AFTER GERMINATION FOR	
			Two days	Four days
		μg/100 gm	*μg/100 gm*	*μg/100 gm*
Kalai	Phaseolus mungo	0.42 ± 0.02 [1]	0.85 ± 0.01	1.20 ± 0.01
Mung	Phaseolus radiatus	0.61 ± 0.01	0.81 ± 0.02	1.53 ± 0.03
Musur	Lens esculenta	0.43 ± 0.01	0.47 ± 0.01	2.37 ± 0.01
Mator	Pisum sativum	0.36 ± 0.02	1.27 ± 0.03	2.36 ± 0.0
Khesari	Lathyrus sativus	0.37 ± 0.0	0.75 ± 0.0	0.86 ± 0.0
Arhar	Cajunus indicus	0.53 ± 0.01	0.68 ± 0.01	0.57 ± 0.01
Chhola	Cicer arietinum	0.35 ± 0.01	1.90 ± 0.00	1.22 ± 0.01

[1] Mean ± standard error of the mean.

15 pounds pressure for 30 minutes. At this pH the excess cyanide was driven off during autoclaving and did not cause inhibition of the growth of the test organism. The pH of the extract was adjusted to 6.8 with 10N NaOH, the volume was made up to 250 ml and filtered. Vitamin B_{12} was estimated in the filtrate microbiologically using *Lactobacillus leichmannii* (NRRL-B 735 W) as the test organism according to the United States Pharmacopia method ('51). The bacteria were rejuvenated by daily transfer in 10 ml basal medium containing 0.5 mμg vitamin B_{12} for 4 days before inoculation. After

incubation for 72 hours at 37°C., the acid produced was titrated with 0.1N NaOH and the vitamin B_{12} content calculated by interpolation from the standard curve in terms of the original weight of seeds before germination. Crystalline vitamin B_{12}[1] was used as the standard. The results are given in table 3.

DISCUSSION

The results show that pulses contain vitamin B_{12}. In the ungerminated condition the vitamin B_{12} content was highest in *Phaseolus radiatus* and lowest in *Cicer arietinum, Pisum sativum* and *Lathyrus sativus. Lens esculenta* and *Cajunus indicus* showed intermediate values. During the process of germination vitamin B_{12} values increased in all the pulses and the increase was maximum in most of the pulses on the 4th day of germination. *Pisum sativum, Lens esculenta* and *Phaseolus radiatus* were found to be good sources of vitamin B_{12} when they were germinated. Consumption of germinated pulses should, therefore, be advocated from the nutritional point of view. Cases of pernicious anemia are not very common in India although Indians are mostly vegetarian in their food habits. Pulses form an important constituent of the daily dietary of Indians and it is no wonder that they receive sufficient vitamin B_{12} from the pulses they consume.

SUMMARY

A method of extraction of vitamin B_{12} from pulses has been described which will release a maximum amount of the vitamin.

The vitamin B_{12} values of 7 varieties of pulses have been determined microbiologically using *Lactobacillus leichmannii* both before and after germination for 48 or 96 hours. In all of the pulses vitamin B_{12} values increased after germination for 48 hours and in most of the cases the increase was highest after 96 hours' germination.

[1] Merck and Co., Inc., Rahway, New Jersey.

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INTERRELATIONSHIP OF SERINE AND GLYCINE FOR CHICK GROWTH [1]

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INTRODUCTION

By the criterion of dietary need for maximum growth, both serine and glycine have been demonstrated to be non-essential amino acids for the rat (Rose et al., '52). However, in the chick, Almquist has shown that glycine supplements increased the rate of growth of chicks on diets containing casein (Almquist and Mecchi, '40, '42) or amino acid mixtures (Almquist and Grau, '44). This finding has been confirmed by Hegsted et al. ('41), and recently by Wietlake and coworkers ('54). For this reason glycine has been classified as an essential amino acid for the chick (Almquist and Grau, '44).

However, these chick diets, due to the addition of the amino acid supplement, contained variable amounts of total nitrogen. In this connection, Rose et al. ('49) have demonstrated that glycine can serve as a source of nitrogen for the synthesis of non-essential amino acids in the weanling rat. Thus the above-mentioned chick experiments might be open to the interpretation that the nitrogen added in the extra glycine is being used for the formation of other non-essential amino acids. Some of the following experiments were designed to evaluate this alternative hypothesis.

In recent years there has accumulated considerable evidence from tracer and enzymatic studies to demonstrate the inter-

[1] Research paper No. 1165, Journal Series, University of Arkansas. This investigation was supported by research grant No. 227, Nutrition Foundation, Inc. A preliminary report was given at the annual meeting of the American Institute of Nutrition at San Francisco, April 11–15, 1955.

conversion of serine and glycine. In general these experiments have shown that serine, by the loss of the β-carbon atom, is the precursor of glycine (Shemin, '46), and that glycine by the addition of a one-carbon unit is converted into serine (Sakami, '48, '49). Pyridoxine (Deodhar and Sakami, '53; Lascelles and Woods, '54) and folic acid (Greenberg, '54; Totter et al., '50; Blakeley, '54) have recently been implicated in these metabolic reactions.

The interest in these two amino acids is enhanced because of their known participation in a wide variety of biosynthetic processes. Aside from their interconversion, glycine has a role in the rat in the formation of porphyrins, purines, bile acids, creatine, glutathione, hippuric acid, sarcosine and protein, while serine is a precursor of ethanolamine, choline, betaine, phosphatidylserine, cysteine and tissue protein. Both the β-carbon of serine and the α-carbon of glycine participate in numerous metabolic reactions involving a one-carbon unit (Greenberg, '54).

In view of the current knowledge of the intermediary metabolism of serine and glycine, it is desirable to inquire about the rate of the *in vivo* interconversion of these two amino acids in the chick as measured by the method of growth. In this respect, though it was earlier stated that the omission or inclusion of serine had no effect on the glycine deficiency of chicks fed amino acid mixtures (Almquist and Grau, '44), experimental evidence to buttress this claim has not yet been found in the nutritional and biochemical literature. The second series of experiments reported here was designed to clarify the interrelationship of serine and glycine in chick nutrition.

EXPERIMENTAL

One-day-old single comb White Leghorn male chicks were wing-banded, weighed, and given the starting diet. They were allowed water and food ad libitum. The cockerels were housed in electrically heated metal battery brooders with raised screen floors in an air conditioned room and with a uniform distribution of artificial light for all groups for 14

hours per day. During the first week, the cockerels were electrically debeaked to prevent feather picking. At one or two weeks of age, some of the slowest and the fastest growing chicks were discarded (about 15 to 20 % of the total). The remaining chicks were distributed so that all experimental groups of 12 chicks had the same mean initial weights and

TABLE 1

Composition of the basal ration

INGREDIENTS	AMOUNT	VITAMINS ADDED PER 100 GM	
	gm		*mg*
Casein [1]	20.0	Thiamine HCl	1
L-Arginine · HCl	0.5	Riboflavin	1
L-Cystine	0.3	Pyridoxine HCl	1
Salts [2]	5.0	Ca-Pantothenate	3
$CaCO_3$	0.6	Niacinamide	5
$MnSO_4 \cdot 4H_2O$	0.1	i-Inositol	100
Cellulose [3]	3.0	Choline Cl	200
Hydrogenated vegetable oil [4]	3.0	Menadione	1
Corn oil	0.9	α-Tocopherol	25
Glucose hydrate [5]	66.2		
		Folic acid	200 μg
		Biotin	20 μg
		Vitamin B_{12}	3 μg
		Vitamin A [6]	3000 I.U.
		Vitamin D [6]	425 I.U.

[1] Labco vitamin-free casein.
[2] Jones and Foster ('42).
[3] Cellu flour, Chicago Dietetic Supply House.
[4] Crisco, Proctor and Gamble Company.
[5] Cerelose, Corn Products Refining Company.
[6] Percomorph liver oil with viosterol, Abbott Laboratories.

the same range of weights. These comparable weight groups were placed on the basal diet with or without supplements for two weeks.

The composition of the basal diet is described in table 1. The casein was supplemented with arginine and cystine. Calcium carbonate was included with the salt mixture to raise the total calcium level from 0.76 to 1.00% and the calcium: total phosphorus ratio from 1.3:1 to 1.7:1. This

modification made the composition of the ions closer to the nutrient requirements for poultry (Committee on Animal Nutrition, '54). The starting diet was prepared by the addition of 10% of gelatin to the basal diet. The nitrogenous supplements in the experimental diets were checked by analysis for the total nitrogen content by the macro-Kjeldahl procedure, and were added to the basal diet by substitution for an equal weight of glucose hydrate. The diets were prepared in two-kilogram batches, and the main supply was kept in a refrigerator.

Records of weekly weight, feed intake, and mortality were kept. The data on the chick growth are reported as the average gain and as the average percentage gain per day; this latter is defined as the average weight gain per day times 100 divided by the average chick weight during the experimental period (Almquist and Grau, '44).

Every experiment included one group of chicks fed a commercial chick starting ration. Since the growth rates of these chicks were consistently high and quite reproducible, this group served as a check on the constancy of the experimental conditions and as an additional reference point for the comparison of successive experiments. Hence the growth rates of the chicks on the commercial starting ration are omitted from the tables.

RESULTS

In two preliminary experiments, the supplement of 1.5% of glycine was the minimum amount that produced maximum growth when added to the basal diet. This finding confirms the value reported earlier (Almquist and Mecchi, '42), and consequently, 1.5% of glycine was used in all subsequent experiments.

The next experiment, for which the data are not reported here, indicated that while 1.5% of glycine led to an increase in the rate of growth, an isonitrogenous supplement of diammonium citrate also produced a comparable increase in the average rate of growth of 12 chicks. This observation led to

the consideration of the earlier-mentioned alternative hypothesis — namely, that perhaps the nitrogen added in the extra glycine is being used for the formation of other non-essential amino acids. However, the extra growth with diammonium citrate could not be duplicated in two subsequent experiments with 12 chicks for each experimental diet. Because of this variable response, all subsequent experiments have been carried out with three groups of 12 chicks on each experimental diet.

The first experiment reported, experiment IX in table 2, compared the growth effect of glycine and diammonium citrate with three times the number of chicks of the earlier experiments. Sufficient diammonium citrate on the basis of Kjeldahl nitrogen analysis of 12.26% (theoretical 12.39%) was added to supply an amount of nitrogen equal to that in 1.5% glycine. Under the earlier conditions, the addition of 1.5% glycine to the basal ration increased the percentage growth rate from 3.97 to 4.74. Application of the "t" test showed that the probability that the observed difference in the mean gain of the basal and the glycine-supplemented group was due to chance was slightly higher than 1%. By contrast, the average gain for the isonitrogenous diammonium citrate-supplemented group was not statistically different from the basal group. In these experiments, the efficiency of the utilization of the feed was highest for the glycine group, whereas the feed efficiency of the diammonium citrate and basal groups were approximately the same. Thus, under these conditions, diammonium citrate did not spare the apparent glycine requirement.

At the present time, glycine has been shown to be a nutritive essential only for chicks under 4 weeks of age. Therefore it is pertinent to inquire whether older chicks also need a dietary source of glycine. Two groups of 18 4-week-old chicks each were placed on the basal ration, and two groups on the experimental diet for two weeks. While one group with glycine supplement did have a higher average gain than the basal group, there was not a significant difference between the mean

gain of both glycine groups and the mean gain of both basal groups. Therefore the need for additional dietary glycine for maximum growth of 4-week-old chicks is less marked than that of younger chicks.

In view of current knowledge of the intermediary metabolism of glycine and serine, it was of interest to determine

TABLE 2

Effect on the growth rate of adding glycine or diammonium citrate to the basal diet of chicks

EXPERIMENT NUMBER	SUPPLEMENT TO BASAL DIET	NO. OF LIVE CHICKS	FEED EFFICIENCY	AVERAGE GAIN ± STANDARD ERROR	AVERAGE GROWTH RATE
				gm	*%*
IX [1]	None	12	.34	93.7 ± 13.1	
	None	12	.36	112.8 ± 13.3	
	None	12	.31	88.8 ± 17.7	
	Mean	36	.34	98.4 ± 8.5	3.97
	1.5% glycine	12	.43	128.6 ± 13.4	
	1.5% glycine	12	.40	120.9 ± 13.0	
	1.5% glycine	12	.43	132.2 ± 10.7	
	Mean	36	.42	127.2 ± 7.0 [2]	4.74
	2.28% $(NH_4)_2HC_6H_6O_7$	12	.35	96.5 ± 13.5	
	2.28% $(NH_4)_2HC_6H_6O_7$	12	.37	112.7 ± 14.4	
	2.28% $(NH_4)_2HC_6H_6O_7$	12	.34	109.6 ± 11.6	
	Mean	36	.35	106.3 ± 7.5	4.19
XIII [3]	None	18	.30	188.9 ± 21.8	
	None	18	.31	185.2 ± 20.7	
	Mean	36	.31	187.1 ± 14.8	3.31
	1.5% glycine	18	.34	195.2 ± 23.9	
	1.5% glycine	18	.43	243.6 ± 18.2	
	Mean	36	.39	219.4 ± 15.1	3.73

[1] The chicks in experiment IX were two weeks old, and had an overall mean initial weight of 127.9 gm.

[2] Significant at the 5% level.

[3] The chicks in experiment XIII were 4 weeks old, and had an overall mean initial weight of 310.2 gm.

whether dietary serine could spare the glycine requirement. The next three experiments, the data for which are presented in table 3, provide evidence to answer this question. The design of experiment X was the same as that for the previous one; three groups of 12 two-week-old chicks were placed on each experimental diet for two weeks. The addition of 1.5% of glycine gave the expected increase in rate of weight gain. Under the same conditions, an isonitrogenous amount of DL-serine — i.e., 2.1% — also gave an increase in the growth rate.

The experiment just described was repeated with one modification, namely, the use of one-week-old chicks. In experiment XI, glycine supplements led to the anticipated increase in growth rate. At the same time, the addition of 2.1% of DL-serine gave an increase in percentage growth from 5.01 to 5.67, an increase that was almost as great as that given by glycine. The difference was statistically significant at the 5% level.

In a recent study of the amino acid deficiencies of casein when used as the sole source of protein for the chick, Wietlake and coworkers ('54) found that supplementation of a 35%-casein diet with 0.5% of DL-methionine, 1.24% of L-arginine and 1.5% of glycine produced excellent growth. With the minor difference of the use of cystine instead of methionine, our diets in experiment XII were based on their findings. As expected, a higher growth rate was found for the glycine-supplemented 35%-casein diet than for the comparable 20%-casein diet in experiment XI; the protein efficiencies were 1.74 and 2.00 respectively. Further evidence for the adequacy of the supplemented 35%-casein diet may be found by comparison of the growth on this diet with that of a group of 12 chicks raised on a commercial starting ration [2] under identical conditions. The average gain of these 12 chicks was

[2] Purina "chick Startena," which contains an antibiotic and 0.0025% 3-nitro-4-hydroxyphenyl arsonic acid, manufactured by Ralston Purina Company, St. Louis, Mo.

173.2 ± 5.6 gm and the average percentage growth rate was 7.50. However, the important point observed in experiment XII is that the basal ration containing arginine- and cystine-supplemented 35% casein is still deficient in glycine. This is demonstrated by the improved percentage growth upon the addition of glycine, i.e., from 6.54 to 6.95. Again, the question was asked whether serine would alleviate the apparent glycine deficiency. Thus it is pertinent to note that, under the same conditions, the supplement of DL-serine also led to a comparable increase in the percentage rate of gain, i.e., from 6.54 to 6.87. Comparison of the mean gains of the glycine and basal groups by the "t" test indicated that the probability that the difference was due to chance was less than 1%. At the same time the difference between the mean gains of the serine and basal groups was statistically significant at the 5% level.

In all three of the experiments just mentioned, the feed efficiency was highest for the glycine-supplemented groups, and lowest for the basal group. The chicks with the serine supplement utilized their feed in an intermediate manner, though in several cases it was as high as that of the glycine-supplemented group.

DISCUSSION

The data presented confirm part of the earlier findings of Almquist and Mecchi ('42), namely, the slow growth found on the basal ration containing casein, which has a low glycine content (Block and Bolling, '51), and the improvement in the growth rate by the addition of dietary glycine. Almquist's observations led to the conclusion that, while the chick can synthesize some glycine, the rate of manufacture of glycine is not commensurate with tissue needs for maximum rate of growth. Therefore glycine was classified as an essential amino acid for the chick (Almquist and Grau, '44). However, in view of the extension of the experimental inquiry in this report, the above conclusions may need modification as indicated below.

The results found in experiment IX indicate that the extra nitrogen in the form of ammonium ion did not lead to increased growth. Thus the preliminary experiment, in which diammonium citrate led to an increase in the rate of growth, was a chance observation. Subsequently no less than 6 groups of 12 chicks each have been placed on the diammonium citrate-supplemented ration. All have been found to have a growth rate of the same order of magnitude as the basal group in the respective experiments. Furthermore, in a preliminary experiment, for which the data are not reported, addition of an isonitrogenous amount of DL-alanine (1.78%) or casein (1.91%) to the basal diet for groups of 12 chicks each did not improve the growth appreciably above that of the basal group. Therefore it is unlikely that the attainment of maximum growth is limited by insufficient total nitrogen, or by the distribution of nitrogen between the essential and non-essential amino acids of the supplemented casein. Apparently then, the rate of synthesis of glycine, or possibly serine, was limiting the rate of growth in these chicks. This limitation is evidently less marked in 4-week-old chicks than in two-week-old chicks.

The three separate experiments, which are presented in table 3, indicate that DL-serine improved the rate of growth of chicks on a glycine-deficient ration. This observation was valid for both the minimum-protein 20%-casein diet, and the high-protein 35%-casein diet. At the present time it is unknown why the growth of the serine-supplemented chicks was slightly less than that of the glycine-supplemented chicks. Three possible explanations might be considered. The first is that the chick growth might be hindered due to the nephrotoxic action of D-serine (Artom et al., '45). In this respect, the earlier observed kidney damage in rats occurred after the administration of 100 mg daily doses of DL-serine by stomach tube and was most evident in pyridoxine-deficient rats. However, no differences were found upon comparison of the growth curves and health of the rats receiving 100 or 200 mg of DL-serine mixed with their daily diet, and of rats on a suitable

TABLE 3

Effect on the growth rate of adding glycine or serine to the basal diet of chicks

EXPERIMENT NUMBER	SUPPLEMENT TO BASAL DIET	NO. OF LIVE CHICKS	FEED EFFICIENCY	AVERAGE GAIN ± STANDARD ERROR	AVERAGE GROWTH RATE
				gm	%
X [1]	None	12	.33	108.0 ± 9.1	
	None	12	.34	100.3 ± 14.1	
	None	12	.33	105.3 ± 20.8	
	Mean	36	.33	104.5 ± 8.7	3.93
	1.5% glycine	12	.46	137.2 ± 17.1	
	1.5% glycine	12	.42	126.2 ± 18.1	
	1.5% glycine	12	.43	127.7 ± 18.4	
	Mean	36	.44	130.4 ± 10.0 [2]	4.58
	2.1% DL-serine	12	.41	130.1 ± 12.4	
	2.1% DL-serine	12	.37	105.1 ± 16.2	
	2.1% DL-serine	12	.36	108.2 ± 14.5	
	Mean	36	.38	114.5 ± 8.3	4.19
XI [3]	None	12	.31	84.2 ± 7.7	
	None	12	.25	79.2 ± 10.4	
	None	12	.33	87.5 ± 10.0	
	Mean	36	.30	83.6 ± 5.3	5.01
	1.5% glycine	12	.43	97.7 ± 9.4	
	1.5% glycine	12	.35	113.5 ± 9.4	
	1.5% glycine	12	.41	102.1 ± 9.6	
	Mean	36	.40	104.4 ± 5.4 [4]	5.75
	2.1% DL-serine	12	.40	110.3 ± 10.6	
	2.1% DL-serine	12	.43	100.1 ± 12.8	
	2.1% DL-serine	11	.37	94.0 ± 9.7	
	Mean	35	.40	101.5 ± 6.6 [2]	5.67
XII [5]	None	11	.52	131.4 ± 7.3	
	None	12	.55	133.0 ± 8.6	
	None	12	.49	135.6 ± 5.7	
	Mean	35	.52	133.3 ± 4.1	6.54
	1.5% glycine	12	.61	153.0 ± 4.1	
	1.5% glycine	12	.60	145.7 ± 6.5	
	1.5% glycine	12	.61	147.0 ± 7.2	
	Mean	36	.61	148.6 ± 3.4 [4]	6.95
	2.1% DL-serine	12	.54	152.1 ± 6.4	
	2.1% DL-serine	12	.56	138.2 ± 7.4	
	2.1% DL-serine	11	.55	144.6 ± 6.1	
	Mean	35	.55	145.0 ± 3.9 [2]	6.87

[1] The chicks in experiment X were two weeks old and had an overall mean initial weight of 137.9 gm.

[2] Significant at the 5% level.

[3] The chicks in experiment XI were one week old and had an overall mean initial weight of 77.4 gm.

[4] Significant at the 1% level.

[5] The chicks in experiment XII were one week old, had an overall average initial weight of 78.5 gm, and had a basal diet containing 35% casein plus 1.24% L-arginine HCl, plus 0.3% L-cystine.

control diet (Artom and Fishman, '44). From this background, it is unlikely that the dietary D-serine was toxic for the chicks in the present experiments.

A second possible explanation is that the interconversion of L-serine and glycine might be slow in the chick. Hsu et al. ('54) have reported that the availability of glycine-2-C^{14} for synthesis of tissue serine is low in the chick. However the rate of the reverse reaction in the chick has evidently not been studied as yet.

Another possible explanation is that the D-serine is not readily utilized for glycine formation. The experiments of Shemin ('46) indicate that, while N^{15}-labeled L-serine is extensively and rapidly converted to N^{15}-labeled glycine and hippuric acid in the rat and guinea pig, the N^{15}-D-serine undergoes considerable dilution, and is not readily utilized for hippuric acid formation. If the D-isomer is also not available in the avian species, then perhaps the growth of chicks on diets containing only the L-isomer of serine, and at a level isonitrogenous with 1.5% glycine, might be comparable with the growth of chicks on diets containing added glycine.

The data presented on the nutritional interrelationships of glycine and serine might be interpreted in two possible ways. If serine is required to a greater extent under the stated experimental conditions, then these observations represent a transformation of glycine to serine. On the other hand, the opposite case might be prevailing, namely, a limited formation of glycine for various metabolic reactions. If this is the case, then the results should be interpreted as a conversion of serine to glycine. In view of the known utilization of glycine for uric acid formation (Greenberg, '54), and for feather production (Hegsted et al., '41), perhaps the latter explanation is the more probable one. Upon consideration of just the growth data in this report, it is impossible at present to differentiate between these two possible interpretations.

The observations reported in this paper are consistent with the earlier mentioned *in vitro* and *in vivo* radioactive tracer experiments (Sakami, '48, '49; Greenberg, '54), which es-

tablished the interconversion of serine and glycine. However these isotopic investigations might be explained by exchange processes, and do not demonstrate *per se* a net synthesis of glycine from serine or *vice versa*. Hainline and Lewis ('53) administered DL-serine and sodium benzoate to rabbits and observed a net production of glycine measured as excreted hippuric acid. By contrast, the semiquantitative nutritional relationship between these two amino acids in the present experiments was observed without the addition of the non-physiological stress of sodium benzoate. The comparable growth response to glycine or serine supplements in the chick may also be interpreted as a net *in vivo* synthesis of one of these amino acids from the other. This nutritional observation is similar, in certain respects, to the well-known sparing effect of cystine for methionine, and of tyrosine for phenylalanine. With this background, it may be necessary to modify the classification of essential amino acids for the chick with respect to glycine and serine.

CONCLUSION

Glycine supplements to a casein-containing diet led to an increase in the growth rate of chicks. This increase was not due to the extra nitrogen in the glycine added to the basal diet, but was due to an inability of the chick to synthesize glycine, or possibly serine, at a rate commensurate with maximum rate of gain. This limited rate of synthesis was found primarily in chicks under 4 weeks of age.

Under the conditions of these experiments, the addition of DL-serine was found to improve the rate of growth of chicks on a glycine-deficient ration. This observation is consistent with the known metabolic interconversion of these two amino acids as demonstrated by evidence from tracer and enzymatic studies, and presents additional confirmation by the method of growth. In summary, the evidence presented suggests a nutritional interrelationship of serine and glycine in the growing chick.

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THE THIAMINE REQUIREMENT OF THE BABY PIG [1]

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Hughes ('40), Van Etten et al. ('40), Ellis and Madsen ('44) and Moustgaard ('53) have reported thiamine requirements of young growing pigs. These reported requirements range from 22 to 75 μg of thiamine per kilogram body weight daily.

Since the only data presented to date relative to the determination of the thiamine requirement of pigs have resulted from study with young growing pigs, it seems important also to consider the requirement of the baby pig for this vitamin. The purpose of the present paper is to submit experimental data relating this requirement to the baby pig's total dietary solids intake and to present observations of thiamine deficiency symptoms appearing in baby pigs fed sub-minimum levels of this vitamin.

EXPERIMENTAL

A triplicated experiment was conducted involving 55 baby pigs. In each of the replications, pigs were taken from the

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[2] The data contained in this paper are a portion of the research and thesis to be presented by the senior author in partial fullfillment of the requirements for the degree of Doctor of Philosophy, School of Graduate Studies, Michigan State College, East Lansing.

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sow at three or 4 days of age and placed into individual cages. Room temperature was thermostatically maintained at 70°F. except in the second replication which was conducted during the summer when day room temperatures often exceeded 80°F. Infra-red heat lamps were used to supply additional heat for the first two weeks of the experimental period.

All pigs first underwent a 4-day depletion-adjustment period. During this period they were fed a synthetic milk diet identical to that used by Miller et al. ('54) except that thiamine was absent from the diet and riboflavin replaced it in equal amount. At the end of this depletion-adjustment period, the pigs were assigned to lots on the bases of sex, size, litter and general appearance.

On the basis of the reported work with older pigs, the minimum thiamine requirement of the baby pig was judged not to exceed 2 mg of thiamine per kilogram of dietary solids intake. Consequently, this level was chosen as the positive control level in this experiment and fed concurrently with other lots receiving levels of 0, 0.5, 1.0 and 1.5 mg of thiamine per kilogram of solids consumed.

All pigs were fed regularly 6 times a day early in the experiment and later on 5 times daily. The amount of diet fed was restricted during the first few days and whenever diarrhea developed. Diarrhea was not usually a particular problem after the first week of experimental feeding. Samples of each diet were assayed frequently by the thiochrome method as described in the Methods of Vitamin Assay of the A. V. C. ('51) to verify the prepared concentration of thiamine in the diet. Pigs were weighed every 4th day just before the final feeding.

Blood samples were taken from an ear vein at two-week intervals for white cell counts and hemoglobin determination. Electrocardiograms were taken weekly throughout the second and third replications. Blood samples were taken from the anterior vena cava during the late stages of the experimental period for blood thiamine assay.

Post mortem examinations were made on all pigs that died during the experiment and on those pigs which were sacrificed while in a semimoribund condition. Several positive control pigs were sacrificed at the end of the experiment to serve as comparative standards in these examinations. Heart, adrenal and thyroid weights were taken and recorded. Blocks of tissues were taken and fixed in formalsaline. Hematoxylin-eosin was used on all sections and myelin sheaths were stained using Weil's ('45) method. Nissl bodies were stained by a thionin technique described by Fletcher ('47).

RESULTS AND DISCUSSION

The results of the experimental feeding period pertaining to pig growth and feed consumption are presented in table 1. Statistical treatment was given, using the method of Snedecor ('46) for analyzing multiple classification variance permitting removal of replication error. Data on growth and feed consumption for lots 1 and 2 were not included in the analysis since several of these pigs either died or received therapeutic treatment during the experiment. Analysis of the data presented indicates that under the conditions of this experiment the minimum thiamine requirement of the baby pig lies between 1.0 and 1.5 mg per kilogram of solids in the diet and further suggests that the 1.5 mg level should be considered a practical minimum thiamine concentration in the baby pig's diet. Since the daily dietary solids intake of each baby pig constituted approximately 5% of its body weight throughout the experiments, this requirement value is equivalent to that of 75 μg per kilogram body weight daily reported by Moustgaard ('53) for older pigs.

Pigs receiving no thiamine ate and gained equally as well as the positive control pigs for the first 12 days of experimental feeding. Then the lot 1 animals developed an increasing degree of anorexia, vomited frequently, gained more slowly, then lost weight, became quite weak and emaciated and finally died after three or 4 weeks on the thiamine-deficient diet. Similar effects appeared somewhat more belatedly in the

experiment in the lot 2 pigs. A few of the pigs in lot 3 developed anorexia and vomited occasionally very late in the experiment and two of the pigs from this lot died on the final day of the trial. There was no appearance of abnormal gait, nor were there any outward manifestations of nervous disorders in any of the pigs. All pigs in lots 4 and 5 were free of the aforementioned deficiency symptoms throughout the experimental period.

TABLE 1

Response of baby pigs to synthetic milk diets containing different levels of thiamine [1]

	LEVEL OF THIAMINE IN DIET, IN MG/KG SOLIDS				
	0	0.5	1	1.5	2
Lot number	1	2	3	4	5
Number of pigs	11	11	11	11	11
Days on test	.. [2]	.. [2]	32	32	32
Av. initial wt. (lb.)	4.77 ± 0.21 [3]	4.68 ± 0.23	4.73 ± 0.18	4.62 ± 0.15	4.68 ± 0.25
Av. final wt. (lb.)	..	..	18.25 ± 0.83	19.96 ± 0.50	20.35 ± 1.12
Av. daily gain (lb.) [4]	..	..	0.42 ± 0.02	0.48 ± 0.01	0.49 ± 0.03
Av. daily solids consumed (lb.)	..	..	0.50 ± 0.02	0.53 ± 0.03	0.53 ± 0.03
Solids per lb. gain (lb.) [5]	..	..	1.20 ± 0.06	1.11 ± 0.03	1.09 ± 0.03

[1] Pigs were taken from the sow when three or 4 days old and placed on a depletion diet containing no thiamine for 4 days. Pigs were then assigned to lots and started on the feeding trial.

[2] Data on growth and feed consumption for lots 1 and 2 are not included since most of these pigs had died, been sacrificed in extremis or received thiamine injections before the end of the experimental feeding period.

[3] Standard error of mean.

[4] Lots 4 and 5 gained significantly faster at the 10% level than lot 3.

[5] Lots 4 and 5 were significantly more efficient in food utilization at the 5% level than lot 3. The pigs in the second and third replications of all three lots were significantly more efficient in food utilization at the 1% level than the pigs in the first replication.

All deficient pigs which died or were sacrificed showed gross symptoms of thiamine deficiency. In animals dying naturally, there was marked cyanosis noticeable in the skin, nose and mucous membranes. The most common gross finding was a pale yellowish-gray mottled heart. The left ventricle was usually contracted and the right ventricle was quite flabby. The whole heart was less firm than normal and in several instances it was rounded and resembled a myxedematous heart. Heart and adrenal weights were greatly increased in relation to the body weight whereas thyroid weight as a percentage of the body weight was not appreciably affected. There was always an excess of pericardial fluid and in many cases the peritoneal and pleural cavities also contained excessive fluid.

In most cases, there was congestion of the liver and the serosa of the small intestine was reddened. The mesenteric vessels were usually injected. In a few of the animals, the liver showed a yellowish mottling suggestive of fat. There was inflammation of the cecum or colon varying from a mild reddening to a severe necrotic enteritis with ulcers and caseous masses adhering to the intestinal wall.

On microscopic examination the hearts of thiamine-deficient pigs showed congestion, focal fragmentation and some necrosis of the muscle fibers. The cells in the inflamed area were primarily macrophages although some lymphocytes and Anitschkow cells were also present. This inflammation was more pronounced in the hearts of pigs from lots 2 and 3 and there was an increase in collagenous fibers as demonstrated by Mallory's aniline blue stain. Sudan IV stains showed extensive fatty degeneration in the heart tissue of all deficient pigs but the fat droplets were small and tell-tale vacuoles were not readily observed by the hematoxylin-eosin stain. All portions of the myocardium were affected but lesions were more marked near the epicardium. The left ventricle was involved to a greater extent than the right. Heart lesions were not noted in pigs from lots 4 and 5 nor in deficient pigs that had recovered after thiamine injections. These findings are es-

sentially those of Follies et al. ('43) and Wintrobe et al. ('42) except that fatty changes in the heart were more marked in this series and the neutrophilic exudate described by Follies et al. ('43) was not observed in this experiment.

Congestion of the mucosa of the gastro-intestinal tract and mucoid degeneration of the cecum or colon were observed in pigs from lots 1 and 2. No changes were noted in the central nervous system nor in the sciatic nerves.

Studies of the white cell counts and the hemoglobin determination values revealed no significant trend that could be related to the level of thiamine in the diet. Data taken from the electrocardiograms obtained from thiamine-deficient pigs (to be presented in another publication) reveal the bradycardic condition resulting primarily from a lengthening of the P-R and S-T intervals. Blood samples taken for thiamine assay late in the experimental feeding period show that blood-thiamine level was increased in those pigs receiving higher dietary levels of this vitamin. The mean values for blood thiamine of the 5 lots in the experiment together with the standard error, expressed in micrograms per 100 milliliters of blood, for lots 1 through 5 are: 4.2 ± 1.0, 4.3 ± 1.2, 7.3 ± 1.9, 9.0 ± 0.9 and 11.8 ± 1.8.

Three deficient pigs received thiamine injections in an attempt to overcome the deficient condition. One of the pigs failed to respond and died shortly thereafter exhibiting most of the symptoms heretofore mentioned. The other two pigs, however, showed an almost immediate response to the injection. Their heart rates returned from bradycardia to normal within a few minutes. Their appetites improved rapidly and within 24 hours they were gaining in body weight. Thereafter intraperitoneal injections of 100 to 500 mg of thiamine were given at weekly intervals and these pigs when placed on the positive control diet continued to gain normally over a three-week recovery period. These pigs were then killed and post mortem examination showed no gross evidence of a deficiency except a few light colored areas on one pig's heart which was no longer flabby.

SUMMARY

Fifty-five baby pigs were used in a triplicated experiment to determine the thiamine requirement. Following a depletion-adjustment period on a thiamine-free, synthetic-milk diet, the pigs were individually fed diets containing 0, 0.5, 1.0, 1.5 and 2.0 mg of thiamine per kilogram of solids.

Data on individual growth response and dietary intake are presented. Analysis of the data indicates that the minimum thiamine requirement of the baby pig for optimum growth and feed efficiency approximates 1.5 mg per kilogram of dietary solids (10% fat) intake. External, gross and microscopic lesions were present in all pigs receiving less than 1.0 mg per kilogram of solids. Blood thiamine levels were positively related to dietary thiamine intake. Good gaining ability was rapidly restored to deficient animals which received thiamine injections.

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A BIOCHEMICAL BASIS FOR THE INTERRELATIONSHIP OF PANTOTHENIC ACID AND METHIONINE[1]

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INTRODUCTION

The nutritional interrelationship of pantothenic acid and methionine was discussed in an earlier report from this laboratory (Dinning et al., '54). Two possible biochemical explanations were suggested. It was pointed out that adequate dietary methionine might result in more efficient utilization of pantothenic acid in the synthesis of coenzyme A. Alternatively, pantothenic acid could be concerned in transmethylation or methyl synthesis reactions and thereby reduce the requirement for dietary methionine. The present experiments were designed to test these hypotheses.

EXPERIMENTAL

Weanling Sprague-Dawley rats of both sexes were given the basal diet previously described (Dinning et al., '54). This diet is deficient in the sulfur-containing amino acids and in pantothenic acid.

In the first experiment, groups of rats were given this basal diet with the various supplements of methionine and pantothenic acid listed in table 1. After 30 days of feeding, each rat was given 50 mg of guanidoacetic acid per 100 gm of body

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weight. The guanidoacetic acid was dissolved in dilute hydrochloric acid and given by stomach tube. The rats were then transferred to metabolism cages and a 24-hour urine sample was collected from each. Creatine and creatinine were determined by the Jaffe reaction (Folin, '14), and guanidoacetic acid by the method of Sims ('45). When guanidoacetic acid is heated in acid solution under conditions employed in the conversion of creatine to creatinine, it is converted to glycocyamidine, a substance which gives a Jaffe reaction. The nec-

TABLE 1

The influence of dietary methionine and pantothenic acid on the metabolism of orally administered guanidoacetic acid by rats

METHIONINE SUPPLEMENT	PANTOTHENIC ACID SUPPLEMENT	NO. OF RATS	AV. DAILY WEIGHT GAIN	URINARY CREATININE	URINARY CREATINE	URINARY GUANIDOACETIC ACID
%	*mg/kg*		*gm*	*mg/100 gm body weight/day*		
0	0	6	0.3	3.4 ± 0.1 [1]	11.2 ± 2.5	4.6 ± 2.2
0.1	0	7	0.7	3.0 ± 0.1	9.0 ± 1.4	20.8 ± 3.8
0.43	0	4	1.4	3.0 ± 0.2	4.5 ± 1.8	21.5 ± 3.6
1.73	0	3	1.6	2.8 ± 0.2	4.5 ± 2.3	17.9 ± 2.2
0	20	7	0.5	2.6 ± 0.1	6.1 ± 1.8	12.0 ± 2.8
0.1	20	3	0.8	2.9 ± 0.2	8.5 ± 0.8	12.4 ± 2.9
0.43	20	4	2.1	2.7 ± 0.3	6.0 ± 2.0	12.8 ± 4.9
1.73	20	4	1.8	2.8 ± 0.3	5.0 ± 1.1	11.1 ± 3.5

[1] Mean ± standard error.

essary correction factor was calculated and applied to the creatine determinations.

In the second experiment, groups of rats were given the basal diet with the various supplements listed in table 2. After 44 days of feeding, the rats were sacrificed and liver coenzyme A was determined by the method of Kaplan and Lipmann ('48).

RESULTS AND DISCUSSION

The data in table 1 show that the animals gave a growth response to both pantothenic acid and methionine. The results agree quite well with those previously reported (Dinning et al., '54). Rats receiving only the basal diet exhibited a con-

siderably higher excretion of creatinine than did rats receiving supplements of methionine or pantothenic acid. This may indicate that animals in the basal group possessed relatively greater quantities of muscle tissue than did the supplemented animals. Since subsequent experiments demonstrated that rats receiving the basal diet exhibted low levels of liver coenzyme A, fatty acid synthesis may have been impaired. Gross observations of liver fat supported this hypothesis. Fatty livers were frequently seen in animals receiving the basal diet supplemented with pantothenic acid but no methionine. In contrast, fatty livers were never seen in animals receiving the basal diet without supplement. The basal diet without supplementary methionine may be deficient in lipotropic factors; in the presence of pantothenic acid with the resulting increase in tissue coenzyme A, fatty acid synthesis may lead to fatty infiltration of the liver. When the diet does not contain pantothenic acid and tissue coenzyme A levels are low, fatty acid synthesis may be impaired. Under these conditions fatty livers do not develop even though the diet is deficient in lipotropic factors.

There was considerable individual variation in creatine excretion as indicated by the relatively large standard errors. The data do indicate quite clearly that pantothenic acid deficiency did not impair the methylation of guanidoacetic acid to creatine.

The excretion of guanidoacetic acid was also subject to considerable individual variation. We have no explanation for the low value obtained for animals receiving the basal diet. In the other groups approximately 30% of the administered dose was excreted as such in a subsequent 24-hour period. The data for creatine and guanidoacetic acid excretion presented in table 1 do not support the hypothesis that pantothenic acid is concerned in methyl synthesis or in methyl transfer.

The data presented in table 2 show that dietary pantothenic acid and methionine influence liver coenzyme A levels. Supplementation of the basal diet with pantothenic acid or methio-

nine separately resulted in an approximately 50% increase in liver coenzyme A levels. When the diet was supplemented with both pantothenic acid and methionine, liver coenzyme A levels were further increased to values comparable to those reported for normal rats (Kaplan and Lipman, '48).

The influence of dietary methionine on liver coenzyme A levels is probably due to its serving as a precursor of the thioethylamine moiety of the coenzyme. The work of Levintow and Novelli ('54) and of Hoagland and Novelli ('54) has shown that cysteine is a precursor of this portion of the coenzyme under *in vitro* conditions.

TABLE 2

The influence of dietary methionine and pantothenic acid on rat liver coenzyme A levels

METHIONINE SUPPLEMENT	PANTOTHENIC ACID SUPPLEMENT	NO. OF RATS	AV. DAILY WEIGHT GAIN	LIVER COENZYME A
%	*mg/kg*		*gm*	*units/gm wet weight*
0	0	7	0.1	58 ± 10 [1]
1.73	0	11	1.0	86 ± 10
0	20	9	0.2	91 ± 11
1.73	20	11	1.3	119 ± 11

[1] Mean ± standard error.

The results of these experiments indicate a biochemical basis for the nutritional interrelationship of pantothenic acid and methionine. In the presence of limiting amounts of pantothenic acid, additional dietary methionine results in increased levels of liver coenzyme A and this in turn would reduce the severity of the pantothenic acid-deficiency manifestations.

SUMMARY

Experiments were designed to determine the biochemical basis of the interrelationship of pantothenic acid and methionine. Sprague-Dawley rats were fed various levels of methionine with and without pantothenic acid. Guanidoacetic acid was administered to the rats by stomach tube. Dietary pantothenic acid or methionine did not significantly affect the extent

of methylation of guanidoacetic acid. Liver coenzyme A levels were markedly reduced when the diet was deficient in both methionine and pantothenic acid. Supplementation of the diet with either of these nutrients significantly increased liver coenzyme A levels; however, both were necessary for maintenance of normal levels of the coenzyme.

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A BACTERIAL METHOD FOR DETERMINING PROTEIN DIGESTIBILITY [1]

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ONE FIGURE

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INTRODUCTION

The development of a satisfactory laboratory method for measuring protein digestibility should reduce the need for feeding trials with small animals, and permit better control of those factors which decrease protein digestibility during the manufacture of processed foods and feeds. Laboratory methods using pepsin alone (Sterling, '29; Almquist et al., '35; Ranganathan and Sastri, '38; Pfeiffer and Clarenz, '36; Stotz and Columbus, '39; Noelle, '41; Schneider et al., '50; Gehrt et al., '55), are useful for certain animal products, but give incomplete splitting of the proteins, and are difficult to relate to animal digestion coefficients. By supplementing the action of pepsin with the proteolytic action of the bacterium, *Pseudomonas aeruginosa*, protein coefficients have been obtained which, in most cases, are within 10% of the animal coefficients determined on the same samples in this laboratory. Dialyzable sugars and certain ether-soluble substances inhibit the proteolytic activity of the bacterium, and must be removed before digesting the sample.

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EXPERIMENTAL

Digestion. Samples are ground to pass a 40-mesh screen and extracted with ethyl ether for 4 hours in a Goldfish extractor at a high heat input. In cases where the sample contains substantial amounts of mono- or disaccharides (e. g. sugar-coated cereals), the sample is dialyzed against running distilled water for 24 hours in a cellophane casing.[3] Triplicate samples containing 6.3 mg of nitrogen (Kjeldahl), to give a nitrogen concentration of 0.03 mg/ml of final reaction mixture, are suspended in 100 ml of distilled water containing 5.0 mg of crystalline, porcine pepsin [4] using a 500-ml Erlenmeyer flask. The pH is adjusted to 1.5 to 2.0 with 8 drops of concentrated sulfuric acid, the flasks are stoppered with aluminum foil-wrapped cotton stoppers, and placed on a shaking machine at 37°C. for 24 hours. At the end of this time, the sulfuric acid is neutralized by adding an excess (3 gm) of calcium carbonate. The digests are buffered to pH 7.8 to 8.0 with 35 ml of 0.2 M tris (hydroxymethyl) aminomethane, and treated with 5 ml of 1:1000 Zepharin chloride.[5] Fifty-five milliliters of distilled water are added, and the reaction mixture is inoculated with 5 ml of a washed bacterial cell suspension (described below) of *Pseudomonas aeruginosa.* Shaking at 37°C. is resumed. At 24 hours (or less frequent) intervals, aliquots are removed and the digestion coefficient is determined. The digestion is continued until the digestion coefficient reaches a plateau (5 to 7 days).

Assay method. At intervals, approximately 3 ml samples of the digest are removed with a sterile pipette, filtered through Whatman no. 3 filter paper, and 0.2 ml aliquots are pipetted into 19 × 150 mm Coleman round cuvettes. One milliliter of ninhydrin reagent is added to each sample, the cuvettes are covered with an aluminum cap,[6] and heated in vigorously

[3] Visking.
[4] Armour.
[5] Winthrop-Stearns.
[6] Catalogue no. 78300, A. S. Aloe Co., St. Louis, Missouri.

boiling water for 20 minutes. After completion of color development, the cuvettes are allowed to cool for 2 to 3 minutes and the contents are diluted with 10 ml of a 1:1 (by volume) water-n-propanol mixture. The percentage transmittancy of the protein digest in the cuvette is determined after setting the spectrophotometer [7] at 100% transmittancy with a zero time color blank described below. All measurements are made at 570 mμ.

Preparation of the zero time color blank. A blank is prepared in order to correct for color development at zero digestion time. The substrate sample, containing 6.3 mg of nitrogen, is suspended in 100 ml of water containing 5 mg of pepsin. The suspension is adjusted to pH 1.5 to 2.0 with 8 drops of concentrated H_2SO_4 and immediately neutralized with an excess of calcium carbonate. Buffer, bacterial suspension, preservative and distilled water to make a final volume of 200 ml are then added. The blank is divided into approximately 3-ml portions and stored in stoppered test tubes in a deepfreeze until ready for use. When needed, a 3-ml portion is thawed, filtered, and a 0.2-ml sample is used as a blank in the colorimetric determination.

Preparation of the color standard representing complete digestion. To determine the color representing complete release of amino acids, casein samples containing 6.3 mg of nitrogen are hydrolyzed with 5 ml of 25% (by weight) H_2SO_4 for 20 hours, in an autoclave at 15 pounds steam pressure. Dilution with 100 ml of H_2O, neutralization with excess $CaCO_3$, filtration into a 200 ml volumetric flask, and dilution to volume gives a solution which contains the same amount of original substrate nitrogen (0.03 mg/ml) as the final bacterial digest. Again, 0.2-ml portions are treated with 1 ml of ninhydrin reagent, and the color is developed as previously described. After setting the spectrophotometer at 100% transmittancy with a blank made by repeating all of the above steps starting with 5 ml of 25% sulfuric acid, but omitting the protein, the

[7] Coleman Junior, Model 6B, with 19 × 150 mm cuvettes all matched to within 1% transmittancy.

percentage transmittancy of the protein acid digest is measured. The percentage transmittancy of the acid digest is assigned the value of 100% digestion on the linear scale, and the 100% transmittance value is assigned the value of 0%

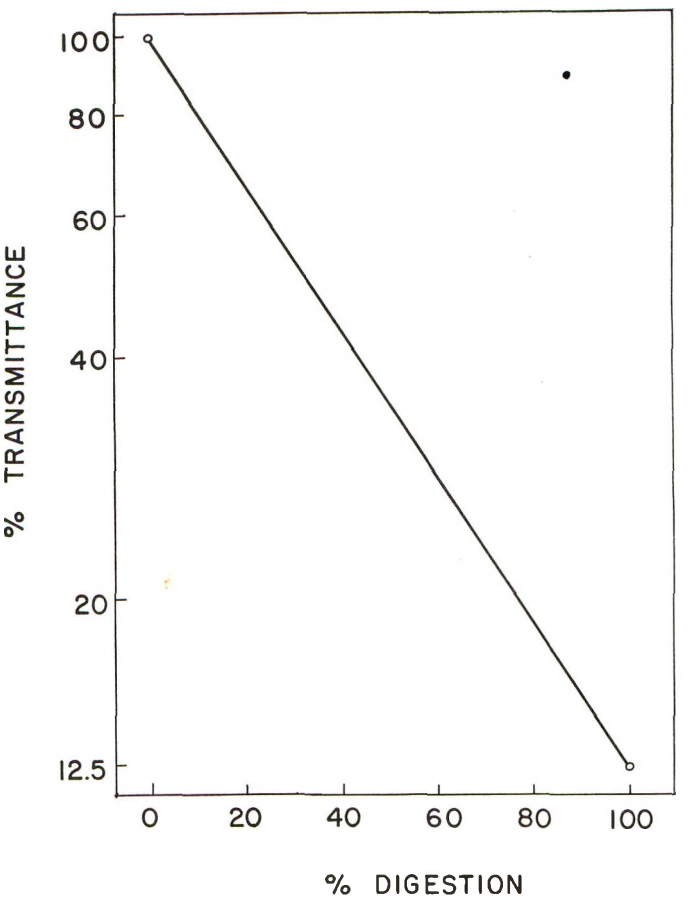

Fig. 1 Plot relating transmittance and digestion.

digestion on the linear scale, of single cycle semilogarithmic graph paper. The 0% and 100% digestion values are connected by a straight line (see fig. 1). Intermediate digestion coefficients for bacterial digests are then read from this line. A self-digestion blank for amino acid releases due to self-

digestion of the enzymes present, is made by carrying out the pepsin-bacterial digestion and analyses described above with no substrate present in the reaction mixture. The blank usually lies in the range of 2 to 4% (as read from the standard curve), and needs to be determined only once by each operator.

Preparation of washed bacterial cell suspension. Six 500-ml Erlenmeyer flasks containing 100 ml of nutrient broth are inoculated with *Pseudomonas aeruginosa* and incubated in shake-culture at 37°C. for 24 hours. Using sterile technique throughout, the broth cultures are transferred to 250 ml centrifuge bottles and the centrifuged cells are suspended in 10 ml of 0.85% NaCl, and pooled and recentrifuged at 2500 rpm for 30 minutes. The supernatant is discarded, and the cells are resuspended in 15 ml of 0.85% NaCl, and used at once; if this is inconvenient, the suspension is quickly frozen in an acetone-dry ice bath. The frozen preparation is stored at — 10°C. for not more than three days, and just prior to use, is thawed at room temperature. The stored or fresh cell suspension is diluted to 100 ml with 0.85% NaCl. Five milliliters of the diluted suspension are used to inoculate each reaction mixture.

Digestion trials with rats. Each of the rat digestion values listed in table 1 for the first 4 feeds [8] is the average value obtained on 10 200–gm rats using the method of Fraps ('45). For the remaining values, white rats weighing approximately 100 gm were fed the test samples at levels providing 7 to 10% of crude protein in the diet. The other major ingredients were cod liver oil 2%, minerals 5%, corn oil 2%, cerelose 50%, metallic oxide marker 1%, and celluflour to make 100%. Water was fed ad libitum. Four animals were used for each feed tested. At the beginning of the test period, each feed container was filled with 50 gm of the experimental feed, which contained 1% of chromic oxide. The nitrogen content of each ration, thus tagged, was determined prior to the start of the experiment by the Kjeldahl procedure. The collection of feces was begun when the green color due to the chromic

[8] We are indebted to Mr. F. O. Christ for conducting these digestion trials.

oxide appeared in the excreta. After 4 days, the feed containers were removed and replaced with ones containing 50 gm of the experimental feed marked with 1% of ferric oxide. The appearance of red feces due to the ferric oxide, marked

TABLE 1

Bacterial and animal protein digestion coefficients

SAMPLE USED	BACTERIAL COEFFICIENT	ANIMAL COEFFICIENT
	%	%
Casein	91 (91 [1])	96
Soybean meal	87 (96 [1])	85
Fish meal	88 (92 [1])	88
Meat and bone scraps	73 (73 [1])	71
Corn	77 (49 [1])	79
Commercial pig starter	89 (74 [1])	81
Commercial broiler feed	91 (75 [1])	82
Commercial cereal A [2]	44	51
Commercial cereal B	64	81
Commercial cereal C	67	67
Commercial cereal D	67 (3 [3])	65
Commercial cereal E	70	63
Commercial cereal F	73 (5 [3])	67
Commercial cereal G	75	76
Commercial cereal H	75	78
Commercial cereal I	93	84

[1] Sample not extracted with ether prior to digestion.

[2] A: Toasted rice product
B: Corn grits
C: Toasted rice product
D: Sugar-coated toasted corn product
E: Bran product
F: Sugar-coated puffed cereal
G: Toasted wheat product
H: Baked wheat product
I: Toasted wheat product

[3] Sample not dialyzed prior to digestion.

the end of the experimental fecal collection period. Feed consumption, total fecal excretion, and the nitrogen content of a 2-gm aliquot of total fecal excreta, were determined. From these values, the protein digestibility of the feed or food was calculated.

RESULTS

Table 1 shows the relatively good agreement between the bacterial and animal coefficients. Most of the bacterial coefficients are within $\pm$ 10% of the animal coefficients. Corn, and products containing corn (commercial pig starter and broiler feed), contain an ether-soluble component which inhibits the proteolytic activity of the bacterium. High protein feeds such as casein, soybean meal, etc. are apparently free of this inhibitor.

The commercial cereals tested show an interesting gradation in protein digestibility by both the bacterial and animal methods. The data show that *in vivo* differences in protein digestibility exist among commercial cereals, and that these differences can be demonstrated with the bacterial method.

The response of *Pseudomonas* to dialyzable sugars was unexpected. Sugar-coated cereals D and F (table 1) were not attacked by *Pseudomonas* unless the sugars were first removed. A similar effect was obtained by mixing glucose or sucrose with casein; inhibition of proteolysis increased with increasing ratios of sugar to casein, with an over-all digestibility of 5% (probably due to pepsin) at a ratio of 10 parts of glucose or sucrose to one part of casein.

DISCUSSION

The protein-splitting powers of *Pseudomonas aeruginosa* were first appreciated by workers in this laboratory when it was found to be a contaminating microorganism in our digests. Positive identification of the microorganism was made by using the procedures of Haynes ('51), and by electron micrographs (rod-shaped polar monotrichous flagellate, dimensions approximately $0.75 \times 1.5\ \mu$). Cultures obtained elsewhere were tested on casein and pig starter, and gave protein digestion coefficients which agreed with those obtained with the Purdue culture. The strains tested were strain B-7 (apyocyanogenic) and B-1361 (pyocyanogenic), kindly supplied by the Northern Regional Research Laboratories, Peoria, Illinois,

and Strain 10145 (American Type Culture Collection, Washington, D. C.). Using standard bacteriological precautions, the routine use of this saprophyte, which is a normal inhabitant of the intestines, should not constitute a health hazard in the laboratory (Cecil and Loeb, '51; Harrison, '51).

Pseudomonas displays optimum proteolytic activity on substrates which have been predigested with pepsin. However, some substrates (for example, casein) are readily acted upon if predigested for 24 hours with the standard volume of distilled water alone at pH 1.5 to 2.0. Other substrates (zein, blood meal) require treatment with pepsin. Under the conditions employed in this method, the digestion coefficient after treatment with pepsin is usually 5 to 10%.

Plating tests show that *Pseudomonas* increases in population as digestion of the substrate progresses, indicating utilization of the substrate. Paper chromatograms of Seitz-filtered *Pseudomonas* digests of casein using two different systems (Levy and Chung, '53; Redfield, '53) revealed no peptide spots, but showed well defined amounts of 10 different amino acids, namely, alanine, aspartic acid, glutamic acid (predominating), proline, serine, valine, threonine, leucine, isoleucine, and tyrosine. Absence of arginine, lysine and phenylalanine suggests their specific destruction by the microorganism. Further evidence for the destruction of some of the free amino acids was obtained by comparing the casein digestion coefficients using our standard ninhydrin color technique, with the ninhydrin carbon dioxide titration method of Van Slyke et al. ('41). Using casein samples varying in weight from 50 mg to 20 gm, the ninhydrin color method gave digestion values of 85 to 92%, the ninhydrin-CO_2 method, values of 48 to 66%. This indicates that about two-thirds of the ninhydrin color is derived from free amino acids; the remainder of the ninhydrin color is apparently contributed by the ammonium ion released from amino acids (*Ps. aeruginosa* has been shown by Stumpf and Green ('44), to possess an L-amino acid oxidase). Ammonia is the major volatile

product of our pepsin-*pseudomonas*-casein digestion mixtures when they are subjected to alkaline aeration.

The use of a single standard, casein, and a fixed amount of sample nitrogen, simplifies the pepsin-*Pseudomonas* method. The feed and food samples are ground to a particle size which permits passage through a 40-mesh screen. Inasmuch as preliminary tests suggest a relationship between particle size and digestibility, this factor is being studied.

It is recognized that the ninhydrin method will not detect the release of the prolines, which give almost no measurable color with ninhydrin at 570 mμ; also, the partial destruction of several amino acids during the acid hydrolysis of the casein standard introduces some error. However, it has been estimated that the combination of these factors does not introduce more than a ± 2% error in the digestion coefficient values.

SUMMARY

A bacterial method for determining protein digestion coeffcients is presented. This method combines the proteolytic action of pepsin with that of the bacterium, *Pseudomonas aeruginosa*. In most cases, the bacterial digestion coefficients of the foods and feeds tested agree within 10% with the protein digestion coefficients obtained in feeding trials with rats.

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