Vitamin Deficiencies in Rations of Natural Feedstuffs

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ABSTRACT

A swine ration consisting of yellow corn, tankage, casein, linseed oil meal, alfalfa meal, vitamin A and D concentrate and mineral supplements, has been fed to rats from the time of weaning through maturity, including reproduction and lactation.

Results indicate that for growth of weanling rats the ration under consideration is deficient in some combination of thiamine, riboflavin, pyridoxine and pantothenic acid. Using paired feeding trials it was shown that no one of these four vitamins is effective alone. Whether or not a simpler combination is effective is undetermined.

After studying reproduction records it was apparent that the ration supported normal reproduction through parturition. However, at 28 days of age, only 16.5 to 63.0 per cent of the young were weaned, and average weaning weights were subnormal. Addition of choline to the basal increased the weaning percentage somewhat, but some unrecognized factor, present in liver, soluble in water and alcohol, and adsorbed on fuller's earth at pH 1, was effective in increasing the weaning percentage to normal. A crude concentrate of folic acid was also effective.

Apparently the ration under consideration is not only deficient in these known growth factors, but also in an unrecognized factor (or factors) similar to, if not identical with, folic acid, and which is of special importance in suckling young. Apparently the deficiency is one affecting milk quality rather than quantity.

Vitamin Deficiencies in Rations of Natural Feedstuffs[1]

Hubert Heitman, Jr.,[2,3]   and A. G. Hogan

The mortality rate in suckling pigs throughout the corn belt is excessively high, and Hogan and Johnson (1940) have estimated that roughly 40 per cent of the pigs farrowed in this district die during the suckling stage. These investigators were of the opinion that a large proportion of the losses are due to nutritional deficiencies, and this opinion was largely confirmed by their experimental data. They supplied brood sows with a basal ration of yellow corn, tankage, linseed oil meal, alfalfa meal, cod liver oil, and minerals which, according to current feeding standards, should be adequate. However, the weaning percentage varied from 30 to 100 in various years. Weaning weights were also variable from year to year, but as a rule they were below normal. When supplied in sufficient quantities, wheat germ, dried skim milk, liver meal and dried yeast had exceptional properties in improving the litter records under their conditions (Hogan and Johnson, 1941). The only feedstuff that was both effective and economical was fresh forage or pasture. Further work has suggested that the inferior litter performance observed was due to vitamin deficiencies (Hogan and McRoberts, 1940). Year to year variability was attributed to variations in the vitamin content of the ration. The Missouri reports have been confirmed and extended by others with swine and rats (Ross and associates, 1942a, 1942b, 1944; Keith and associates, 1942; Fairbanks and associates, 1945; Krider and associates, 1946; and Van Landingham and Lyon, 1947). Addition of 10 per cent alfalfa meal (basal diet contained 5 per cent alfalfa meal) permitted normal reproduction and improved lactation (Ross and associates, 1942a, 1942b, 1944).

The object of the investigations described in the following pages was to determine whether the rations used by Hogan and Johnson (1941) are deficient in vitamins, either recognized or unrecognized. Investigations of this type with swine are expensive and time-consuming and it was decided to use rats as experimental animals in a pilot trial. It was hoped that the results would find practical application later in studies with brood sows.

EXPERIMENTAL

A. Rations. - A modification of ration 214 of Hogan and Mc-Roberts (1940) was used as a basal ration and will be referred to as ration A (Table 1).

TABLE 1. - Composition of Rations, grams

Ration	214	A	B	C	D	E	F
Constituents							
Ground yellow corn	77	70	70	70	70	70	70
Linseed oil meal, 37% protein	5	5	5	5	5	5	5
Tankage, 60% protein	10	10	10	10	10	10	10
Casein, acid-washed	--	7	7	7	7	7	7
Alfalfa meal, leafy, No. 1	5	5	5	5	5	5	5
Cod liver oil	1	--	--	--	--	--	--
Lard mixture* of vitamins A and D	--	1	1	1	1	1	1
NaCl	.87	.87	.87	.87	.87	.87	.87
Limestone, finely ground	.60	.60	.60	.60	.60	.60	.60
Steamed bonemeal	.50	.50	.50	.50	.50	.50	.50
$FeSO_4$	.03	.03	.03	.03	.03	.03	.03
$MnSO_4 \cdot 4H_2O$	--	.04	.04	.04	.04	.04	.04
Liver fraction 4303#	--	--	4	--	--	--	--
Liver fraction 4912#	--	--	--	4	--	--	--
Liver fraction 5528#	--	--	--	--	4	--	--
Liver fraction 5529#	--	--	--	--	--	6	--
Liver fraction 6448#	--	--	--	--	--	--	4

*Lard mixture consisting of 98% lard and 2% Oleum Percomorphum (Mead-Johnson, labeled to contain 60,000 USP units vitamin A and 8,500 USP units vitamin D per gram).
#Weights of liver fractions on a dry matter basis.

A few liver extract fractions were used as supplements, and the combinations in which they were supplied are shown in Table 1. Preparation 4303 was obtained by extracting dried beef liver with 95 per cent ethanol at 70° C. The alcohol was evaporated off, and the residue was diluted with water at the same temperature in order to obtain a sharp separation of the lipoids from the water-soluble fraction. The lipoid layer was removed by decantation, and the aqueous material was then concentrated under vacuum to a suitable dry matter content.

Fraction 4912 was prepared as follows: Dried beef liver was first extracted with hot 95 per cent ethanol and then with boiling water. The ethanol extract was discarded. The aqueous extract was concentrated to a small volume, and 95 per cent ethanol was added to make a final concentration of 50 per cent by volume. The precipitate was discarded and the filtrate was concentrated in vacuum to a desired consistency yielding 4912.

Preparation 5528 was made from fresh beef liver. The fresh liver was ground and extracted with water at 90° C. The extract was brought to a pH of 1 and treated four or five times with fuller's earth. The combination of the first two adsorptions was preparation No. 5528. Combination of the two subsequent adsorptions yielded fraction 5529.

Preparation 6648 was a liver fraction designated as a "folic acid concentrate".[4]

All vitamin[5] supplements were synthetic or crystalline except biotin which was a highly purified preparation.[6] The combinations in which they were supplied are described in Table 2.

TABLE 2. - Combinations of Synthetic Vitamins Added to Basal Diet A

(Milligrams per 100 grams of diet)

Added Vitamins	Ration								
	G	H	J	K	L	M	N	P	Q
Thiamine hydrochloride	---	0.8	0.8	1.5	--	--	--	1.5	1.5
Riboflavin	---	1.6	1.6	--	1.6	--	--	1.6	1.6
Pyridoxine hydrochloride	---	1.2	1.2	--	--	1.2	--	1.2	1.2
d-Calcium pantothenate	---	1.0	1.0	--	--	--	5.0	5.0	5.0
Choline chloride	400	400	--	200	200	200	200	200	200
Biotin	---	--	--	--	--	--	--	--	*
Nicotinic acid	---	5	5	--	--	--	--	--	5
Ascorbic acid	---	25	25	--	--	--	--	--	25
P-Aminobenzoic acid	---	30	30	--	--	--	--	--	30
i-Inositol	---	200	200	--	--	--	--	--	200
Alpha-tocopherol	---	--	--	--	--	--	--	--	2.5
2-Methyl-1, 4-naphthoquinone	---	--	--	--	--	--	--	--	1.0

*2 micrograms per rat, twice weekly.

B. Care of Animals.

- Albino rats were used in all experiments, and the females were placed on experimental diets at weaning (21 days of age in stock colony) and reared in cages with screen bottoms. Some received the supplemented diets from weaning, but usually the females were reared on the basal ration. A week before females were placed in breeding cages (weighing at least 165 grams) they were given their supplemented diets. The males were reared on a stock ration.

When the females became pregnant they were isolated in individual cages on clean wood shavings. The females were left with their litters until the young were weaned (28 days) or dead, and then they were immediately returned to a breeding cage. Litters were reduced to 8 young at parturition when more than that number

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[4]This fraction was generously supplied by Dr. Thomas H. Jukes of Lederle Laboratories, Pearl River, N. Y. Dr. Jukes indicates that Lederle Fraction "AB" contained about 30,000 Snell-Peterson units (Snell and Peterson, 1940) of folic acid, or as much as in about 66 grams of fresh liver, and only about 0.03 gamma of biotin per gram.

[5]Generous supplies of synthetic alpha-tocopherol and crystalline thiamine, riboflavin, pyridoxine, calcium pantothenate, choline, nicotinic acid, inositol, ascorbic acid and 2-methyl-1, 4-naptho-quinone were kindly supplied by Merck and Co., Inc., Rahway, N.J.

[6]S.M.A. Corp., Chagrin Falls, Ohio, concentrate No. 200 labeled to contain 20 gamma of biotin per cc.

were born alive. The 8 young were selected at random except that insofar as possible equal numbers of each sex were retained. Experimental litters were weaned at 28 days of age.

GROWTH OF STOCK COLONY RATS

Even though the primary object of this problem was to find the cause of reproductive inefficiency, it soon became evident that the rate of growth gave helpful suggestions. In order to interpret the differences in growth responses, it became necessary to construct an average growth curve. Assuming that out stock colony rats were normal, their growth records were used to calculate this average growth curve.

Weekly weights of more than 79 rats of each sex were averaged. The stock colony rats received a mixed dry ration (Steenbock, 1923), and, in addition, they received fresh raw milk daily (approximately 25 cc. per rat, or 35 cc. when lactating). These rats were originally of the Wistar strain and have been kept in this colony for over 15 years. This colony supplied all the experimental rats for this study.

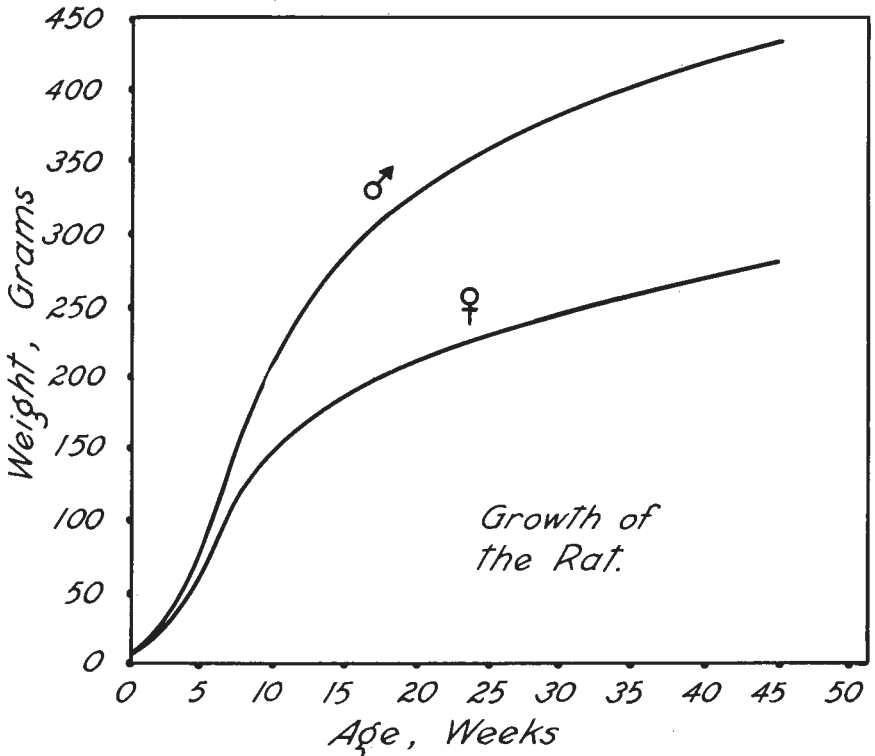The average growth curves for each sex are illustrated in Figure 1.

Fig. 1 — Growth curves for stock colony rats.

RESULTS

Reproduction in all groups appeared normal. Young were vigorous at birth, and weighed about 5 grams per rat which is similar to that in the stock colony litters. However, when the litter records following birth are considered, it is equally clear that they did not respond as did the stock colony litters.

A. Growth and Survival of Litters. - A total of 6325 young distributed among 630 litters has been observed. To conserve space only representative results will be discussed. Some of these are given in Table 3.

TABLE 3. - Litter Results

Ration	No. females	No. litters	Weaning percentages	Average weaning weight, grams
Fall, 1940				
A	11	54	63.0	46.2
G	9	38	79.8	47.4
H	7	27	76.3	54.0
B	10	32	84.8	55.9
C	7	21	81.4	55.8
Spring, 1941				
A	8	34	53.8	39.8
G	2	4	62.5	40.1
J	4	12	53.6	50.3
D	5	13	92.6	50.3
Winter, 1941				
A	4	18	41.5	39.6
E	4	14	72.8	47.9
Spring, 1942				
F	5	14	94.6	53.6
Fall, 1942				
A	5	13	16.5	37.3
G	4	8	53.2	40.2
P	4	9	78.7	45.7

The basal ration A is inadequate as is indicated both by growth rate and litter survival. Average weaning weights of litters in basal groups varied from 37.3 to 46.2 grams per rat while stock rats average 53.8 grams at this age (28 days). Weaning percentages in basal groups varied from 16.5 to 63.0.

It will be observed that the addition of choline alone (ration G) had little effect on the weaning weight, but it was decidedly effective in increasing the weaning percentage. The least effect was observed in the spring of 1941, when the weaning percentages were 53.8 on ration A and 62.5 on ration G, a gain of 8.7. The largest effect was observed in the fall of 1942, when the weaning percentages were 16.5 on ration A and 53.2 on ration G, a gain of 36.7.

Addition of vitamins other than choline (ration J) increased the average weaning weight about 10.5 grams but had little effect on weaning percentage. This was also true on ration H where the increase in weaning percentage was no greater than with a supplement of choline alone. However, on ration P addition of other vitamins increased the weaning percentage beyond the effect of choline alone. This was the only trial where this was true, and it is suggested that the first limiting vitamin deficiency of the basal ration may have changed by the fall of 1942. In this run it will be noted that the basal rats did more poorly than in any other trial. Similar observations with the sow ration have been made by Hogan and McRoberts (1940). A ration containing a supplement of all 14 vitamins known at that time (ration Q) was no more beneficial than ration P (synthetic folic acid was unavailable at the time of these experiments).

Biotin was fed as a supplement, alone and in combination with various vitamin mixtures, and it gave negative results. In a few cases addition of inositol or p-aminobenzoic acid yielded results suggesting that they were somewhat beneficial as regards weaning percentage. However, in most combinations this was not true (note results of ration H).

In the last series of experiments addition of various vitamins along with choline was tried to see which affected litter growth. Addition of thiamine, riboflavin or pantothenic acid gave weaning weights as large as did ration P. Addition of pyridoxine alone gave negative results. All were ineffective as regards weaning percentage.

From the results discussed, it seemed evident that combinations of known vitamins as supplements failed to sustain a normal rate of litter survival. These observations suggested that rations composed of natural foods must ordinarily contain other factors yet unrecognized. As liver was known to be an excellent source of vitamins, 10 per cent dried beef liver was fed as a supplement and found to be effective in increasing both litter weaning weights and survival. Growth appeared normal and about the same as on the vitamin supplemented rations, but the survival rate was still subnormal. Nevertheless, survival was better than with choline alone. It appeared, therefore, that known vitamin deficiencies were concerned with growth, whereas this observation confirmed the hypothesis that there were yet unknown factors in liver concerned with litter survival.

Aqueous and alcohol extracts of liver were used as supplements (rations B and C) and found to confirm the findings with dried whole liver. Growth was excellent, but no better than that with known vitamins as a supplement, and litter survival was better than with

the choline supplement or any supplements of known vitamins. The unknown factor or factors could be concentrated by adsorption on fuller's earth from an aqueous liver extract at pH of 1, as the adsorbate was an excellent supplement as concerns survival of litters (ration D). The second fuller's earth adsorbate was also effective, though less so than the first (ration E). Fuller's earth would adsorb riboflavin, thiamine, pyridoxine and folic acid under these conditions and growth was improved by the adsorbates. The folic acid concentrate (ration F) proved to be an excellent source of the litter survival factor as well as the factors required for growth. Again there is no reason to believe the latter was due to anything but known vitamins.

B. Symptomatology. - Young rats from mothers on the basal rations did not develop any spectacular symptoms. Many litters became lethargic before death, especially in those litters which had reached 10 days or two weeks of age. At autopsy a large proportion of the young had milk in their stomachs, and it is not believed that they died from a lack of milk. As the young died and were autopsied, yellowish livers were observed. In 850 twenty-eight day old rats the amount of crude fat in the dried livers was determined indirectly by loss of weight due to anhydrous ether extraction.

There was no appreciable difference in weights of fresh livers from various rations. However, the ether-soluble material content was quite variable. The highest level of ether-soluble material was found in the livers of rats raised on the basal diet, the average being 11.6 per cent on a dry matter basis. The lowest level was in rats fed ration H, and it was 7.3 per cent. However, the level of ether-soluble material in the livers was not related either to the weaning weight of the rats or their weaning percentage. Rather it appeared to be related to the choline content of the supplement. It is of interest that the level of ether-soluble material in the livers of rats receiving the fuller's earth adsorbate was about the same as that of the basal rats, even though the adsorbate yielded excellent litter records.

C. Growth of Weanling Rats. - Usually females were not fed a supplement until they were sexually mature. However, some from stock colony litters were fed test rations from weaning. It soon became evident that rats fed the basal ration from weaning grew at a somewhat slower rate than stock colony rats (Figure 2), even though at 12 weeks of age they weighed the same. Weanling rats, which received a supplement of thiamine, riboflavin, pyridoxine and pantothenic acid in addition to the basal, grew at a somewhat greater

rate than even the stock rats. At 12 weeks the vitamin-supplemented rats were still larger, but the mature weights were the same on all diets.
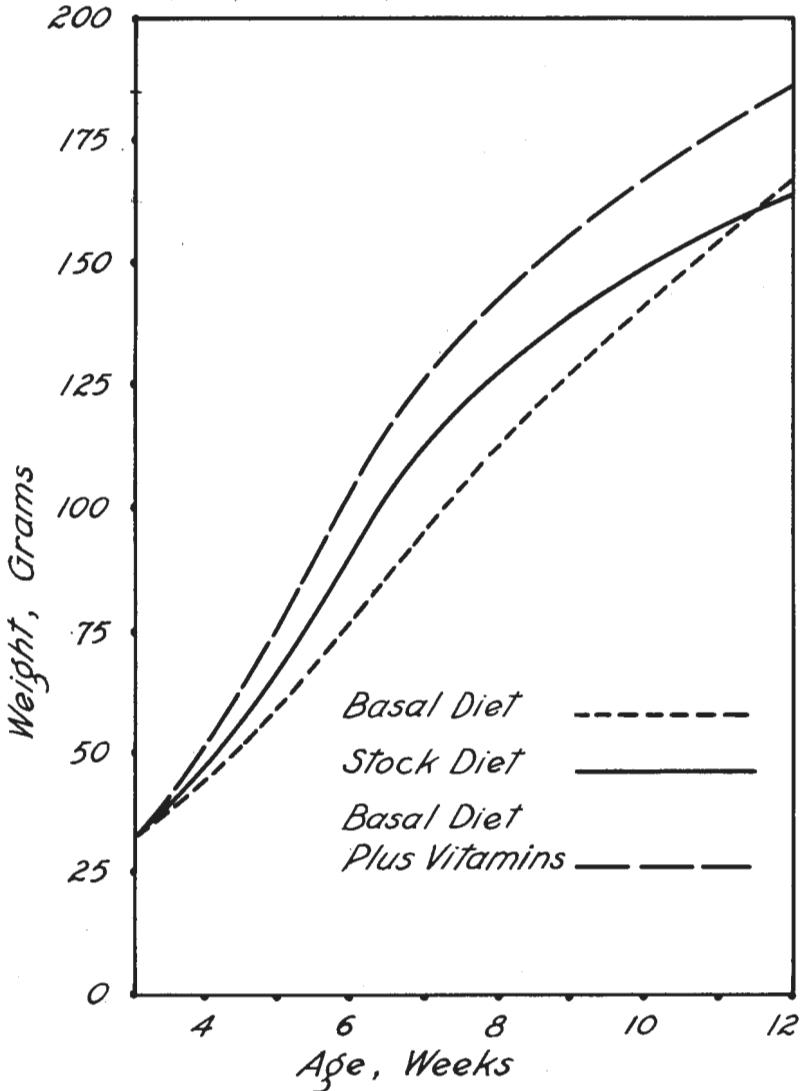

Fig. 2 — The rate of growth of female rats on the basal diet is accelerated by the addition of thiamine, riboflavin, pyridoxine and pantothenic acid.

Addition of aqueous or alcoholic liver extracts, or dried beef liver, supported growth comparable to that of stock colony females.

D. Growth of Triads of Rats. - From observations made on growth of weanling rats, it seemed that growth, under these con-

ditions, is limited on the basal ration by deficiencies of known vitamins. This was unexpected as the swine work had indicated that weaned pigs grew at about the normal rate on rations similar to 214. These growth factor deficiencies were studied by use of triads of weanling litter mate rats selected on the basis of uniformity of sex and weight. Feed intake was ad libitum. Ration P (basal plus thiamin, riboflavin, pyridoxine, pantothenic acid and choline) was compared to ration G (basal plus choline) and ration Q (supplement of all known vitamins).

On the choline-supplemented ration 8 triads produced an average gain of 81.6 grams in 24 days, whereas rations P and Q supported gains of 98.0 and 99.6 grams, respectively. The difference in gains between rations P and Q are not statistically significant, whereas both P and Q gave significant increases in gains over ration G.

To demonstrate that choline had no growth effect, the basal (ration A) was compared with the basal plus choline (ration G). Over a 24 day period in 8 pairs of rats the basal supported an average gain of 72.8 grams while the rats receiving a supplement of choline gained 71.4 grams. This difference is not significant statistically. In 6 of these pairs there was in addition a third rat receiving ration P and these averaged over 20 grams greater gain. It appears certain that choline has no growth-promoting properties under these conditions.

Trials were next run with single vitamins added to the basal plus choline. Triads were given ration G, P, and either K, L, M, or N (see Table 2 for composition). Table 4 shows the gains of these rats. Statistical analyses were made as before and not one

TABLE 4. - Average Gains (in grams) on Various Rations Through a 24-day Period (8 triads per trial)

Ration under Test	Gain in Weight on Indicated Ration		
	Test Ration	Ration G	Ration P
K	81.6	82.5	106.5*
L	83.6	83.6	103.4
M	80.6	81.9	97.1
N	85.8	82.9	104.4

*This group received ration Q instead of ration P which yields similar results (see text).

of the test rations was significantly better than ration G whereas in every case ration P gave significant increases. We conclude that the addition of thiamine, riboflavin, pyridoxine and pantothenic acid to ration G brings the rate of growth up to normal, but that no one of these when added alone gives any significant growth response. It should be emphasized that feed intake was ad libitum.

DISCUSSION

Of the recognized vitamins known to be required for growth of weanling rats, the swine ration under consideration appears to be

deficient in some combination of thiamine, riboflavin, pyridoxine and pantothenic acid. The deficiency is not of a single vitamin, but it is not certain that all four are involved. It appears that under our experimental conditions no unknown vitamin deficiency is concerned with growth, and this is not surprising because rats grow at approximately a normal rate when they receive only known vitamins (Richardson and associates, 1941).

Poor reproduction records obtained under these conditions appear to be due to poor quality of milk. Addition of choline increased the number of survivors. It would appear then that the ration was deficient in choline. However, survival was still below that expected. Addition of other known vitamins appeared to be of no more benefit than choline alone as concerns survival of litters. It is possible that either p-aminobenzoic acid or inositol is somewhat beneficial, but proof is insufficient. And, if either is effective, the ration is still inadequate.

Various liver fractions also improved litter survival, and they seemed to make the ration entirely adequate. All fractions were so crude that they contained enough known vitamins to allow good growth. It seems certain that these liver fractions contain a factor or factors that are unrecognized. Even though choline is of benefit, it seems unlikely that it is the active factor in liver. Choline was not as effective as various liver preparations, and, even if it were the active factor, the fractions would have to contain more than 10 per cent choline chloride. This seems unlikely. More decisive evidence is furnished by the observation that the active liver fraction is adsorbed on fuller's earth from acid solution, whereas choline is not.

In Figure 3 our observations on survival are presented graphically. Any point on the curve represents the percentage of the litter which would be alive at that age. Results of the several trials were combined to give smooth curves. It is clearly shown that a large part of the differences in weaning percentages is due to mortalities which occur when the young are supposedly beyond the danger stage. There is a high mortality rate during the first week of life, but there is another peak in mortalities from about 18 to 22 days of age on the inadequate diets. This would confirm our observation that rats do not die of starvation, i.e., lack of sufficient milk. In the majority of cases autopsied the stomachs were filled with milk.

Daniel and associates (1942) have reported that pyridoxine deficiency can be developed in suckling rats without impairing lactation itself. We feel that poor litter survival in this study reflects a similar mechanism.

Sure (1941) has worked extensively on lactation of rats. He has

named an unknown factor present in rice bran and liver, and which greatly improves lactation, factor B_X. Sure believes p-aminobenzoic acid is a component of B_X, and also suggests that inositol may be a component. The activity of p-aminobenzoic acid and inositol
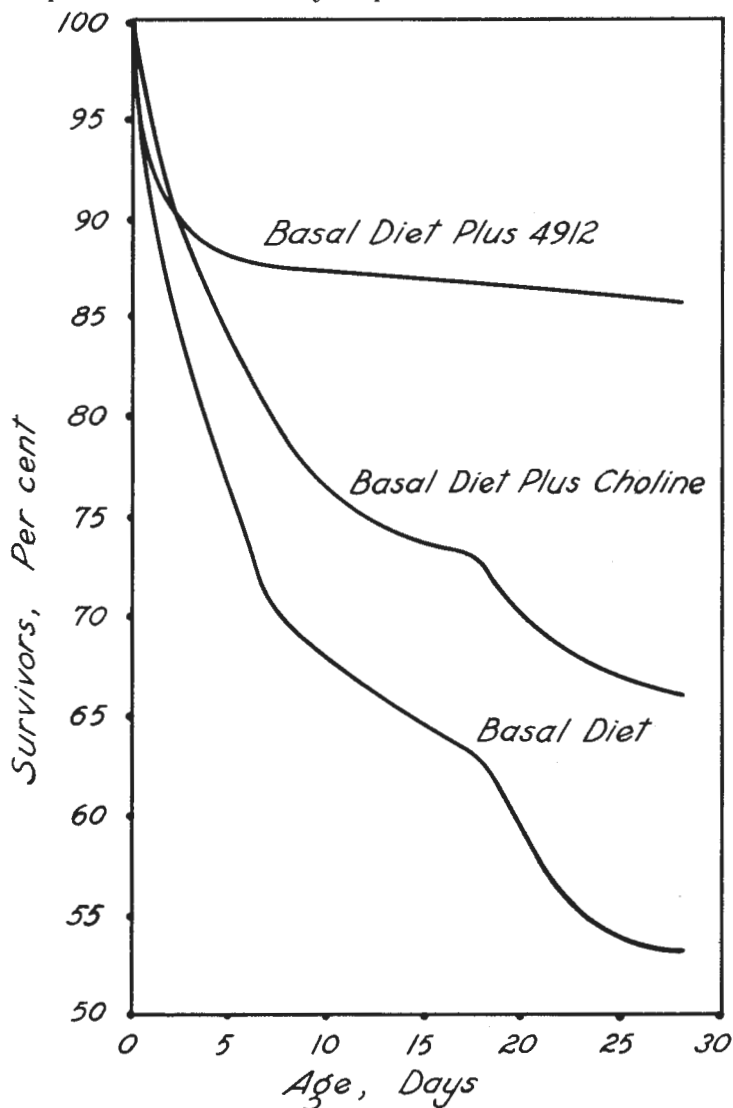

Fig. 3 — Percentage of suckling rats surviving on the basal and supplemented rations.

in our work is indecisive. It would not be surprising if inositol were inactive, because corn is a good source of it in the form of its phosphoric acid ester, phytin. We are under the impression that Sure's factor B_X is not identical with the litter survival factor

for these reasons. Other evidence pointing to this conclusion is offered by the difference in properties with regard to fuller's earth. The litter survival factor is adsorbed on fuller's earth from acid medium, as is folic acid (Hutchings and associates, 1941). We believe the litter survival factor is identical with, or closely related to, folic acid (Snell and Peterson, 1940; Mitchell and associates, 1941) and with the factor of Richardson and associates (1942), which is adsorbed on fuller's earth and eluted with 0.2 N ammonia.

Beneficial effects of folic acid on lactation have been reported with rats (Cerecedo and Vinson, 1944b) and mice (Cerecedo and Vinson, 1944a; Cerecedo and Mirone, 1947; Fenton and Cowgill, 1947).

Foster and associates (1943) have raised mice to the fourth generation on a highly purified diet containing the known vitamins (including inositol and p-aminobenzoic acid). Fertility was normal but litter growth prior to weaning was subnormal and the mortality rate was high.

No evidence is reported in this work to indicate whether the litter survival factor is composed of one or more substances. There is no reason to believe it is a single substance, and subsequent work in other laboratories would indicate that there are further unidentified substances essential during lactation (Bowland and associates, 1948). Undoubtedly many natural feedstuffs contain the litter survival factor, but apparently it is not present in large amounts in the concentrates commonly supplied swine.

SUMMARY

1. A modified swine ration of natural feedstuffs, consisting of yellow corn, linseed oil meal, tankage, casein, alfalfa meal, a concentrate of vitamins A and D and salts, was supplied to female rats.

2. Although this ration was entirely adequate as judged by current feeding standards, the growth and survival rates of litters born to these females were subnormal. Poor survival was apparently due to milk quality. No outstanding symptoms were observed in these litters.

3. Poor survival under these conditions was not caused by deficiencies of vitamins A, D, E, K, C, thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, or biotin. Addition of inositol and p-aminobenzoic acid gave indecisive results. Addition of choline was somewhat beneficial, but some factor (or factors) present in beef liver, and as yet unidentified, was more potent and allowed normal weaning percentages.

4. This unidentified litter survival factor can be extracted from liver by water or alcohol, and it is adsorbed on fuller's earth from solutions at pH of 1.

5. Evidence is presented to show that the litter survival factor is similar to, if not identical with, folic acid.

6. The slow growth rate of litters was caused by deficiencies of some combination of thiamine, riboflavin and pantothenic acid. There is no reason to believe that deficiencies of unidentified vitamins are concerned with poor growth under these conditions.

7. ' Growth rate of weanling rats was subnormal when they received only the modified swine ration. The nature of its deficiencies were studied by means of paired feeding trials in which weanling rats were fed the diets ad libitum.

8. Weanling rats grew at a normal rate when supplied with the basal ration and a supplement of thiamine, riboflavin, pyridoxine, pantothenic acid and choline. Choline alone did not improve growth. Growth of weanling rats was not limited to a deficiency of any one of the other 4 vitamins, and it is not known if any simpler combination is as adequate as a combination of all four.

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