# ABSORPTION OF TOPICALLY APPLIED VITAMINS

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TWO FIGURES

It is well known that in ulcerative colitis, obstructive jaundice, enteritis and various non-specific diarrhoeas and dysenteries, absorption of essential nutrients from the intestinal tract may be greatly impaired. This condition may be associated with a weakening of the metabolic body processes due to the lack of absorption of the essential dietary nutrients.

Medical literature contains reports of the failure of patients to respond to either the oral or parenteral administration of nutrients and drugs. Vilter et al. ('53) demonstrated that pyridoxine when applied topically in an ointment was effective in the treatment of seborrheic dermatitis of the sicca type, whereas oral or parenteral treatment was ineffective. It was reported by Dainow ('52) that niacin topically applied to the skin lesions of pellagrins caused a dramatic response in healing of the lesions. This type of lesion requires a long period of oral therapy to produce complete remission. Sobel and Rosenberg ('53) stated that topical application of vitamin A was nearly as effective in overcoming vitamin A deficiency in rats as when the vitamin was given orally.

The present work is an extension of experiments which were recently the subject of a preliminary report (Greene et al., '54). Due to the comparative ease with which vitamin de-

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ficiencies can be produced in the rat, this animal was used to measure the skin absorption and effectiveness of various ointments of the following vitamins: thiamine, riboflavin, riboflavin, pantothenic acid, pyridoxine, and vitamin D. Our experiences with the absorption of radioactive vitamin $B_{12}$ are to be reported in a subsequent paper.

## EXPERIMENTAL

Weanling rats of the Squibb strain, weighing 40 to 50 gm, were placed in individual cages and uniformly grouped with respect to number, weight and sex. In general each treatment was determined on 6 to 12 rats per group. Food and water were supplied ad libitum. For the B complex vitamin experiments the rats were fed a basal diet as follows: alcohol extracted casein,[2] 20; sucrose, 70; mineral mixture,[3] 4; cod liver oil, 1; hydrogenated vegetable oil [4] 5. Vitamins were added (milligrams/kilogram of diet) as follows: thiamine nitrate, 4; pyridoxine hydrochloride, 4; riboflavin, 8; calcium pantothenate, 20; niacin, 40; biotin, 0.4; inositol, 400; folic acid 2; 2-methyl-1,4-naphthoquinone, 10; alpha tocopherol acetate, 50; vitamin $B_{12}$, 0.015; choline, 2,000. The vitamin whose absorption was being measured was omitted from the basal diet. In the vitamin D experiments, the rats were made rachitic by the procedure reported by Numerof et al. ('54). Treatment, orally and topically, was instituted and the criterion for success of this procedure was the degree of calcification as measured by the U.S.P. XIV line test ('50).

For topical treatment, an area of about 2 $cm^2$ on the back of the neck of each rat was denuded of hair with a non-irritating hair remover. Ten milligrams of the ointment containing the total dose was then applied to the denuded area with a spatula. During the period of skin absorption of two hours,

[2] The casein was extracted continuously with boiling 95% alcohol (ethanol-methanol 90:10) for 72 hours and then dried in an oven at 65°C.

[3] The mineral mixture had the following percentage composition: $Ca_3(PO_4)_2$, 44.8; $K_2HPO_4$, 22.3; $CaCO_3$, 13.9; $MgCO_3$, 4.3; NaCl, 10.8; Fe Citrate, 2.8; $MnSO_4 .4H_2O$, 0.54; $ZnCO_3$, 0.22; $CuSO_4 .5H_2O$, 0.11; KI, 0.11; $CoCl_2$, 0.046.

[4] Primex, Procter and Gamble Company, Cincinnati, Ohio.

the rats were confined in a tight-fitting wire enclosure to prevent any access to the ointment with their feet or tongues. During this period food and water were withdrawn. After the absorption interval, the area of ointment application was washed with soap and water and then with alcohol. At the times indicated in the tables the urinary excretion of the vitamins was measured. For this, the rats, after treatment as above, were placed in metabolism cages with food removed and a 16-hour urine sample collected for assay.

To demonstrate that the results obtained are a measure of skin absorption and not of rupture of the epidermal skin layer during denuding, 100 times the lethal dose of curare was administered in a manner similar to that used for the vitamins. None of the animals so treated exhibited any symptoms of curare poisoning.

## RESULTS

### *Transfer of vitamins through skin by topical application of thiamine*

To test the efficacy of different ointment bases or diluents as vehicles for topical application of individual vitamins, Plastibase Hydrophilic [5] and a Carbo-wax ointment base CMC-120,[6] were compared with a 3.75% solution of vanillin in 25% alcohol. Ten milligrams of ointment were applied to the denuded area, dosage of the vitamin being as indicated in table 1. The results of a typical experiment indicate that Plastibase Hydrophilic is an excellent medium for absorption of topically applied thiamine and is superior to either Carbo-wax CMC-120 or vanillin solution. In view of the small dosage of thiamine applied to the skin, and the fact that only two hours was allowed for absorption, the utilization of the topically applied thiamine as measured by body weight gain and urinary excretion is considered highly efficient. Rats hav-

[5] Plastibase Hydrophilic is a Squibb Trade name. The preparation used was an oleaginous type of base, consisting of 5% polyethylene and 95% mineral oil, to which 5% of glycerol monooleate was added to render it hydrophilic.

[6] This ointment is a water-soluble mixture of polyethylene glycol and carboxymethyl cellulose.

TABLE 1

*Skin absorption of thiamine*

| TREATMENT | DOSAGE OF VITAMIN $B_1$ TWICE WEEKLY | STARTING WEIGHT | AV. BODY WT. GAIN [1] AT WEEKS | | MORTALITY | AV. 16-HOUR VITAMIN $B_1$ EXCRETION AT WEEKS | |
|---|---|---|---|---|---|---|---|
| | | | 2 | 4 | | 3 | 4 |
| | *μg* | *gm* | *gm* | *gm* | *No. died/ total* | *μg/ml* | |
| None | 0 | 46 | 23 | 0 | 2/6 | 0.013 | 0.07 |
| None | 0 | 45 | 22 | — 1 | 2/6 | 0.02 | 0.10 |
| Oral | 200 | 47 | 66 | 140 | 0/6 | 0.45 | 0.49 |
| Plastibase hydrophilic, skin | 400 | 44 | 49 | 102 | 0/6 | 0.31 | 0.40 |
| Plastibase hydrophilic, skin | 200 | 44 | 38 | 68 | 0/6 | 0.31 | 0.25 |
| Vanillin solution, skin | 200 | 46 | 37 | 57 | 0/6 | 0.09 | 0.18 |
| Carbowax CMC-120, skin | 400 | 42 | 24 | 28 | 1/6 | 0.05 | 0.03 |

[1] Six rats in each group.

TABLE 2

*Skin absorption of riboflavin*

| TREATMENT | DOSAGE OF RIBOFLAVIN TWICE WEEKLY | STARTING WEIGHT | AV. BODY WT. GAIN [1] AT WEEKS | | 16-HOUR URINARY EXCRETION TAKEN AT 5 WEEKS |
|---|---|---|---|---|---|
| | | | 2 | 5 | |
| | *μg* | *gm* | *gm* | *gm* | *μg/ml* |
| None | .. | 43 | 30 | 67 | 0.05 |
| Oral | 250 | 44 | 64 | 158 | 0.46 |
| Plastibase hydrophilic, skin | 250 | 44 | 39 | 107 | 0.08 |
| Plastibase hydrophilic, skin | 500 | 45 | 51 | 116 | 0.37 |
| Vanillin solution, skin | 250 | 44 | 46 | 129 | 0.20 |
| Vanillin solution, skin | 500 | 46 | 57 | 148 | 0.64 |
| Carbowax CMC-120, skin | 500 | 47 | 43 | 97 | 0.04 |

[1] Six rats per group.

ing symptoms of polyneuritis treated in this manner rapidly recovered and gained in body weight at a normal rate.

*Riboflavin.* The utilization of riboflavin after topical application is illustrated by the data in table 2. The results, as measured by body weight gain and urinary excretion, indicate that either Plastibase Hydrophilic or vanillin solution is a satisfactory vehicle for the adsorption of riboflavin. The dose of 500 μg of riboflavin administered two times per week in the Plastibase Hydrophilic was not quite as efficient as

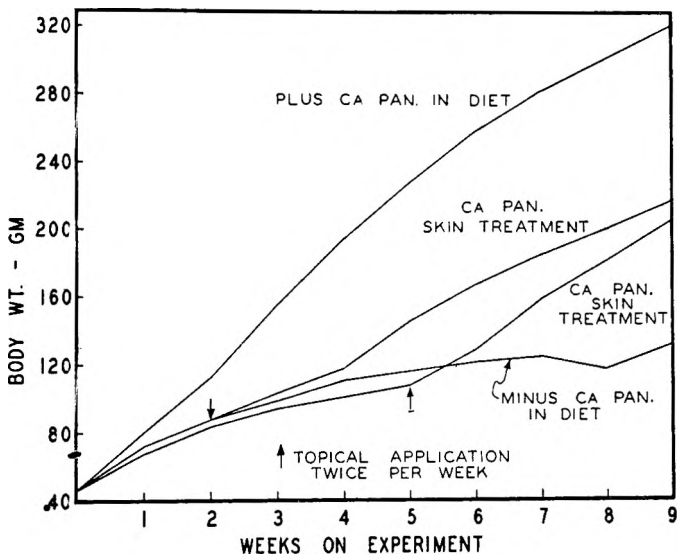


Fig. 1 Skin absorption of calcium pantothenate.

250 μg fed orally. As was noted in the absorption of thiamine, Carbo-wax CMC-120 as a vehicle for riboflavin is inferior to either Plastibase Hydrophilic or vanillin solution.

*Pantothenic acid.* Unpublished experiments in this laboratory using hooded rats fed a semipurified complete diet, with only calcium pantothenate omitted, resulted in a gradual cessation of growth accompanied by a greying of the dark hair in two to 5 weeks. Addition of calcium pantothenate (20 mg per kilogram of diet) to the above deficient diet prevented this syndrome, or cured the syndrome after its manifestation.

Figure 1 shows that the topical application of 20 mg of calcium pantothenate, twice per week, in a 3.75% solution of vanillin caused a resumption of growth. The grey hair in evidence during the vitamin-deficient period was gradually replaced with the dark hair normally found on the hooded rat. The reversal of the syndrome demonstrates that calcium pantothenate was absorbed through the skin in sufficient quantity to have an effect on growth and grey hair. Each curve in figure 1 represents the average of 12 rats.

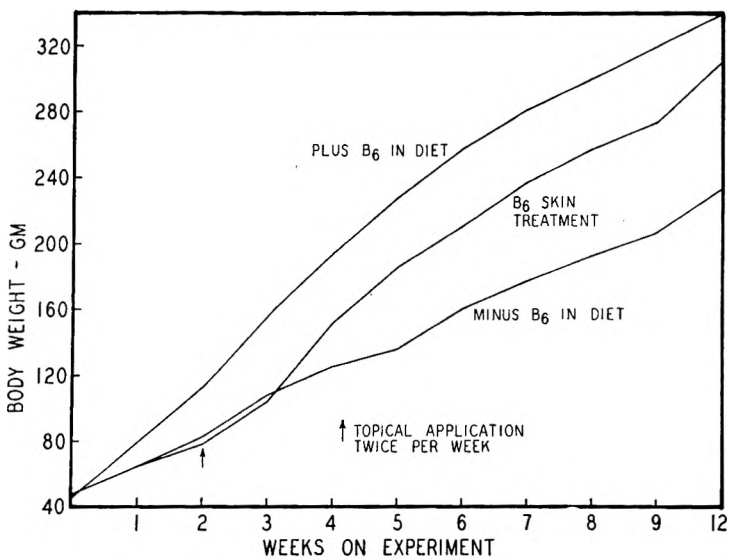


Fig. 2 Skin absorption of vitamin $B_6$.

*Pyridoxine.* The three lots of 12 rats in figure 2 show typical results when pyridoxine was omitted, fed to the rats or applied topically. One lot received the semipurified complete diet containing pyridoxine (4 mg per kilogram of diet). A second lot received the above diet minus pyridoxine and the third lot received the same diet but when a plateau in weight and visual evidence of acrodynia appeared, the topical application of pyridoxine was started.

In figure 2, the result of the topical application of 20 mg of pyridoxine, twice per week, in a 3.75% solution of vanillin, is shown. Topical application was made at the point where the weight of the rats plateaued for three days, and when scaly lesions on the front feet and about the mouth appeared. Increase in weight with a gradual disappearance of the scaly lesions in 14 days resulted. The resumption of growth and the cure of acrodynia symptoms show that the pyridoxine was effectively transferred through the skin.

In the pyridoxine and calcium pantothenate experiments, about 2 $cm^2$ of hair was removed from the scapular region,

TABLE 3

*Effect of topically administered vitamin D to vitamin D-deficient rats*

| TEST DOSE U.S.P. UNITS | NO. OF RATS | METHOD OF DOSING | AVERAGE DEGREE OF HEALING |
|---|---|---|---|
| 5 | 9 | Alcoholic solution, topical | 5.44 + |
| 10 | 10 | Alcoholic solution, topical | 6.60 + |
| 20 | 9 | Alcoholic solution, topical | 9.00 + |
| 40 | 10 | Alcoholic solution, topical | 11.00 + |
| 5 | 10 | Plastibase hydrophilic, topical | 4.20 + |
| 10 | 9 | Plastibase hydrophilic, topical | 7.10 + |
| 20 | 10 | Plastibase hydrophilic, topical | 8.20 + |
| 40 | 10 | Plastibase hydrophilic, topical | 10.80 + |
| 3.2 | 10 | Standard D oil, oral | 4.55 + |
| 4.5 | 9 | Standard D oil, oral | 5.33 + |

then the vitamin was applied by dropping the dose from a calibrated syringe. The rats were immediately immobilized in a wire enclosure for two hours. After the absorption period the rats were removed from the enclosure and the region of topical application was thoroughly washed.

*Vitamin D.* In the vitamin D experiments, doses of calciferol theoretically containing 40,000,000 U.S.P. units of vitamin D per gram were taken up in a 20% alcoholic solution or mixed in Plastibase Hydrophilic. The effect of administering vitamin D topically was compared to feeding vitamin D orally as prescribed by the U.S.P. XIV line test ('50), where the standard reference sample contained 400 U.S.P. units of vita-

min D per gram. As can be seen by the data in table 3, the degree of healing responses at the higher dosage levels of the topically applied preparations were beyond the limits of the reference sample. However, extrapolation curves based on the U.S.P. reference sample estimated the potency of the calciferol sample as being close to 36,000,000 U.S.P. units of vitamin D. The results of this test demonstrated that vitamin D is utilized as efficiently when topically administered as when fed orally. The efficiency, rapidity and uniformity of the effects of vitamin D applied on the skin are obvious when one considers that the dose was administered on a small hairless area of the neck and that the absorption period was only two hours.

## DISCUSSION

Our interest in this type of study was stimulated initially by the observations of Schreiner et al. ('52). These workers observed that patients with seborrheic dermatitis of the "sicca" type failed to respond to oral or parenteral administration of pyridoxine in dosages of 300 to 1,000 mg per day for three to 4 weeks. After discontinuation of parenterally administered pyridoxine this vitamin was applied either in the form of an ointment or as a solution in 20% alcohol, both at a concentration of 10 mg per gram. By this type of therapy the treated lesions cleared within 5 to 21 days while control areas were unchanged.

These results indicated that in certain diseases there may be a metabolic defect in the epidermal cells so that oral or parenteral administration of the vitamin fails to reach the affected site.

Since our studies were undertaken Dainow ('52) reported the use of an ointment containing 4% of nicotinamide in a nonfatty neutral excipient. Nineteen cases of pruriginous dermatosis and 20 cases of hyperkeratosis were treated with this ointment. When applied to the skin, the nicotinamide ointment exerted a rapid and pronounced effect on the pruritus and hyperkeratosis.

SUMMARY

The absorption of topically applied vitamins by the rat has been determined. In view of the small doses of the vitamins (thiamine, riboflavin, pantothenic acid, pyridoxine and vitamin D) administered and the limited time allowed for absorption, the utilization of the vitamins studied is considered highly efficient. In addition to the body weight gain responses, and the prevention or cure of deficiency symptoms, the data on urinary excretion of thiamine and riboflavin substantiate the conclusion.

In general, a plasticized hydrocarbon water-absorbing gel ointment base was superior to a carbowax preparation as a vehicle for skin transfer of the vitamins studied. An alcoholic vanillin solution was also an effective vehicle for the skin absorption of riboflavin, pantothenic acid, pyridoxine and vitamin D.

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# VITAMIN K ACTIVITY OF MENADIONE SODIUM BISULFITE IN CHICKENS[1]

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TWO FIGURES

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Experimental use of a water-soluble menadione sodium bisulfite complex in outbreaks of the chick hemorrhagic syndrome was described by Goldhaft and Wernicoff ('54) and by Frost and Spruth ('55). Although adequate controls were not used in these field studies, there appeared to be little doubt that menadione sodium bisulfite had a positive effect in reducing clotting time and mortality in the treated flocks. As cited by Goldhaft and Wernicoff, "The only product which gave any uniform results was Menadione bisulphite added to the drinking water at a level of 10 mg per gallon of water." The authenticity of these reports received independent support from Bornstein and Samberg ('54). These workers reported dramatic reduction in clotting time within 4 hours in commercial chickens in Israel following injections of menadione sodium bisulfite. Examination of these three reports strongly suggests that a marginal deficiency of vitamin K may have been aggravated by the use of sulfonamide drugs at levels and for time periods much in excess of those recommended. In line with these findings, Frost and Spruth ('55) found the requirement for vitamin K greatly increased in chicks fed a vitamin K-low diet with 0.1% of sulfaquinoxaline and discovered the vitamin K activity of menadione sodium bisulfite complex to be at least 4 times that of menadione.

[1] This work was reported in part to the American Institute of Nutrition in San Francisco, April, 1955. Federation Proc., *14:* 434.

Under these conditions, even 0.72 gm of menadione per ton, twice the National Research Council ('54) estimated requirement for vitamin K in poultry feed, was ineffective to support normal blood clotting time.

Studies are described herein to establish more exactly the ratio of the activity of menadione sodium bisulfite to that of menadione, both in the presence and absence of 0.1% of sulfaquinoxaline in the diet. The first of these studies was conducted as before using a simplified vitamin K-low corn-soy ration. Because the growth of the chicks was poor on this type of ration, we next tested the ability to produce an uncomplicated vitamin K deficiency on a modification of the Animal Nutrition Research Council (A.N.R.C.) reference broiler ration. This ration was devised in 1954 by a committee headed by Dr. George M. Briggs as a modern type, high energy ration to support near-maximum growth. In addition to 2% of alfalfa, this ration contains 2 gm of menadione per ton as a source of vitamin K. Unlike the simplified corn-soy ration, the A.N.R.C. ration contains fish solubles and condensed whey as sources of unidentified factors, plus procaine penicillin and arsanilic acid as growth stimulants.

Experiment showed that vitamin K deficiency (prothrombin less than 10% of normal) was readily produced on the A.N.R.C. reference ration when the alfalfa and menadione were omitted. At the same time the growth rate was rapid. This ration was chosen therefore as the base for precise comparisons of vitamin K potency of materials, both in the presence and absence of 0.1% of sulfaquinoxaline.

## EXPERIMENTAL

The menadione sodium bisulfite complex [2] used in these studies contains 63% of menadione sodium bisulfite, U.S.P., equal to 33% of 2-methyl-1,4-naphthoquinone (menadione). Chemically, the highly active antihemorrhagic product of sodium bisulfite with menadione is sodium 2-methyl-1,4-dioxo-

[2] Klotogen F, trademark Abbott Laboratories, contains by definition 63% of menadione sodium bisulfite, U.S.P.

tetraline-2-sulfonate (Carmack et al., '50). The menadione used met U.S.P. specifications. Both vitamin K supplements were carefully premixed in finely ground limestone. Additions of premix were made to appropriate aliquots from a single batch of basal ration for each experiment. The composition of the basal rations used is shown in table 1.

TABLE 1

*Experimental chick rations*

| | SIMPLIFIED VITAMIN K LOW BASAL | A.N.R.C. REF. BROILER RATION |
|---|---|---|
| | % | % |
| Ground yellow corn | 66.35 | 60.7 |
| Soybean meal (solvent — 44% prot.) | 30 | .. |
| Soybean meal, 50% prot. | .. | 28 |
| Bone meal | 2 | .. |
| Limestone | 1 | .. |
| Iodized salt | 0.5 | .. |
| Manganese sulfate | 0.5 | .. |
| Vit. A and D (3000 A + 600 D/gm) | 0.1 | .. |
| Methionine | 0.05 | 0.05 |
| Alfalfa meal, 17% prot.[1] | .. | 2 |
| Fish solubles, 50% solids | .. | 2.5 |
| Dried whey, 50% lactose | .. | 2.5 |
| Calcium carbonate, 39% Ca | .. | 1.6 |
| Dicalcium phosphate, 20% P, 24% Ca | .. | 1.75 |
| Trace mineral mix, Del-A-mix[2] | .. | 0.1 |
| Salt | .. | 0.5 |
| Vitamin A, stabilized, 4000 I.U./gm | .. | 0.2 |
| Vitamin $D_3$, 1500 I.U./gm | .. | 0.05 |
| Choline chloride, 25% mix | .. | 0.15 |
| | *mg/lb.* | *mg/lb.* |
| Choline chloride | 150 | .. |
| Riboflavin | 2 | 1.5 |
| Calcium DL-pantothenate | 1.5 | 2.5 |
| Niacin | 10 | 15 |
| Vitamin $B_{12}$ | 0.01 | 0.003 |
| Vitamin E acetate | .. | 2 |
| Menadione[1] | .. | 1 |
| Procaine penicillin | .. | 2 |
| Arsanilic acid | .. | 45 |

[1] Alfalfa and menadione omitted except as shown in the text and in table 4.

[2] A trace mineral mixture supplied by Dr. H. W. Titus of Limestone Products Company, Newton, New Jersey.

White Leghorn or New Hampshire day-old chicks, 10 to 20 per group, were raised on wire in electrically heated brooders. Transfer to batteries was made if the experiment continued longer than 4 weeks. Water was changed daily. Otherwise no precautions appeared necessary throughout to obtain clear-cut vitamin K deficiency in the control group.

The chicks were weighed each week. Blood clotting times were taken by piercing the exposed wing vein and catching about 1 ml of free-flowing blood in 2 × 15 cm test tubes. The tubes were suspended in a water bath at 38°C. and shaken mechanically until the blood clotted. The data were expressed as previously (Frost and Spruth, '55) both in terms of average blood clotting times and numbers of chicks with clotting times over 30 minutes.

Determinations of whole blood prothrombin time were made by a method quite similar to that of Almquist ('41), except that a commercial lyophilized chick embryo extract [3] (Difco EE100) was used as the source of thromboplastin, rather than the freshly prepared extract of chick muscle used in the A.O.A.C. method. Prothrombin clotting times of blood from normal control chicks was in the prescribed range, 18 to 30 seconds. In severe vitamin K deficiency prothrombin time ranged from 80 to 180 seconds. The method is as follows: Each 2 ml vial of chick embryo extract [3] is diluted with 10 ml of 0.025 molar calcium chloride in 0.85% sodium chloride. This thromboplastin · $CaCl_2$ solution is held in the water bath at 37°C. Blood is obtained by heart puncture whereby 0.1 ml of 1.34% sodium oxalate in the syringe is simultaneously mixed with 0.9 ml of blood. Two-tenths milliliter of the oxalated blood is immediately incubated at 37°C. for one minute, after which 0.14 ml of thromboplastin $CaCl_2$ mixture is added. The time required for this mixture to clot on gentle tilting of the tube is accurately observed.

*Menadione bisulfite vs. menadione in simplified vitamin K-low basal with 0.1% sulfaquinoxaline.* In the first studies with the simplified vitamin K-low basal diet (Frost and

[3] Difco EE100.

TABLE 2

*Comparative activities of menadione and menadione sodium bisulfite in chicks on simplified vitamin K-low ration*

16 White Leghorn cockerels per group

| ADDITION TO VITAMIN K-LOW DIET WITH 0.1% SULFAQUINOXALINE | AV. 4-WEEK WT. GAIN | AV. CLOTTING TIME [1] | NO. CHICKS > 30 MINUTES [2] |
|---|---|---|---|
| | *gm* | *minutes* | |
| None | 108 | 28 ± 2.4 [3] | 9/10 |
| Menadione sodium bisulfite, U.S.P., 45 mg/ton | 149 | > 30 | 14/14 |
| Menadione sodium bisulfite, U.S.P., 180 mg/ton | 151 | 23 ± 2.6 | 9/15 |
| Menadione sodium bisulfite, U.S.P., 720 mg/ton | 168 | 7.5 ± 1.5 | 0/16 |
| Menadione, 720 mg/ton | 189 | 27 ± 1.9 | 11/14 |
| Menadione, 1440 mg/ton | 161 | 21 ± 2.8 | 7/13 |
| Menadione, 2880 mg/ton | 150 | 12 ± 2.6 | 3/15 |
| Basal diet without sulfaquinoxaline | 181 | 29 ± 1.3 | 15/16 |

[1] Chicks with clotting times longer than 30 minutes considered as 30 minutes in this computation. Standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

[2] Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

[3] Standard error.

TABLE 3

*Comparative activities of menadione and menadione sodium bisulfite in chicks on simplified vitamin K-low ration*

15 White Leghorn cockerels per group

| ADDITION TO VITAMIN K-LOW DIET WITH 0.1% SULFAQUINOXALINE | AV. 4-WEEK WT. GAIN | AV. CLOTTING TIME [1] | NO. CHICKS > 30 MINUTES [2] | GIZZARD LESIONS |
|---|---|---|---|---|
| | *gm* | *minutes* | | |
| None | 153 | > 30 ± 0.0 [3] | 12/12 | Severe |
| Menadione sodium bisulfite, U.S.P., 0.1 gm/ton | 157 | 27.1 ± 2.9 | 11/13 | .... |
| Menadione sodium bisulfite, U.S.P., 0.3 gm/ton | 136 | 12.3 ± 2.9 | 3/13 | .... |
| Menadione sodium bisulfite, U.S.P., 0.6 gm/ton | 157 | 3.7 ± 0.3 | 0/15 | Slight |
| Menadione sodium bisulfite, U.S.P., 1.0 gm/ton | 169 | 4.2 ± 0.3 | 0/15 | .... |
| Menadione, 1.0 gm/ton | 182 | 19.6 ± 2.9 | 6/13 | .... |
| Menadione, 2.0 gm/ton | 161 | 22.9 ± 2.4 | 8/14 | .... |
| Menadione, 4.0 gm/ton | 169 | 6.9 ± 0.7 | 0/12 | Slight |
| Menadione, 6.0 gm/ton | 163 | 7.3 ± 1.1 | 0/15 | .... |
| Basal diet without sulfaquinoxaline | 140 | 26.6 ± 2.4 | 8/10 | Moderate |

[1] Chicks with clotting times longer than 30 minutes considered as 30 minutes.

[2] Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

[3] Standard error.

Spruth, '55) blood clotting time plotted against the log of the concentration gave a straight line in the range of 0.045 to 0.72 gm of menadione sodium bisulfite, U.S.P., per ton of diet. Menadione was largely ineffective in this range, showing a measurable effect only at the level of 0.72 gm per ton.

An experiment was next set up to estimate the critical effective feed concentration of menadione in the range 0.72 to 2.88 gm per ton. Table 2 shows the data for this experiment. Menadione sodium bisulfite, U.S.P., was used again as a control, with results similar to those previously reported. Here a plot of clotting time against the log of menadione concentration falls on a straight line, indicating this to be a critical response range, even though the highest level was still inadequate.

A further comparison was run similarly using concentrations of menadione 6 to 10 times those of menadione sodium bisulfite, U.S.P. As a further point of interest chicks in certain groups were examined for incidence of gizzard lesions. The gizzard lesions were seen as severe (more than two areas of ulceration or one or more large ulcers with crater-like appearance), moderate (one or two roughened areas with discoloration), and slight (small roughened areas with no discoloration). The data are seen in table 3.

*Studies with modified A.N.R.C. reference ration.* Initial studies with this ration were made with and without 0.1% of sulfaquinoxaline in the diet. The adequacy of different sources of vitamin K is more clearly revealed with two planes of requirement than with one. Both whole blood clotting and prothrombin times were determined to assess the vitamin K adequacy of the diet more critically. A standard prothrombin dilution curve was used to estimate the group averages as a percentage of normal. A prothrombin time of 29 seconds was considered 100% normal in this test. Samples of blood were taken from the wing vein for the clotting time and subsequently by heart puncture for prothrombin diterminati-

TABLE 4

*Comparison of vitamin K sources in two vitamin K-low diets with and without 0.1% sulfaquinoxaline*

| RATION | VITAMIN K SOURCE | AV. WEIGHT 4 WEEKS | AV. CLOTTING TIME [1] | PROTHROMBIN GROUP AV. | TIME % OF NORMAL |
|---|---|---|---|---|---|
| | | *gm* | *minutes* | *seconds* | *%* |
| A.N.R.C. minus alfalfa and menadione | None | 362 | 22 ± 2.6 [2] (12/18) [3] | 177 ± 11.8 | < 10 |
| | 2% alfalfa + 2 gm menadione/ton | 393 | 2.2 ± 0.3 | 34 ± 0.8 | 67 |
| | Menadione sod. bisulfite, U.S.P., 1 gm/ton | 361 | 2.8 ± 0.2 | 29 ± 0.6 | 100 |
| As above + 0.1% sulfaquinoxaline | None | 211 | > 30 ( 9/9 ) | ... | .. |
| | 2% alfalfa | 346 | 4.1 ± 0.5 | 56 ± 3.2 | 29 |
| | 2% alfalfa + 2 gm menadione/ton | 327 | 3.5 ± 0.4 | 47 ± 1.9 | 37 |
| | Menadione sod. bisulfite, U.S.P., 1 gm/ton | 324 | 2.6 ± 0.3 | 35 ± 0.8 | 62 |
| Simplified corn-soy | None | 140 | 26 ± 2.3 ( 5/7 ) | 78 [4] | .. |
| As above + 0.1% sulfaquinoxaline | None | 132 | > 30 ( 5/5 ) | ... | .. |
| | Menadione sod. bisulfite, U.S.P., 1 gm/ton | 148 | 4 ± 0.9 | 27 ± 1.5 | 100 |

[1] Chicks with clotting times longer than 30 minutes considered as 30 minutes.

[2] Standard error.

[3] Ratio of chicks with clotting time over 30 minutes to total number surviving to 4 weeks.

[4] Prothrombin time taken on only one chick because of poor condition of group.

Fast growing New Hampshire Reds were used to take full advantage of the growth promoting power of the A.N.R.C. ration. Three parallel groups were run on the simplified corn-soy ration to compare both growth and extent of vitamin K deficiency on these rations.

As shown in table 4, the growth rate of chicks on the A.N.R.C. ration was more than twice that on the simplified ration. Vitamin K deficiency appeared about equally severe on both rations. Growth rate was apparently not affected except by the extreme vitamin K deficiency exhibited by the group on the modified A.N.R.C. ration with 0.1% of sulfaquinoxaline. In each case the presence of 0.1% of sulfaquinoxaline aggravated the deficiency to the point where all chicks bled to death when the first blood sample was taken for determination of clotting time. Prothrombin determination could be obtained on only one chicken on the simplified ration because of the small size and poor condition of these birds. Hemorrhages were seen in the tissues of all chicks where the clotting time was longer than 30 minutes.

Menadione sodium bisulfite, U.S.P., 1 gm per ton, appeared more effective than 2% of alfalfa plus 2 gm of menadione per ton with respect to the maintenance of prothrombin level, both in the presence and absence of sulfaquinoxaline. Calculation showed these differences to be significant ($P. < 0.01$). As found previously (Frost and Spruth, '55) the standard error of the mean for blood clotting time was small in the normal range and considerably larger when clotting time was prolonged. This was true also of the data for prothrombin clotting time.

*Comparison of menadione sodium bisulfite complex and menadione at critical levels.* Our earlier studies on White Leghorn chickens using the simplified corn-soy ration had shown that 90 to 180 mg of menadione sodium bisulfite, U.S.P., per ton was needed to maintain normal blood clotting time. Only partial protection was provided by 30 to 45 mg per ton. Stamler et al. ('43) have indicated that only one-twentieth as much menadione is required to protect birds from hemor-

rhagic manifestations as to maintain normal prothrombin time. This is in line with the general evidence that blood clotting time is not seriously affected until prothrombin level falls below about 30% of normal. Hemorrhages were invariably found on autopsy in various tissues of birds in our tests where prothrombin concentrations were as low as 10% of normal. Occasionally hemorrhages accompanied prothrombin concentrations which were 40 to 50% of normal.

TABLE 5

*Comparative activity of menadione sodium bisulfite complex and menadione*

10 Straight-run Production Reds per group

| ADDITION TO VITAMIN K-LOW DIET [1] | | AV. 4 WEEK WEIGHT | PROTHROMBIN | |
|---|---|---|---|---|
| | | | Average time | % of normal |
| | | *gm* | *seconds* | |
| None | | 306 | 92.5 ± 10.2 [2] | 10 |
| Alfalfa, 2% + menadione, 2 gm/ton [3] | | 352 | 22.3 ± 0.6 | 100 |
| Menadione sodium bisulfite complex | 30 mg/ton | 293 | 73.3 ± 6.4 | 14 |
| | 60 mg/ton | 334 | 51.7 ± 3.2 | 23 |
| | 120 mg/ton | 357 | 33.1 ± 1.4 | 49 |
| | 240 mg/ton | 289 | 24.2 ± 1.4 | 77 |
| Menadione | 45 mg/ton | 326 | 79.6 ± 7.6 | 13 |
| | 90 mg/ton | 285 | 64.2 ± 3.5 | 18 |
| | 180 mg/ton | 385 | 51 ± 2.3 | 25 |
| | 360 mg/ton | 314 | 34.2 ± 2.1 | 44 |

[1] A.N.R.C. Reference ration minus alfalfa and menadione.

[2] Standard error.

[3] A.N.R.C. Reference diet.

We were interested then to use the more critical prothrombin determination in assessing the relative values of the menadione sodium bisulfite complex and menadione as sole sources of vitamin K in the modified A.N.R.C. ration. The commercial premix containing 2.52 gm menadione sodium bisulfite, U.S.P.,[4] per pound in calcite was used in this experiment. The experiment was set up with 10 straight-run New Hampshire (Production Red) chicks per group. The

[4] Four grams of Klotogen F.

complete A.N.R.C. ration was used as a positive control (prothrombin 100% normal). Four levels each of the menadione sodium bisulfite complex and menadione, thought to be in the critical range, were selected and carefully mixed into appropriate aliquots of the vitamin K-low feed. The experiment was run for 4 weeks. Blood samples by heart puncture were taken for prothrombin determination only at the 4th week in these birds. The results are shown in table 5.

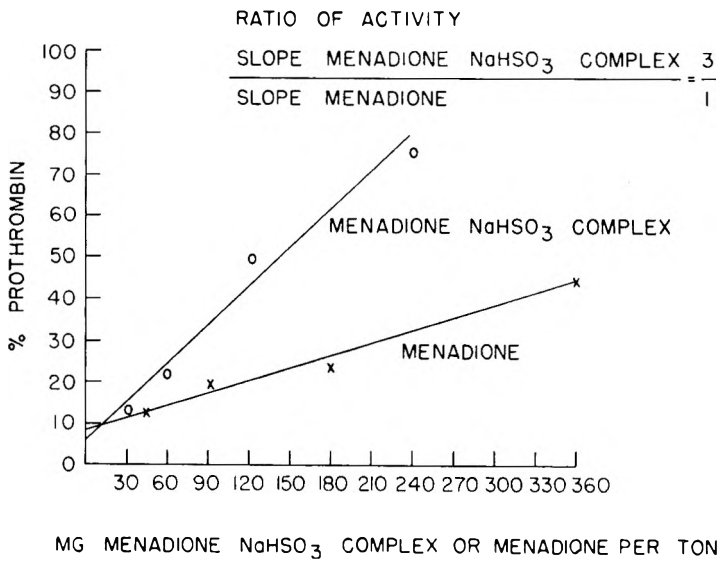


Fig. 1 Response to graded levels of menadione sodium bisulfite complex (Klotogen F) and menadione in the critical requirement range. Vitamin K-low A.N.R.C. ration. Note: Prothrombin clotting time of the group on the complete A.N.R.C. ration (see table 5) was arbitrarily designated 100% normal.

Comparison of the data shows that the menadione sodium bisulfite complex at 60 and 120 mg per ton is almost exactly as effective as 180 and 360 mg per ton respectively of menadione. Figure 1 shows the concentration of each form of the vitamin plotted against prothrombin, as the percentage of normal. This, in effect, shows the desired straight line log-dose response. Calculating the relative slopes of the

two lines shows the menadione sodium bisulfite complex to be three times as potent as menadione under these conditions.

## DISCUSSION

The menadione sodium bisulfite complex used in these studies was found to be three times as effective as menadione as a source of vitamin K in the A.N.R.C. reference ration. This was not expected from work in the literature comparing menadione against its water-soluble forms. The classic work of Quick et al. ('54) in dogs operated upon to produce complete absence of bile from the intestinal tract showed a 5000-fold increase in requirement for vitamin K, with no increase in requirement for menadione sodium bisulfite.

The experiments in this laboratory indicate that when 0.1% of sulfaquinoxaline is added to vitamin K-low rations the requirement for vitamin K increases markedly. The requirement for vitamin K in terms of the fat-soluble menadione increases disproportionately to that of the water-soluble menadione bisulfite. This suggests that massive sulfa therapy interferes with utilization of menadione, possibly by interfering with normal production of bile.

A point which has not been generally recognized is how significantly sulfa drugs may increase the need for vitamin K. In preliminary laboratory experiments 2 gm of menadione sodium bisulfite complex per ton of feed proved inadequate to protect prothrombin levels in the presence of 0.06% of sulfaquinoxaline in the drinking water. Other experiments, to be described elsewhere, indicate that at least 10 gm of menadione sodium bisulfite complex per ton may be needed under these conditions, more than 10 times the requirement in the absence of sulfaquinoxaline. Moreover the need for menadione under similar conditions appeared to be well in excess of 40 gm per ton. Goldhaft and Wernicoff ('54) reported that 4 gm of menadione per ton did not prevent field hemorrhagic disease, whereas 10 mg of menadione bisulfite per gallon of drinking water appeared to effect a cure. Again, the field cases with which we have had direct contact (Frost and

Spruth, '55) were ones in which sulfa drugs had been used in gross excess over recommended levels. The appearance of afflicted birds in the field, both inwardly and outwardly, was the same as that of some of our vitamin K-low laboratory birds given very high levels of sulfa drug. The appearance of such birds is seen in figure 2. At autopsy these birds had large internal hemorrhages. Birds similarly treated, but re-

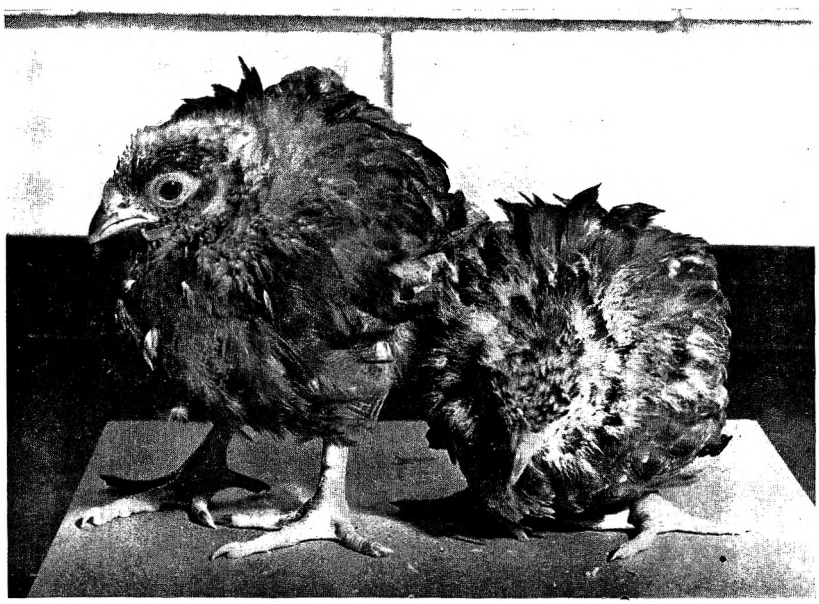

Fig. 2 Acute vitamin K deficiency induced in 5-week-old chicks by feeding a diet deficient in vitamin K for 4 weeks, followed by the addition of 0.1% of sulfaquinoxaline for one week.

ceiving adequate menadione sodium bisulfite appeared normal, although there was some inhibition of growth, and more detailed examination revealed abnormal bone marrow, slight anemia and, in some cases, petechial hemorrhages.

As described by Seeler et al. ('44) sulfaquinoxaline is unique among the sulfonamides in the speed with which it produces hypoprothrombinemia in rats and dogs on normal diets. Scott ('55) confirms this effect in the chick and cites

the equally rapid recovery following vitamin K therapy. The symptoms of sulfaquinoxaline poisoning described by Delaplane and Milliff ('48) and by Davies and Kendall ('53) are not unlike those which characterize the hemorrhagic syndrome. The latter authors suggest that the clinical picture in the field is generally complicated by other disease. They cite the observation recently confirmed by Yacowitz et al. ('55) that toxic symptoms are more likely to develop in older chicks, and also cite the great variance among birds in susceptibility to the toxicity, a fact which again has a direct parallel in flocks with "hemorrhagic disease."

Research is needed under a variety of conditions of diet and disease to establish more clearly the vitamin K needs of poultry. Hill ('55) has recently indicated that resistance to fowl typhoid is increased by appropriate high levels of certain vitamins. He states, "Some vitamins, such as vitamin K, must be increased over the requirement for growth many times more than others in order to bring about increased resistance to typhoid." Fowl typhoid and many other infectious diseases of poultry lead to hemorrhagic manifestations (Goldhaft and Wernicoff, '54); again suggesting that the so-called "hemorrhagic syndrome" is not a single entity but rather a complex.

The high vitamin K potency of soybean oil was first reported by Almquist and Stokstad ('37). It is again worthy of note in the light of our findings that emergence of the "hemorrhagic syndrome" as a disease entity (Gray et al., '54; Goldhaft and Wernicoff, '54; Bornstein and Samberg, '54; and Cover et al., '55) followed the general shift from expeller to solvent extracted soybean oil meal in feeds. Attention has been called (Anderson et al., '54, '55) to the resultant decrease in vitamin K in poultry rations.

The results of the study of the effect of adding animal fat to the above rations have been quite inconsistent. In one experiment the addition of 2% of animal fat to the simplified vitamin K-low diet appeared to enhance the value of menadione. In a subsequent experiment, however, the addition

of 4% of animal fat to the vitamin K-low A.N.R.C. ration actually decreased the vitamin K value of both menadione sodium bisulfite and menadione. Further work is needed therefore to establish the role of fat in this regard.

Although data from only one experiment are shown here, there was a close relation in 4 other experiments between the incidence and severity of gizzard lesions and the adequacy of vitamin K. Lesions were extensive only when vitamin K deficiency was severe. Here again accentuation of vitamin K need in the presence of 0.1% of sulfaquinoxaline markedly increased the incidence and severity of gizzard lesions. Further studies, employing these two planes of vitamin K requirement, may be of value to establish this role of vitamin K.

## SUMMARY

Vitamin K deficiency occurred in two to 4 weeks in chickens on a simplified corn-soy ration, or on the modified vitamin K-low Animal Nutrition Research Council reference broiler ration. A water-soluble menadione sodium bisulfite complex containing 63% of menadione sodium bisulfite, U.S.P., proved three times as effective as menadione as a sole source of vitamin K in the modified A.N.R.C. reference ration. Menadione at 0.18 mg per pound of feed, the National Research Council recommended level for vitamin $K_1$, failed to give normal prothrombin levels in chicks up to 4 weeks of age on this ration. Sulfaquinoxaline at the unusually high level of 0.1% increased the requirement for vitamin K markedly on these rations. This increase was disproportionately greater for menadione than for menadione sodium bisulfite, U.S.P. Menadione sodium bisulfite, U.S.P. proved 6 to 10 times as effective as menadione in the simplified ration with 0.1% of sulfaquinoxaline. These differences are thought to be due to the better absorption of the water-soluble form.

The addition of 2 to 4% of animal fat to these fat-low rations did not consistently improve utilization of either form of the vitamin. Incidence of gizzard lesions appeared to be

related to the severity of vitamin K deficiency. Growth was inhibited only when vitamin K deficiency was profound.

The incidence of the field hemorrhagic syndrome in various areas of the world since 1951–52 is thought to be related to a decreased content of vitamin K in feeds, coupled in part with overmedication with drugs which markedly increase the need for vitamin K. Hypoprothrombinemia caused by intensive sulfa medication was shown in these studies to create unusual requirements for vitamin K. High intake of a readily available source of vitamin K was found to greatly minimize the over-all injury resulting from excessive sulfa drug.

## ADDENDUM

Recently we have observed petechial hemorrhages in commercial flocks where the blood and prothrombin clotting times were normal. These flocks showed high incidence of enteritis, slight anemia, and some abnormalities in the spleen and other tissue. It is not yet clear whether this condition results from infectious disease or dietary cause. In any case adequate vitamin K must be supplied as a prerequisite to the search for other factors which may cause hemorrhage.

## ACKNOWLEDGMENTS

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# REPRODUCTION AND LACTATION OF RATS RECEIVING CORN OIL OR BUTTERFAT IN THE PRESENCE OF SULFATHALIDINE [1]

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From the several studies that have been reported concerning the effects of various fats and oils on the reproductive and lactation performance of the rat it is difficult to draw any definite conclusions. Miller ('43) found that the survival rate of rats at weaning was much improved if the lard or soybean oil in the mother's diet were replaced by hydrogenated cottonseed or soybean oil. On the other hand, Loosli et al. ('44) reported that better lactation was obtained with corn oil or coconut oil than with hydrogenated coconut oil. Deuel et al. ('44) noted that corn, cottonseed, peanut, and soybean oils, and a vegetable oil margarine were just as satisfactory as butterfat in insuring successful pregnancy and lactation. In an extensive series of experiments, von Euler and his associates ('46, '47a, b) found the weaning weights of the young to be consistently higher when the mothers were fed margarine in place of butterfat.

Viswanatha et al. ('54) of this laboratory have previously reported that butterfat supported better growth in rats than

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corn oil when two per cent sulfathalidine [3] (ST) had been incorporated into the diet. It was tentatively suggested that butterfat contained a growth-promoting factor normally synthesized by intestinal bacteria which are inhibited by ST. Since the dietary requirements for reproduction and lactation are believed to be more exacting than for growth (Sure, '24, '41; Vinson and Cerecedo, '44), reproduction and lactation studies were undertaken in an attempt to accentuate the nutritive differences between butterfat and corn oil in the presence of ST.

## EXPERIMENTAL

The basal ration employed in these studies has already been described in detail (Viswanatha et al., '54) and contains 28% fat (butterfat [4] or corn oil [5]), 20% casein,[6] 48% sucrose, 4% mineral salts, plus all the known water-soluble and fat-soluble vitamins. Unless indicated otherwise, two per cent ST was added to the ration at the expense of sucrose. All rations were prepared at intervals not exceeding one week and were stored in the refrigerator at 4°C. Food and water were provided ad libitum at all times.

The rats used in this study were of a strain which has been maintained in this laboratory for many years. The general procedure was to mate randomly selected females with proven males when the former were 10 weeks old or weighed 150 gm, whichever occurred later. Three to 4 females were housed with one male in cages provided with raised-screen bottoms except during the last few days of pregnancy and during lactation when the females were kept in individual cages containing clean wood shavings. Females which did not become pregnant after breeding with two or three different proven

[3] Trade name for phthalylsulfathiazole produced by Sharp and Dohme, Inc., Philadelphia, Pa.

[4] Unsalted winter product obtained from the Department of Dairy Husbandry, University of Minnesota. The water was removed by melting the butter and separating the water from the oil phase in a separatory funnel.

[5] Mazola, Corn Products Refining Co., Argo, Ill.

[6] General Biochemicals, Inc., Chagrin Falls, Ohio.

males over a period of 5 to 6 weeks were recorded as being unable to conceive. Each mother and her litter were weighed daily up to 21 days post-parturition at which time the young were weaned. Except in experiment I all litters were standardized by random selection to 6 pups on the third day following birth. Those females who possessed less than 6 young by the third day were given additional foster pups from the females who had more than 6 surviving young. The mothers were rebred following a rest period of about two weeks subsequent to the weaning of the young.

The principal criterion of the reproductive performance was the survival rate of the young within the first three days following birth (Mirone et al., '48), and the adequacy of lactation was judged primarily on the survival and weight of the young at weaning. Other observations that were employed to evaluate the reproductive and lactation performance of the females were litter size, weight of the young at birth, and the change in the weight of the mother during lactation (Vinson and Cerecedo, '44; Nelson and Evans, '47). Post mortem examinations were conducted on a number of young which had died shortly after birth, but gross inspection failed to reveal the cause of death.

## RESULTS

### *Reproduction and lactation following the first gestation period*

*Experiment I.* The female rats used in this experiment were those which had received ST-containing rations in the growth experiment previously reported (table 5, Viswanatha et al., '54). These animals were maintained on the same diet, containing either corn oil or butterfat, and were bred when they had reached maturity. Their subsequent reproductive and lactation performance is shown in table 1. All of the females in both groups successfully conceived and cast litters of comparable size and weight. By the third day, however, about 27% of the young from mothers on the corn oil diet had

TABLE 1

*Reproduction and lactation following the first gestation period*

| CATEGORY OF INTEREST | EXPERIMENT I [1] | | EXPERIMENT II [2] | | | |
|---|---|---|---|---|---|---|
| | | | Corn oil | | Butterfat | |
| | Corn oil | Butterfat | −ST | +ST | −ST | +ST |
| Females bred | 8 | 9 | 12 | 23 | 10 | 20 |
| Pregnant females | 8 | 9 | 12 | 23 | 10 | 20 |
| Litters east | 8 | 9 | 12 | 23 | 10 | 20 |
| Young per litter [3] | 6.5 ± 0.8 | 7.9 ± 1.1 | 6.6 ± 0.9 | 7.6 ± 0.5 | 8.2 ± 0.6 | 6.8 ± 0.5 |
| Birth weights (per young), gm [3] | 5.3 ± 0.2 | 5.6 ± 0.2 | 5.9 ± 0.2 | 5.3 ± 0.1 | 5.8 ± 0.2 | 5.7 ± 0.1 |
| Mortality, 0–3 days,[4] % | 27 (14/52) | 0 (0/71) | 1 (1/79) | 30 (52/176) | 0 (0/82) | 0 (0/135) |
| Mortality, 3–21 days,[5] % | 3 (1/38) | 9 (7/71) | 1 (1/72) | 4 (4/96) | 0 (0/60) | 0 (0/114) |
| Weaning weights (per young), gm [3] | 24.4 ± 4.0 | 31.2 ± 1.9 | 33.0 ± 1.0 | 22.5 ± 0.09 | 36.0 ± 1.4 | 29.5 ± 0.8 |

[1] Both rations contained 2% sulfathalidine. Mothers were allowed to nurse all their young.

[2] No sulfathalidine: — ST; 2% sulfathalidine: + ST. Litters were adjusted to 6 on third day after birth.

[3] Mean ± standard error of the mean.

[4] $\frac{\text{No. of young dead on 3rd day}}{\text{No. of young born}} \times 100$, based on values shown in parentheses.

[5] $\frac{\text{No. of young dead 3–21 days}}{\text{No. of young given mother on 3rd day}} \times 100$, based on values shown in parentheses.

succumbed compared to the complete survival of the offspring from the butterfat group. At this time, the mothers were allowed to nurse all their young with no restriction in the size of the litter. As judged by the weaning weights of the young, there was also a significant[7] difference ($t = 2.5$; $P < 0.05$) in the lactation performance of the two groups of females in favor of butterfat.

*Experiment II.* In table 1 are also presented the results of another experiment in which the parent females had been on a stock ration[8] until they weighed about 120 gm. At this time the animals were divided into 4 groups: Corn oil, with and without two per cent ST, and butterfat, with and without two per cent ST. These animals were bred three weeks thereafter.

A statistical comparison of the data obtained with the two fats in the absence of ST failed to reveal any significant differences by any of the criteria listed in the first column of the table. In the presence of ST, however, the incidence of mortality of the young by the third day after parturition was 30% in the corn oil group compared to 0% in the butterfat group, thus confirming the observations of experiment I. In this experiment as well, the weights of the young at weaning were significantly higher in the litters fed butterfat ($t = 4.5$; $P < 0.01$).

### *Reproduction and lactation in successive gestation periods*

The females, which had successfully borne and weaned one litter on the ST-free rations in experiment II, were maintained on the same basal ration to which 2% ST had

[7] Significance is based on the calculation of "t" values and the corresponding levels of probability ("P") (Snedecor, '46). Only "P" values $< 0.05$ have been considered significant.

[8] A mixture of 31.49% yellow corn; 10% dried skim milk; 9% each of alfalfa leaf meal, soybean oil meal and fish meal; 1.5% salt mix; and 0.01% irradiated yeast.

TABLE 2

*Reproduction and lacation following three successive gestation periods on diets containing 2% sulfathalidine*

| CATEGORY OF INTEREST | CORN OIL | | | BUTTERFAT | | |
|---|---|---|---|---|---|---|
| | Litter 1 [1] | Litter 2 | Litter 3 | Litter 1 | Litter 2 | Litter 3 |
| Females bred | 12 | 11 | 10 | 10 | 10 | 10 |
| Pregnant females | 12 | 11 | 7 | 10 | 10 | 8 |
| Litters cast | 11 | 10 | 7 | 10 | 10 | 8 |
| Females dead at parturition | 1 | 1 | 0 | 0 | 0 | 0 |
| Resorptions | 0 | 3 | 2 | 0 | 0 | 0 |
| Young per litter [2] | 7.4 ± 1.2 | 6.7 ± 0.9 | 7.7 ± 0.9 | 8.1 ± 0.8 | 8.8 ± 0.9 | 6.9 ± 1.0 |
| Birth weight per young, gm [2] | 5.1 ± 0.2 | 5.1 ± 0.3 | 5.5 ± 0.2 | 5.6 ± 0.2 | 5.5 ± 0.2 | 6.0 ± 0.2 |
| Mortality, 0–3 days,[3] % | 9 (7/82) | 48 (33/69) | 26 (14/54) | 0 (0/81) | 2 (2/88) | 9 (5/55) |
| Mortality, 3–21 days,[4] % | 2 (1/60) | 0 (0/36) | 3 (1/30) | 2 (1/60) | 0 (0/60) | 0 (0/48) |
| Weaning weight per young, gm [2] | 27.3 ± 0.9 | 23.8 ± 1.9 | 27.0 ± 2.8 | 33.8 ± 2.0 | 31.7 ± 0.7 | 32.0 ± 2.7 |
| Weight loss of mother during lactation, gm [2] | 41.7 ± 6.9 | 58.0 ± 9.4 | 63.0 ± 3.5 | 25.4 ± 5.1 | 36.9 ± 3.9 | 25.7 ± 4.9 |

[1] This sequence does not take into account the first litters shown in table 1 on the sulfathalidine-free rations.

[2] Mean ± standard error of the mean.

[3] See footnote 4, table 1.

[4] See footnote 5, table 1. Litters adjusted to 6 young on third day.

been added. The data pertaining to the reproduction and lactation performance which followed after three successive gestation periods are summarized in table 2.

No serious interference with the ability of the females to conceive or cast litters was evident with either fat, although the ability to conceive after the second gestation period was somewhat impaired with both fats. There were also several instances of resorptions and maternal deaths at parturition; these, however, were confined to the corn oil group.

TABLE 3

*Statistical comparison [1] of corn oil versus butterfat with respect to weight of the young at weaning and loss in weight of the mother during lactation*
*(Based on the data shown in table 2)*

| LITTER SEQUENCE | WEANING WEIGHT OF YOUNG | | LOSS IN WEIGHT OF MOTHER DURING LACTATION | |
|---|---|---|---|---|
| | t | P | t | P |
| First | 3.0 | $< 0.01$ | 2.6 | $< 0.05$ |
| Second | 4.2 | $< 0.01$ | 2.3 | $< 0.05$ |
| Third | 2.8 | $< 0.05$ | 5.3 | $< 0.01$ |

[1] See footnote 7.

The most striking feature of these data is the sharp increase in the three-day postnatal mortality of the young after the second gestation period of the mothers receiving the corn oil diet, from a mortality of 9% in the first litter to almost 50% in the second litter. Rather unexpectedly the mortality rate in the third litter had dropped to 26%. The young from the mothers fed butterfat, on the other hand, continued to thrive through the third litter. Statistical analysis of the data pertaining to the weight of the young at weaning and the loss in weight of the mother during lactation reveals that the lactation performance of the animals of the butterfat group was also superior to that of the corn oil group in each successive litter (table 3).

TABLE 4

*Reproduction and lactation in successive generations on diets containing 2% sulfathalidine*

| CATEGORY OF INTEREST | $F_1$ GENERATION | | | | $F_2$ GENERATION | | | |
|---|---|---|---|---|---|---|---|---|
| | Corn oil | | Butterfat | | Corn oil | | Butterfat | |
| | Litter 1 | Litter 2 | Litter 1 | Litter 2 | Litter 1 | Litter 2 | Litter 1 | Litter 2 |
| Females bred | 28 | 18 | 18 | 14 | 19 | 18 | 9 | 5 |
| Pregnant females | 27 | 18 | 18 | 14 | 19 | 18 | 9 | 5 |
| Litters cast | 27 | 18 | 18 | 14 | 19 | 18 | 9 | 5 |
| Young/litter [1] | 8.2 ± 0.5 | 7.7 ± 0.7 | 7.9 ± 0.6 | 8.8 ± 0.8 | 8.0 ± 0.4 | 8.4 ± 0.7 | 8.7 ± 0.8 | 8.4 ± 0.8 |
| Birth weight per young, gm [1] | 5.2 ± 0.1 | 5.0 ± 0.2 | 5.5 ± 0.1 | 5.4 ± 0.1 | 5.4 ± 0.1 | 5.7 ± 0.2 | 5.3 ± 0.2 | 6.0 ± 0.4 |
| Mortality, 0–3 days,[2] % | 12 (27/221) | 42 (49/118) | 3 (4/142) | 3 (3/111) | 1 (2/152) | 1 (2/151) | 0 (0/78) | 0 (0/42) |
| Mortality, 3–21 days,[3] % | 2 (3/138) | 2 (1/66) | 0 (0/108) | 0 (0/78) | 2 (2/114) | 1 (1/108) | 0 (0/54) | 0 (0/30) |
| Weaning weight per young, gm [1] | 26.0 ± 0.5 | 26.2 ± 1.1 | 26.4 ± 1.0 | 27.9 ± 1.2 | 25.6 ± 1.1 | 28.5 ± 1.0 | 28.3 ± 1.7 | 28.4 ± 2.1 |
| Weight loss of mother during lactation, gm [1] | 29.4 ± 8.9 | 30.0 ± 3.7 | 30.3 ± 3.1 | 38.2 ± 7.5 | 34.1 ± 3.8 | 38.2 ± 7.0 | 31.2 ± 3.4 | 39.2 ± 4.8 |

[1] Mean ± standard error of mean.

[2] See footnote 4, table 1.

[3] See footnote 5, table 1. Litters adjusted to 6 young on third day.

*Reproduction and lactation in successive generations*

The $F_1$ females originating from the first litter of the parent generation shown in table 2 were allowed to mature and were then bred to produce two successive litters. The $F_2$ females from the first of these two litters were likewise bred and studied through two gestation periods. In all cases the progeny were maintained on the same rations which had been received by their predecessors. The reproduction and lactation performance observed during the course of these studies is summarized in table 4.

With respect to the $F_1$ generation the mortality of the young by the third day followed the same trend observed in the parent generation, namely a rather low death rate in the first litter (12%) followed by an increase (42%) in the second litter. The $F_1$ females on butterfat again produced litters which survived the first three critical days of life in both litters. In contrast to the parent generation, however, no significant difference in lactation, as judged by the weight of the young at weaning or by the loss in weight of the mothers during the nursing period, was apparent between the two fats.

A rather surprising result was the almost complete absence of infantile mortality in both litters produced by the $F_2$ females on the corn oil diet. This was in sharp contrast to the poor survival rate in litters of the parent and $F_1$ females on the same ration. As in the case of the $F_1$ females, no difference in lactation performance between the mothers on the corn oil and butterfat diets was revealed.

## DISCUSSION

Since the mortality of the young was the principal point of difference between the corn oil and butterfat diets, it is important to point out that the mortality figures have been based on the *total* number of young which failed to survive in any one group. This, of course, gives no indication of the variations in the mortality rate of individual litters within

each treatment. Rather than present a detailed breakdown of each litter, the general observation has been that the mortality of the young was not uniform among all litters, but usually whole litters would fail to survive rather than a few in each litter. While this may be a reflection of individual reproductive difficulties rather than of the diet, it is nevertheless significant that, in spite of random selection of females, a high incidence of infantile mortality was in every case associated with the corn oil + ST diets. Moreover, many of the females in the corn oil group, which had successfully raised one litter in the absence of ST (exp. II, table 1), subsequently lost their entire litters when transferred to the same ration containing ST (table 2).

The failure to observe any difference in the reproduction and lactation performance of rats receiving corn oil or butterfat in the absence of ST is in substantial agreement with the results reported by Deuel et al. ('44). The relatively high incidence of mortality of the young within the first three days following birth suggests an impairment in the reproductive performance of mothers receiving a diet containing corn oil in the presence of ST. This abnormality was particularly evident after the second gestation period of these mothers, the death rate being as high as 48%. Replacing corn oil with butterfat led to almost complete survival of the young under similar circumstances.

If the weight of the young at weaning and the change in weight of the mother during lactation are accepted as criteria of lactation performance, it may be likewise concluded that, with the exception of the females of the $F_1$ and $F_2$ generations, the mothers consuming the butterfat rations were more efficient in nursing their young than those receiving corn oil.

Because it was contrary to expectations, the low incidence of mortality of the young from the $F_2$ females fed corn oil deserves special comment. An explanation is provided if one presumes, as suggested earlier (Viswanatha et al., '54), that the action of ST is directed towards the intestinal flora whereby the microbial synthesis of an essential nutrient,

present in butterfat but absent in corn oil, is suppressed. The capacity of certain microorganisms to develop a resistance to sulfa drugs is not unknown (Sevag and Green, '44), and it is conceivable that the continued ingestion of ST over a period of several generations may have led to the gradual establishment of a ST-resistant microflora. The improvement in the lactation performance of the $F_1$ and $F_2$ females fed corn oil over the preceeding parent generation may also be a manifestation of this adaptation phenomenon on the part of the intestinal flora. The fact that the $F_1$ females on corn oil reproduced so poorly and yet managed to lactate as satisfactorily as their butterfat counterparts indicates that the nutritional requirements for a normal reproduction may be more stringent than those required for lactation, a view previously expressed by Sica and Cerecedo ('48).

An alternative explanation which does not invoke the concept of an unrecognized nutrient in butterfat is suggested by the recent paper of Thomasson ('55) relating to the possible presence of growth-inhibiting substances in certain vegetable oils. In order to explain the observations recorded here on this basis, it becomes necessary to postulate that ST must in some way potentiate the effects of the harmful substances present in corn oil. The manner in which ST would exert this effect is not readily apparent.

## SUMMARY

Corn oil and butterfat in the presence of 2% sulfathalidine have been compared with respect to their ability to promote reproduction and lactation of female rats in successive litters and generations.

In studies on the parent generation, the postnatal mortality of the young from mothers on the corn oil diet ranged from 9 to 48% within the first three days, tending to be highest after the second gestation period. When corn oil was replaced with butterfat almost complete survival of the young was obtained. Lactation performance, as judged by the weight of

the young at weaning and the weight change of the mother during lactation, was also significantly in favor of butterfat.

The $F_1$ females on corn oil produced litters in which the first three-day mortality rate was 12 and 42% in the first and second litters respectively, whereas the $F_2$ females on the same diet had litters in which very few of the young succumbed. Essentially complete survival of the young from all of the females receiving butterfat was again observed. No difference in lactation performance could be noted between the $F_1$ and $F_2$ females on corn oil and those on butterfat.

Possible explanations for these results are considered.

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# RESPONSES OF RATS TO UREA AND RELATED SUBSTANCES [1]

## THE USE OF A SPACED-FEEDING TECHNIQUE

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The practice of feeding rats for limited portions of the day has been investigated by Werthessen ('37) and Barker ('49), and has been used in studies of the respiratory quotient (Tepperman et al., '43) and of fatty acid synthesis (Dickerson et al., '43). More recently Lepkovsky et al. ('55a,b) have employed spaced feeding in investigations of glycogen synthesis. We have used this procedure in proteinuria studies in an attempt to reduce the contamination of urine by spilled food (Rumsfeld and Baumann, '55; Finlayson and Baumann, '56). In the latter studies 12.5% of urea in the diet depressed growth severely in rats fed two hours a day, but when the rats were regrouped and the urea diet was fed ad libitum, no depression occurred. The present experiments deal with the toxicity of urea when fed two hours per day and include attempts to use spaced feeding for determining the biological values of proteins and for altering the effects of mildly toxic compounds.

### METHODS

Holtzman male albino rats were used in all series. They were housed in individual wire-bottom cages and were given

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water ad libitum. Rats were trained for spaced-feeding experiments as follows: weanlings, 21 days of age, were fed a training diet consisting of alcohol-extracted casein 20, corn oil 5, Wesson salts 4, L-cystine 0.2, and glucose monohydrate [3] to 100 with vitamins added in the following amounts in milligrams per kilogram: thiamine 2, pyridoxine 2.5, riboflavin 2, niacin 10, calcium pantothenate 20, inositol 100, choline chloride 1000, biotin 0.1, menadione 10, α-tocopherol 50, vitamin $B_{12}$ 0.02, and folic acid 0.02. In addition each rat received one drop of halibut liver oil [4] per week. After receiving this diet for 10 days ad libitum, the animals were offered the ration for two hours at approximately the same time every day during a two-week period. At this time those rats which had returned to their 31-day weight were grouped and placed on experimental diets, while those which had failed to do so (usually about 40%) were discarded.

Experimental rations were similar to the training diet except that the level of casein in the basal diet was 12% and the L-cystine was in some instances omitted. In all cases where spaced feeding was used (except in the determination of biological values), parallel experiments were performed in which the rats were fed ad libitum. Twenty-one-day-old rats were used in these orthodox series. The rats were weighed weekly throughout all experiments and daily food consumption was measured at comparable stages of growth.

Blood samples were obtained by cardiac puncture from rats under ether anesthesia. Ammonia was measured by a Nessler method; urea, by the method of Archibald ('45). Both determinations were carried out on protein-free filtrates of whole blood prepared by the method of Somogyi ('29).

## EXPERIMENTAL

When rations were fed for two hours per day, as little as 5% of urea produced a considerable growth depression, whereas in ad libitum experiments a statistically significant

[3] Cerelose.

[4] Haliver oil, Abbott Laboratories, North Chicago, Illinois.

depression was usually not apparent until the level of dietary urea exceeded 25%. Moreover, resistance to urea on either regime was not altered by the nutritional adequacy (presence or absence of added cystine) or the level (12 or 20%) of the protein in the diet (Finlayson, '55).

To equate the two methods of feeding, rats received 0, 5, or 10% of urea for two hours per day or 0, 20, or 30% of

TABLE 1

*Growth effects of urea fed ad libitum or two hours per day*

| FEEDING METHOD | UREA [1] | 5 WK. GAIN | % DEPRESSION [2] | | DAILY FOOD INTAKE | UREA INTAKE | |
|---|---|---|---|---|---|---|---|
| | | | 5 wk. | Average [3] | | | |
| | % | *gm* | | | *gm* | *gm/day* | *gm/hr.* [4] |
| Spaced | 0 | 36 ± 9 [5] | 0 | 0 | 8 | 0 | 0 |
| | 5 | 24 ± 9 | 33 | 38 | 8 | 0.4 | 0.2 |
| | 10 | 7 ± 4 | 81 | 85 | 7 | 0.7 | 0.35 |
| Ad libitum | 0 | 148 ± 12 | 0 | 0 | 17 | 0 | 0 |
| | (20) [6] | 144 ± 9 | 3 | 3 | 22 | 0 | 0 |
| | (30) [6] | 141 ± 10 | 5 | 7 | 23 | 0 | 0 |
| | 20 | 128 ± 12 | 14 | 21 | 18 | 3.6 | 0.15 |
| | 30 | 93 ± 6 | 37 | 39 | 16 | 4.8 | 0.2 |

[1] Added at expense of entire diet.

[2] $\frac{\text{Gm depression from controls}}{\text{Gm gain of controls}} \times 100.$

[3] Average of weeks 2–5.

[4] Daily urea intake divided by hours food was available per day.

[5] Standard error of 5 rats.

[6] Values in parentheses are cellulose (Solka Floc) levels.

urea ad libitum. Growth was depressed progressively as the level of dietary urea was increased, the percentage of urea required to produce a given depression being much lower when the feeding was spaced (table 1). Although 5% of urea, using the spaced-feeding technique, depressed growth as much as did 30% ad libitum, the daily urea intake of rats on the latter regime exceeded the former by a factor of 12. Growth depression appeared to be determined by the rate at which urea was ingested: rats in each of these groups

consumed an average of 200 mg of urea for each hour the food was available (table 1). Thirty per cent of cellulose, added as a control against the possible dilution of a critical nutrient by urea, had no appreciable influence on growth or on caloric intake (table 1).

In a subsequent experiment blood studies were carried out on adult rats (190 to 220 gm) fed 5% of urea two hours per day or 30% ad libitum for 6 days. Before being placed on experimental diets the first group received the training ration two hours per day for one week; during this time the others

TABLE 2

*Blood urea after eating urea two hours per day or ad libitum*

| UREA IN DIET [1] | FEEDING METHOD | FOOD EATEN [2] | BUN [3] |
|---|---|---|---|
| % | | *gm* | *mg %* |
| 0 | | 9 | 14.3 ± 2.3 [4] |
| | Spaced | | |
| 5 | | 9 | 40.4 ± 5.0 |
| 0 | | 20 | 11.2 ± 3.0 |
| | Ad libitum | | |
| 30 | | 21 | 40.6 ± 0.9 |

[1] Added at expense of entire diet.

[2] On day immediately prior to taking blood sample.

[3] Blood urea nitrogen. Blood was withdrawn from "spaced-fed" rats two hours after eating.

[4] Standard deviation for three rats.

received it ad libitum. The level of urea in the blood of those fed 5% of urea two hours per day was measured two hours after eating. At this time it equalled that of rats fed 30% of urea ad libitum (table 2).

The study was next extended to other mildly toxic compounds known to depress growth when fed ad libitum. The growth-depressing effects of glycine were not greatly altered by spaced feeding (table 3); those of L-leucine, diammonium citrate, ammonium carbonate, and 2,4-dinitrotoluene were increased, whereas those of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and ethanol were substantially lessened

TABLE 3

*Effect of spaced feeding on the toxicity of various compounds*

| DIETARY ADDITION | 5-WEEK GAIN WITH STANDARD ERROR | | | | PER CENT DEPRESSION [1] | | | |
|---|---|---|---|---|---|---|---|---|
| | Ad libitum | | Spaced | | Ad libitum | | Spaced | |
| | Series A | Series B | Series A | Series B | Series A | Series B | Series A | Series B |
| | *gm* | *gm* | *gm* | *gm* | *gm* | *gm* | *gm* | *gm* |
| None | 169 ± 2 | 177 ± 8 | 72 ± 9 | 74 ± 9 | 0 | | 0 | |
| Glycine [2] | 75 ± 14 | | 46 ± 6 | | 55 | | 36 | |
| 2.5% L-Leucine | 134 ± 7 | 144 ± 10 | 19 ± 5 | 38 ± 5 | 21 | 19 | 74 | 49 |
| 10% Diammonium citrate | 123 ± 4 | | 32 ± 4 | | 27 | | 56 | |
| 5% Ammonium carbonate | 124 ± 20 | 171 ± 6 | 12 ± 12 | 37 ± 13 | 27 | 3 | 83 | 50 |
| 20% Ethanol in drinking water | 131 ± 5 | | 68 ± 9 | | 22 | | 6 | |
| 0.05% 3'-Methyl-4-dimethylaminoazobenzene | | 74 ± 8 (4/5)[3] | | 48 ± 5 | | 58 | | 35 |
| 0.3% 2,4-Dinitrotoluene | | 56 ± 8 | | 12 ± 9 (3/5)[3] | | 68 | | 84 |
| 10 ppm Selenium [4] | | ... (0/5)[3] | | 8 ± 4 | | (96)[5] | | 89 |

[1] $\frac{\text{Gm depression from controls}}{\text{Gm gain of controls}} \times 100.$

[2] Eight per cent of ad libitum ration; 5% of spaced; all compounds added at expense of the entire diet.

[3] Figures in parentheses indicate survival when less than 100%.

[4] Added as $Na_2SeO_3$.

[5] Reflects gain at three weeks, when all rats were alive.

(table 3). Results with 10 ppm of selenium were inconclusive, since the rats fed ad libitum were younger (wealings) than those that had been trained for spaced feeding. With 3′-Me-DAB, growth depression was less in the spaced-fed group than in that fed ad libitum, even though the latter consumed less food per rat per day and hence less carcinogen than its spaced-fed counterpart. In another series the omission of biotin, vitamin $B_{12}$ and folic acid from the vitamin mixture did not alter growth on either regime.

Blood studies carried out on rats fed the two ammonium salts revealed no alterations in the level of ammonia, but indicated marked changes in the blood urea of the spaced-fed rats (table 4). In series A the blood urea of rats fed ammonium carbonate for two hours per day was increased by 25.4 mg %; that of those fed diammonium citrate, by 16.5 mg %. The ratio of these elevations approximates that of the growth depressions shown by these two groups, 83 vs. 56% (table 3). In series B, ammonium carbonate depressed growth approximately as much as did diammonium citrate in series A (table 3), and the levels of blood urea were also very similar (25.9, 26.4 mg %, table 4).

Since spaced feeding exerted its greatest influence on the growth of animals fed nitrogenous compounds, it was hoped that this technique might reveal sparing effects of antibiotics on proteins that are not readily demonstrated in orthodox feeding experiments. The effect of spaced feeding on antibiotic-protein interrelationships was therefore tested in the following combinations: sulfasuxidine or streptomycin at 12% of casein; aureomycin, penicillin, or chloramphenicol at 9, 12 or 18% of casein. In 15 groups fed ad libitum, only one suggestion of a significant growth stimulation was noted viz., in the case of aureomycin fed with 12% of casein; in the series in which the feeding was spaced, however, this stimulation was not seen, nor did the latter technique enhance the effects of antibiotics fed in other combinations.

The spaced-feeding technique was then used to determine biological values of proteins. Rats were fed the basal diet

TABLE 4

*Blood urea and ammonia levels after eating ammonium salts* [1]

| DIETARY ADDITION | BLOOD AMMONIA NITROGEN | | | | BLOOD UREA NITROGEN | | | |
|---|---|---|---|---|---|---|---|---|
| | Ad libitum | | Spaced | | Ad libitum | | Spaced | |
| | Series A | Series B | Series A | Series B | Series A | Series B | Series A | Series B |
| | *mg %* | *mg %* | *mg %* | *mg %* | *mg %* | *mg %* | *mg %* | *mg %* |
| None | 2.2 ± 0.2 [2] | 1.9 ± 0.1 | < 2.4 [3] | 2.0 ± 0.2 | 9.6 ± 1.8 | 9.3 ± 0.8 | 9.9 ± 1.9 | 7.3 ± 1.9 |
| 5% Ammonium carbonate | 2.0 ± 0.1 | 2.0 ± 0.3 | < 2.4 | 1.9 ± 0.1 | 10.6 ± 1.9 | 7.8 ± 1.5 | 35.3 ± 4.1 | 25.9 ± 3.8 |
| 10% Diammonium citrate | 2.8 ± 0.6 | | < 2.4 | | 9.1 ± 2.9 | | 26.4 ± 3.5 | |

[1] Blood withdrawn from "spaced-fed" rats two hours after eating.

[2] Standard deviation.

[3] Samples available were too small for precise determinations.

(without added cystine) plus 38% of casein, lactalbumin [5] or α-protein [6] added at the expense of the carbohydrate. Biological values were calculated from the following formula:

$$\text{Biol. value}_P = 73 \times \frac{\text{2-wk. gain of group fed protein P} - \text{2-wk. gain of controls}}{\text{2-wk. gain of group fed 50\% casein} - \text{2-wk. gain of controls}}.$$

Values determined in this manner were lactalbumin 84, casein 73, and α-protein 56, as compared with 84, 73, and 59 (biological value of raw soybeans) reported by Mitchell ('48).

## DISCUSSION

The merit of spaced feeding as a nutritional technique lies in the increased sensitivity of rats on this regime to dietary additions of certain nitrogenous substances. Outstanding among these is urea, the potential growth-retarding effect of which deserves attention in view of its wide use in ruminant nutrition. Urea, though often regarded as a "neutral and non-toxic substance" (Peters and Van Slyke, '46), can produce headaches and dizziness in humans (Zuntz, '10; Hewlett et al., '16), vomiting and intoxication in dogs (Marshall and Davis, '14) as well as all human uremic symptoms (Leiter, '21; Streicher, '28); and death in cows (Kling and Jürgens, '29). The mechanism of urea toxicity in lambs has been studied extensively by Repp et al. ('55) and Hale and King ('55) who regard the toxicity as due to carbamate formation. Fitzgerald and Murphy ('50) suggested that urea could be converted through ammonia to a compound of mild toxicity, while Harrison and Mason ('37) postulated that an excess of urea could facilitate the accumulation of other metabolic waste products. The present results appear analogous to an early observation of Kling and Jürgens ('29) that an amount of urea, harmless when mixed evenly with the daily feed, caused death when given in a single dose; and they provide quantitative support for the statement of Hew-

[5] Borden 15–42.

[6] Alpha protein of soybeans, Soya Products Division, Glidden Co., Chicago, Illinois.

lett et al. ('16) that to produce toxic effects, a "large quantity of urea must be taken within a brief period of time."

Toxicities of L-leucine and dinitrotoluene were also increased by spaced feeding. Growth retardation by leucine is apparently caused by an isoleucine antagonism (Harper et al., '55), but the reasons for an enhancement of this effect by spaced feeding are obscure. In rats fed dinitrotoluene, those which failed to survive had eaten relatively little, suggesting that the apparent increased toxicity may have been complicated by starvation, as previously observed in mice by Clayton and Baumann ('44; '48). The use of spaced feeding for determining the biological values of proteins appears to have several advantages despite the care necessary in training the animals. The protein under study need be fed for only two weeks which, on a two hour per day feeding regime, would require only a small amount of the test material.

## SUMMARY

Spaced feeding, the practice of feeding rats for only two hours per day, has been found to increase the growth-depressing action of several nitrogenous compounds. Dietary urea depressed growth in both spaced and orthodox experiments; 5% of urea fed two hours per day was as effective as 30% fed ad libitum. The depression in growth has been correlated with the rate of urea intake and the level of urea in the blood, and was not affected by the level or adequacy of the dietary protein.

Spaced feeding increased the toxicity of L-leucine, diammonium citrate, ammonium carbonate, and 2,4-dinitrotoluene. Growth depressions by the ammonium salts varied directly with blood urea. This regimen lessened the toxicity of 3'-methyl-4-dimethylaminoazobenzene and ethanol but had little effect on relative growth rates when biotin, vitamin $B_{12}$ and folic acid were omitted from the diet or when glycine or antibiotics were added. The procedure shows promise in measuring the biological value of small amounts of protein.

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# QUANTITATIVE RELATIONSHIPS OF TRYPTOPHAN AND NICOTINIC ACID IN THE BABY PIG [1]

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ONE FIGURE

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Many reports of a qualitative nature have succeeded that of Krehl et al. ('45), in which it was shown that tryptophan would promote growth in nicotinic acid-deficient rats (Woolley, '46; Krehl et al., '46a; Henderson et al., '47; Rosen et al., '46). Nicotinic acid in the diet has been shown to decrease the requirement of the rat for tryptophan (Krehl et al., '46b). Salmon ('54) has shown that the tryptophan requirement of the rat is enhanced with increases in dietary protein or by the omission of nicotinine acid. Studies with the chick have demonstrated an L-tryptophan requirement of 0.15% and a nicotinic acid requirement of 10 mg%. In the absence of nicotinic acid the L-tryptophan increased to 0.20% of the diet (Fisher, '54). The first study of the requirements of the pig for nicotinic acid (Hughes, '43), set the requirement at 5 to 10 mg/100 lbs. body weight or 0.11 to 0.22 mg/kg body weight per day. Subsequent work (Powick et al., '47), using younger pigs on high protein diets raised the estimated requirement to 0.6 to 1.0 mg/kg body weight per day. High protein diets and consequently high levels of tryptophan have been shown to completely satisfy the nicotinic acid requirement (Powick et al., '48; Cartwright et al., '48). Age and

[1] The data reported in this paper are taken from a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Nutrition.

weight appear to be important factors, as it is difficult to produce a nicotinic acid deficiency in pigs over 60 lbs. Similarly diets containing more than 25% of casein will prevent symptoms of the deficiency (Luecke et al., '48).

Tentative quantitative requirements of the weanling pig for DL-tryptophan have been established (Beeson et al., '49; Shelton et al., '51). The utilization of the D isomer of tryptophan has been investigated to a limited extent in the baby pig (Reber et al., '51; Thompson et al., '52), and was shown to be partially available for protein synthesis.

TABLE 1

*Composition of the basal diet*

| CONSTITUENT [1] | AMOUNT | VITAMINS | AMOUNT/ LITER |
|---|---|---|---|
| | % | | *mg* |
| Hydrolyzed casein | 24.6 | Thiamine HCl | 0.65 |
| Methionine | 0.4 | Riboflavin | 1.30 |
| Gelatin | 3.0 | Pyridoxine HCl | 1.30 |
| Cerelose | 30.09 | Calcium pantothenate | 7.80 |
| Lard | 30.77 | Choline chloride | 130.0 |
| Minerals | 11.14 | Folic acid | 0.052 |
| | | Biotin | 0.010 |
| | | Vitamin A | 2000 I.U. |
| | | 2 methyl-1,4-naphthoquinone | 0.26 |
| | | Vitamin D | 200 I.U. |
| | | Vitamin $B_{12}$ | [2] |

[1] These materials were made into a "milk" containing 13% solids according to a modification of method of Clark ('27) and the vitamins were added at the time of feeding.

[2] Weekly intramuscular injection at the rate of 0.8 mg/kg body weight/day.

Since many factors can affect the relationship of tryptophan and nicotinic acid it seemed advisable to ascertain quantitatively the L-tryptophan and DL-tryptophan requirement of the pig as affected by nicotininc acid using one diet with a constant level of protein.

The overall experimental plan was to determine by growth studies the L- and DL-tryptophan requirements in the presence of excess nicotinic acid, the nicotinic acid requirement in the presence of this minimum DL-tryptophan level and the

DL-tryptophan requirement when there was no nicotinic acid in the diet.

## EXPERIMENTAL

Two- to three-day-old baby pigs were obtained from a commercial source for use in these experiments. They were housed individually in wire bottom metal cages and fed ad libitum a hydrolyzed casein synthetic milk assayed to be nicotinic acid and tryptophan free. The composition of the basal diet is given in table 1. The feeding and care of the animals was similar to that described by Johnson et al. ('48). All experiments were of 4 weeks duration which was sufficient time for

TABLE 2

*Effect of 7 levels of* DL-*tryptophan on growth and feed efficiency in baby pigs*[1,2]

| ITEM | % DL-TRYPTOPHAN | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.1 | 0.125 | 0.15 | 0.175 | 0.20 | 0.25 | 0.30 |
| Av. initial wt., kg | 2.33 | 2.37 | 2.48 | 2.47 | 2.50 | 2.30 | 2.28 |
| Av. final wt., kg | 3.13 | 3.37 | 4.42 | 6.02 | 6.28 | 7.73 | 8.68 |
| Av. gain, kg | 0.80 | 1.00 | 1.93 | 3.55 | 3.78 | 5.42 | 6.40 |
| Feed consumed, kg | 3.61 | 3.47 | 4.60 | 5.83 | 6.13 | 7.69 | 8.21 |
| Feed/gain, kg | 4.51 | 3.47 | 2.38 | 1.65 | 1.62 | 1.42 | 1.28 |

[1] Nicotinic acid was added to the diet at 50 mg per kilogram of dry matter.
[2] Three pigs in each group.

wide differences to occur between treatments in all tests. Differences usually became apparent at 10 to 14 days and widened through the remainder of the test period.

The individual experiments are given in tables 1 through 7.

## RESULTS

### DL-*tryptophan requirement in presence of excess nicotinic acid*

*Experiment 1.* The data from experiment 1, as shown in table 2, indicated that the DL-tryptophan requirement in the presence of an excess of nicotinic acid was higher than had been anticipated.

Both gain and feed efficiency showed a nearly linear increase up to the 0.30% level of DL-tryptophan, indicating that the requirement was at 0.30% or above.

Marked differences in weight were evident as early as one week and increased during the subsequent three weeks. Pigs in the groups receiving less than 0.20% of DL-tryptophan were very rough in appearance and were obviously deficient. Differences between the 0.20%, 0.25% and 0.30% levels were reflected in the increased growth and food consumption.

*Experiment 2.* The results of experiment 2 are summarized in table 3. Growth and feed efficiency showed less variability in this experiment than in the preceding one, since the levels

TABLE 3

*Effect of 6 levels of* DL-*tryptophan on growth and feed efficiency of baby pigs* [1,2]

| ITEM | % DL-TRYPTOPHAN | | | | | |
|---|---|---|---|---|---|---|
| | 0.25 | 0.275 | 0.30 | 0.325 | 0.350 | 0.40 |
| Av. initial wt., kg | 2.00 | 1.86 | 1.92 | 1.79 | 1.77 | 1.80 |
| Av. final wt., kg | 7.68 | 7.98 | 8.37 | 6.10 | 8.05 | 8.34 |
| Av. gain, kg | 5.66 | 6.00 | 6.42 | 4.47 | 6.27 | 6.54 |
| Feed consumed, kg | 7.65 | 8.48 | 9.61 | 6.60 | 9.75 | 8.02 |
| Feed/gain, kg | 1.32 | 1.41 | 1.50 | 1.48 | 1.55 | 1.23 |

[1] Nicotinic acid was added to the diet at 50 mg per kilogram of dry matter.
[2] Four pigs in each group.

were near or above the requirement. The growth data of experiments 1 and 2 are plotted in figure 1 a. The slope of the line representing the lower levels of tryptophan supplementation is represented by the equation $Y = 28.9X - 1.98$ and was found to vary significantly from the horizontal. The horizontal line is described by the equation $Y = 0.93X + 6.09$ and had a slight slope which was not significant. Therefore the mean weight gain of the points used to determine this line was used to calculate the point of intersection of the lines, which may be regarded as an estimate of the requirement for DL-tryptophan when adequate nicotinic acid is present in the diet. The point of intersection as shown in figure 1 is at the 0.29% level of supplementation.

### *Nicotinic acid requirement in presence of required level of* DL-*tryptophan*

*Experiment 3.* Results of this experiment are summarized in table 4. Both growth and feed efficiency reached a maximum at the 17 mg/kg level. The gains (fig. 1 b) are best described by a straight line fitted through all of the points indicating that a plateau may not have been reached. However, the feed required per unit of gain, as shown in table 4, had reached a minimum at the 17 and 20 mg/kg levels.

In view of the lack of groups receiving higher levels of nicotinic acid supplementation the requirement has not been

TABLE 4

*Nicotinic acid requirement of the baby pig* [1,2]

| ITEM | NICOTINIC ACID (MG/KG DRY MATTER) | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 8 | 11 | 14 | 17 | 20 |
| Av. initial wt., kg | 1.79 | 1.85 | 1.83 | 1.81 | 1.80 | 1.75 |
| Av. final wt., kg | 4.00 | 4.62 | 4.05 | 5.38 | 6.09 | 5.58 |
| Av. gain, kg | 2.21 | 2.77 | 2.23 | 3.56 | 4.29 | 3.83 |
| Av. feed consumed, kg | 4.25 | 4.35 | 4.46 | 4.93 | 5.46 | 4.91 |
| Feed/gain, kg | 1.92 | 1.57 | 2.00 | 1.38 | 1.27 | 1.28 |

[1] DL-Tryptophan was added to the diet at 0.3%, the level indicated as required in experiments 1 and 2.

[2] Four pigs in each group.

conclusively demonstrated. However, on the basis of the feed required per unit of gain and the lack of increase in gain at the level of 20 mg/kg it seems probable that the requirement is not over 20 mg/kg of diet when the diet contains the minimum amount of tryptophan necessary for normal growth (0.3% of DL-tryptophan).

### L-*tryptophan requirement in the presence of excess nicotinic acid*

*Experiment 4.* Gains reached a plateau at the 0.2% L-tryptophan level, which represents the approximate requirement

of the baby pig for L-tryptophan (table 5). This was also the lowest level at which feed efficiency was at a maximum. As shown in figure 1c, the data may be represented by two straight lines which intersect at about the 0.20% level of L-tryptophan. A line representing the weight gains of the pigs on the two lower levels of supplementation is represented by the equation $Y = 66.8X - 8.15$ and has a slope which is significantly different from the horizontal. The line fitted to the data obtained on the 0.20%, 0.225% and 0.25% levels is described by the equation $Y = 8.33X + 2.37$ and has a slope which is not significant from zero. Therefore the mean of the

TABLE 5

*The* L-*tryptophan requirement of the baby pig* [1,2]

| ITEM | % DL-TRYPTOPHAN | | | | |
|---|---|---|---|---|---|
| | 0.15 | 0.175 | 0.20 | 0.225 | 0.25 |
| Av. initial wt., kg | 1.62 | 1.57 | 1.48 | 1.50 | 1.93 |
| Av. final wt., kg | 3.48 | 5.10 | 5.63 | 5.52 | 6.50 |
| Av. gain, kg | 1.87 | 3.53 | 4.15 | 4.00 | 4.57 |
| Av. feed consumed, kg | 3.93 | 4.61 | 5.27 | 5.05 | 5.97 |
| Feed/gain, kg | 2.10 | 1.31 | 1.27 | 1.26 | 1.31 |

[1] Nicotinic acid was added to the diet at 50 mg per kilogram of dry matter.
[2] Three pigs in each group.

weight gains used to calculate this line was used to determine the intersection of the two lines. This point of intersection was 0.19% L-tryptophan which may be considered as the approximate requirement.

### DL-*tryptophan requirement in the absence of nicotinic acid*

*Experiment 5.* Several factors complicated this experiment during its course of 4 weeks. The pigs learned to drink in one day but consumed very little "milk" the second day and appeared to be very dehydrated. Nicotinic acid was added to the diet at the next two feedings at the rate of 50 mg/kg of dry matter in the diet. Food consumption returned to normal and recovery was immediate. Each pig had

received approximately 1.3 mg of nicotinic acid. Subsequent supplementation with nicotinic acid was not necessary during the course of the experiment. Apparently the 2- to 3-day old pig is unable to convert tryptophan to nicotinic acid and has a very low reserve of this vitamin.

TABLE 6

*The* DL-*tryptophan requirement of the baby pig in the absence of nicotinic acid (Experiment 5)* [1]

| ITEM | % DL-TRYPTOPHAN | | | | |
|---|---|---|---|---|---|
| | 0.3 | 0.35 | 0.4 | 0.45 | 0.5 |
| No. pigs | 2 | 3 | 4 | 2 | 3 |
| Av. initial wt., kg | 1.25 | 1.33 | 1.39 | 1.35 | 1.30 |
| Av. final wt., kg | 4.05 | 4.39 | 5.35 | 4.70 | 4.00 |
| Av. gain, kg | 2.85 | 3.30 | 3.96 | 3.35 | 2.70 |
| Av. feed consumed, kg | 5.83 | 6.00 | 6.71 | 6.27 | 5.97 |
| Feed/gain, kg | 2.05 | 1.82 | 1.69 | 1.87 | 2.21 |

[1] A different source of hydrolyzed casein which was not entirely salt-free was used and is the cause of the lower gains in this experiment.

TABLE 7

*The* DL-*tryptophan requirement of the baby pig in the absence of nicotinic acid (Experiment 6)*

| ITEM | % DL-TRYPTOPHAN | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.3 + NA [1] | 0.3 | 0.35 | 0.4 | 0.45 | 0.5 | 0.6 |
| No. pigs | 3 | 1 | 3 | 3 | 3 | 3 | 2 |
| Av. initial wt., kg | 1.35 | 1.47 | 1.37 | 1.40 | 1.40 | 1.43 | 1.35 |
| Av. final wt., kg | 4.62 | 3.50 | 3.88 | 4.83 | 5.70 | 5.82 | 4.90 |
| Av. gain, kg | 3.27 | 2.03 | 2.51 | 3.43 | 4.30 | 4.39 | 3.55 |
| Av. feed consumed, kg | 4.63 | 3.36 | 4.19 | 4.75 | 5.25 | 5.23 | 4.93 |
| Feed/gain, kg | 1.42 | 1.65 | 1.67 | 1.38 | 1.22 | 1.19 | 1.39 |

[1] Group 1 pigs received in addition to 0.3% DL-tryptophan 50 mg of nicotinic acid per kilogram of dry matter in the diet.

Growth was erratic and suboptimum throughout the experiment due to the excess salt in the hydrolyzed casein. The pigs scoured continually and 6 died during the 4 weeks. The results are summarized in table 6.

It is evident from table 6 that growth was below normal and that feed efficiencies were suboptimal. However, these

two criteria did indicate a maximum at the 0.4% level of tryptophan and were used to locate the proper range of tryptophan supplementation for a repetition of this experiment.

*Experiment 6.* This experiment was terminated at the end of 4 weeks. However, only three-week data are used in the evaluation. The pigs went off feed and scoured during the 4th week and growth was depressed. Results at three weeks are summarized in table 7.

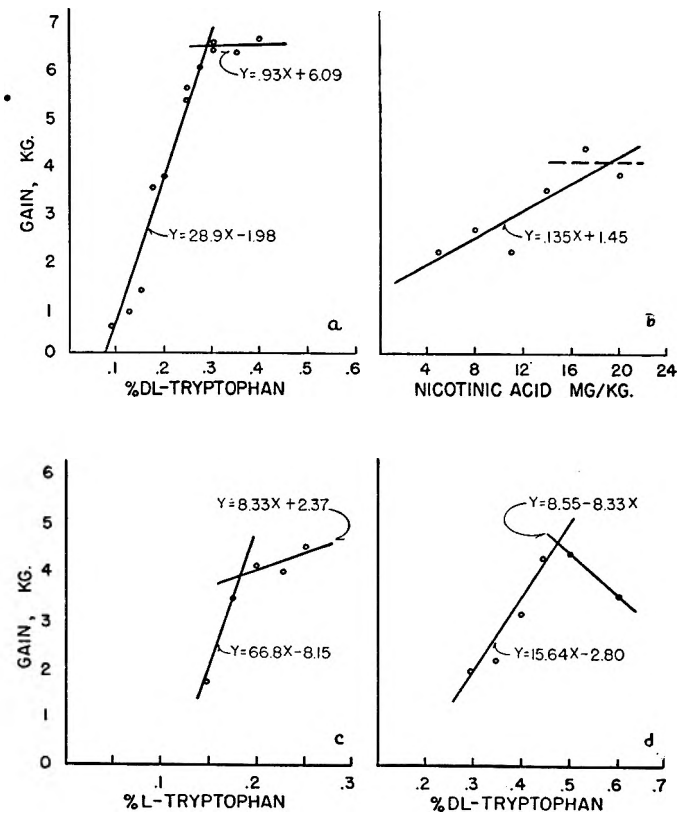


Figure 1

a Average gains of pigs in experiments 1 and 2.
b Average gains of pigs in experiment 3. The dotted line indicates the mean gains of the pigs at the 17 and 20 mg levels.
c Average gains of pigs in experiment 4.
d Average gains of pigs in experiment 6.

The average gains were linear to the 0.45% level of tryptophan, at which they reached a plateau. The level of 0.6% appears to be approaching toxicity and growth was depressed somewhat in this group. The linearity of gains up to the 0.45% level of supplementations would seem to imply that this point should be taken as the requirement for DL-tryptophan in the absence of nicotinic acid. Failure of the control group receiving 0.3% of DL-tryptophan and 50 mg of nicotinic acid per kilogram of diet to equal the gains of the 0.45% tryptophan group is unexplainable.

The weight gains are plotted in figure 1 d. It is obvious that more data are needed between 0.5% and 0.6% levels of supplementation in order to establish a plateau. A plateau cannot be obtained from the data of this experiment since the 0.3%, 0.35%, 0.40% and 0.45% levels of supplementation must be used to obtain a significant positive slope. A line fitted to the data of the remaining 0.5% and 0.6% levels has a significant negative slope and therefore its intersection at 0.47% with the positive slope does not necessarily designate the point at which the response is maximum. However, it does give an estimate of the requirement.

The feed required per unit gain as shown in table 7 is at a minimum at the 0.45 and 0.5% levels of DL-tryptophan. This together with the weight gains suggests that the requirement for DL-tryptophan in the absence of nicotinic acid is approximately 0.45% of the diet.

## DISCUSSION

The data indicate that the tryptophan requirement of the baby pig on the diet used in this work is approximately 0.2% of L-tryptophan or 0.3% of DL-tryptophan in the presence of excess nicotinic acid and thus that one-third of the D-tryptophan is utilizable by the pig for growth.

The data indicate that approximately 0.45% of DL-tryptophan (equal to 0.3% of L-tryptophan) is required to supply

the requirement of the animal for both tryptophan and nicotinic acid.

The diet used in these baby pig experiments was a liquid simulated milk ration containing approximately 30% fat on the dry basis, and this fact should be considered in comparing these data on tryptophan requirements to those obtained by others.[2]

In agreement with the finding of Fisher et al. ('55) for the chick it was found that for the baby pig the tryptophan requirement was markedly increased when nicotinic acid was omitted from the diet. This is in contrast to the results of Oesterling and Rose ('52) for the rat. The replaceability of nicotinic acid by tryptophan approximates 0.1% of L-tryptophan, equal to 20 mg of nicotinic acid per gram of diet, i.e. 100 mg of tryptophan equals 2 mg of nicotinic acid which is in agreement with the results of Harris and Kodicek ('50) and Woolley ('47) with the rat.

A surprising phase of the results was the very rapid development of a nicotinic acid deficiency in the new born pig, even in the presence of a 0.3% level of DL-tryptophan. The symptoms which developed in two to three days consisted of diarrhea, vomiting, dehydration and weakness. The animals responded within 24 hours to a dose of 1.3 mg of nicotinic acid and thereafter developed no symptoms of the deficiency on this same tryptophan level.

[2] If the requirements are recalculated to a 5% fat diet using the Atwater factors of 9,4,4 for the metabolizable energy of fat, carbohydrate and protein, then on this basis the L-tryptophan requirement becomes approximately 0.15% of the diet and the nicotinic acid requirement (assuming it is also related to the energy, and not to grams of food intake) becomes approximately 15 mg/kg dry matter of the diet. This value of 0.15% tryptophan, while slightly lower than the value of 0.2 proposed for the rat (Oesterling and Rose, '52) and the chick (Fisher et al., '55), is above the requirement of 0.115% for weanling pigs as given by Becker et al. ('55). When considered as a percentage of the protein the present work indicates a tryptophan requirement of 0.75% of the protein, which is in agreement with the data of Fisher et al. and Becker et al. Thus, their different tryptophan requirements are only reflections of the different total protein levels required because of differences in age and species of experimental animals.

SUMMARY

The relationship between the requirements of the baby pig for L-tryptophan, DL-tryptophan and nicotinic acid were studied using a hydrolyzed casein synthetic milk diet containing 28% of protein.

1. The requirement of the baby pig for DL-tryptophan is approximately 0.29% of the dry matter of the diet when an excess of nicotinic acid is present.

2. The requirement of the baby pig for L-tryptophan in the presence of an excess of nicotinic acid is 0.19% of the dry matter of the diet. The difference between the L and DL requirements implies that about $\frac{1}{3}$ of the unnatural isomer can be used for growth.

3. The nicotinic acid requirement for maximum growth is near 20 mg per kilogram of diet when 0.3% of DL-tryptophan is present in the diet.

4. The DL-tryptophan requirement is increased to approximately 0.45% of the diet when nicotinic acid is absent.

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# EFFECT OF GENISTIN ON GROWTH AND DEVELOPMENT OF THE MALE MOUSE [1,2]

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The discovery of the estrogenic activity of genistein, 4′,5,7 trihydroxyisoflavone (Bradbury and White, '51) isolated from subterranean clover focused attention on the distribution and physiological effects of estrogenic-like compounds in feeds. Carter et al. ('53) and Cheng et al. ('53) found that the estrogenic activity of soybean oil meal, as measured by the uterine weight of immature female mice, is due to the presence of genistin, the glucoside of genistein. Work in our laboratory (Carter et al., '55) also has shown that when genistin, isolated from soybean oil meal, was fed to mice at a level of 0.2% of the diet fewer litters were born.

The present study is concerned with the further exploration of some of the effects produced by genistin isolated from soybean oil meal. The first objective was to determine the effect of genistin on growth of the male mouse and on the testicular development as measured by testes weights and spermatogenesis. A second objective was to compare the

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[2] Supported in part by Tennessee Valley Authority, Knoxville, Tennessee.

effects of genistin with those of a known estrogen to aid in differentiating between estrogenic effects and non-estrogenic effects.

## EXPERIMENTAL

Diethylstilbestrol was used for the comparative study since it had been used as the standard of reference in previous experiments (Carter et al., '53). Several levels of genistin and of diethylstilbestrol were studied in order to describe the response curve of each substance.

TABLE 1

*Basal diet used in growth study*

| CONSTITUENTS | AMOUNT PER KILOGRAM OF DIET |
|---|---|
| | *gm.* |
| Casein (Vitamin test) | 180.0 |
| Starch (corn)[1] | 630.0 |
| Vegetable oil[2] | 100.0 |
| Mineral mix[3] | 40.0 |
| Cellulose[4] | 20.0 |
| Cod liver oil[5] | 10.0 |

[1] Added water-soluble vitamins and methionine replaced an equal weight of starch.

[2] Wesson Oil and Snowdrift Sales Co., New Orleans.

[3] Wesson's modification of the Osborne and Mendel salt mixture.

[4] Alphacel.

[5] U.S.P. cod liver oil (850 units A/gm, 85 units D/gm).

The design of the experiment was a randomized block having 10 treatments (4 levels of genistin, 5 levels of diethylstilbestrol and one control, table 2) and 10 replications. The levels of genistin and the first 4 levels of diethylstilbestrol selected for study were equilibrated on the basis of their relative estrogenic potency (9 mg genistin $\simeq$ 0.04 µg diethylstilbestrol) as calculated from data of a previous study (Carter et al., '53). One hundred male mice[3] approximately three weeks of age were stratified into 10 uniform weight

[3] Supplied by M. P. Bailey of the North Carolina Laboratory of Hygiene, Raleigh, North Carolina.

groups of 10 animals each and then each animal within a group was assigned at random to one of the 10 treatments until a complete replication had been formed. This process was repeated for each of the remaining weight groups.

The mice were housed individually in wire cages with screen floors.

The composition of the basal diet is given in table 1. Vitamins and methionine [4] were provided as reported by Carter et al. ('55). Each animal was fed its assigned daily dose of genistin [5] or stilbestrol premixed in 1 gm of the basal diet. After this was consumed, untreated basal diet was fed ad libitum. This procedure was repeated daily throughout the experimental period of 6 weeks.

Body weights were recorded weekly. At the termination of the experiment the mice were sacrificed; fresh weights were determined and histological studies were made on the testes, adrenals, spleen and kidneys.

## RESULTS

Ten mice died during the experiment; 4 were receiving the 4th level (highest) of genistin, two were receiving the third level of genistin and 4 were scattered, singly, among some of the other levels of the two test substances (table 2).

A regression analysis of the weight gains as the dependent variable and the logarithm of the dosage level as the independent variable indicated that there was a significant ($P \leq 0.01$) linear decrease in growth rate associated with increasing levels of genistin in the diet. In fact, on the average, the mice receiving the 4th level of genistin lost weight. On the other hand all mice receiving stilbestrol gained weight; only the group receiving the highest level of stilbestrol gained significantly less ($P \leq 0.01$) than the control group (table 2).

[4] Vitamins and methionine were contributed by Merck and Company.

[5] Genistin was isolated from commercial soybean meal as described by Carter et al. ('53). The soybean meal was contributed by members of the North Carolina Feed Manufacturers' Association.

Genistin also had a depressing effect on testes weight (table 2). The linear decrease in testes weight associated with the logarithm of the dose levels of genistin, moreover, remained significant when the data were adjusted by covariance to a mean body weight, indicating that genistin depressed testes weight directly. The depressing effect of stilbestrol on testes weight was manifested by the mice on the third level of stil-

TABLE 2

*Results of growth study with mice*

| DOSE | NO. DIED [1] | FINAL BODY WEIGHT | GAIN | MEAN WT. AT END OF EXPERIMENTAL PERIOD | | | |
|---|---|---|---|---|---|---|---|
| | | | | Testes | Adrenal | Kidney | Spleen |
| | | *gm* | *gm* | *mg* | *mg* | *mg* | *mg* |
| Control | | | | | | | |
| 0 | 0 | 24.8 | 13.5 | 175.4 | 4.7 | 386.3 | 96.5 |
| Genistin, mg | | | | | | | |
| 9 | 0 | 20.5 | 9.3 | 163.4 | 5.3 | 324.9 | 90.7 |
| 18 | 1 | 15.6 | 4.3 | 140.4 | 5.6 | 231.9 | 80.2 |
| 36 | 2 | 12.0 | 0.5 | 57.8 | 4.4 | 192.8 | 49.3 |
| 72 | 4 | 10.0 | — 1.5 | 16.4 | 4.5 | 192.9 | 36.0 |
| Diethylstilbestrol, $\mu$g | | | | | | | |
| .04 | 0 | 24.1 | 12.7 | 173.5 | 5.4 | 360.7 | 93.8 |
| .08 | 1 | 24.2 | 12.7 | 173.8 | 5.0 | 389.1 | 85.7 |
| .16 | 1 | 24.3 | 13.1 | 134.6 | 5.0 | 396.7 | 100.6 |
| .32 | 0 | 22.6 | 12.6 | 146.8 | 5.6 | 359.1 | 49.3 |
| .64 | 1 | 21.2 | 9.7 | 122.8 | 5.5 | 334.5 | 36.0 |
| L.S.D. | 0.05 [2] | 2.3 | 2.6 | 32.7 | 0.8 | 52.5 | 58.2 |
| L.S.D. | 0.01 | 3.6 | 3.4 | 43.5 | 1.1 | 69.7 | 77.3 |

[1] Died before completion of experiment.

[2] Least significant difference at specified probability levels.

bestrol although the body weight of this group of animals was not significantly different from that of the controls. The histological examination of the testicular tissue showed that no spermatozoa were present in the testes of the groups receiving the two higher levels of genistin. Spermatozoa, though present in the testes of the group receiving the highest level of diethylstilbestrol, were fewer in number than in testes of control animals.

As is shown in table 2, there was an increase in adrenal weights associated with the first two levels of genistin, followed by a drop in adrenal weights in animals receiving the two highest levels of genistin. All the groups of mice receiving stilbestrol showed an increase in adrenal weight as compared to the control group. There was only a slight correlation between body weight and adrenal weight ($r = +0.027$).

The differences observed in kidney weights could be explained by differences in body weight. The correlation between body weight and kidney weight was $r = +0.769$. Necrotic areas were observed on the kidneys of animals receiving the three higher levels of genistin.

The spleen weights, table 2, were quite variable. There were no significant differences in weight of the organ that could be attributed to differences in treatment of the animal.

## DISCUSSION

The evidence presented indicates that genistin at certain dose levels has a detrimental effect on survival, growth rate and spermatogenesis in mice. Undoubtedly the effects observed could be partially explained by the probable coincident decrease in food intake but it is unlikely that taste *per se* was a significant factor, because of the feeding procedure used. It appears, therefore, that the results obtained were due, in part at least, to an effect of genistin other than nutrient intake.

One of the questions remaining is whether or not the effects of genistin are associated with its estrogenic properties. The depressing effects of exogenous estrogens on growth and testicular development are considered to be mediated via the pituitary. All the naturally-occurring estrogens and stilbestrol apparently decrease the output of gonadotrophin and growth hormone of the pituitary (Richards and Kueter, '41; and Emmens and Parks, '47).

A comparison of the results obtained on growth and testicular development indicate that the physiological action of genistin is different from that of stilbestrol. As is shown in

table 2, a significant ($P \leq 0.01$) effect of genistin on growth is manifested at a lower dose (9 mg/day) than it is on testes weight (18 mg/day), whereas the effect of stilbestrol is in the reverse order (0.64 μg to depress growth and 0.16 μg to depress testes weight). Furthermore, if a comparison is made between the group of mice on the highest dose level of stilbestrol (0.64 μg/day) and the group on the lowest dose level of genistin (9 mg/day), two groups which are comparable in body weight, it is observed that the testes weight of the genistin-treated mice is significantly greater ($P \leq 0.05$) than that of those of the stilbestrol-treated group. The kidney weights of these two groups, however, are not significantly different. These data indicate, therefore, that the action of genistin on testes weight is different from that of stilbestrol.

Morrell and Hart ('41) reported that immature female rats continued to grow, although at a lower rate than controls, despite the administration of relatively enormous doses of stilbestrol (1.07 mg/day). These authors also reported there was no direct quantitative relationship between size of dose and decrease in growth. Richards and Kueter ('41) reported that immature male and female rats administered 2 mg daily of stilbestrol continued to grow for approximately two weeks before growth was arrested. Deaths attributable to massive doses of stilbestrol were not reported in either of the two papers mentioned. In contrast to these results reported in the literature and to those obtained with stilbestrol in this study, a direct relationship was found between the dose level of genistin and the retardation in growth. Moreover, the higher dose level of genistin appeared to be lethal. It appears from these results, therefore, that genistin, in relation to its estrogenic activity, has a greater depressing effect on growth than does stilbestrol.

The differences in the results between genistin and stilbestrol on growth and testicular development indicate that the effects obtained with genistin in this study may be primarily non-estrogenic.

SUMMARY

When genistin was fed at levels of 9, 18, 36 and 72 mg per day per mouse, the following results were obtained:

1. An inverse linear relationship was found between the logarithm of dose levels of genistin and growth rate.

2. Genistin appeared to have a depressing effect on testes weight beyond that attributable to differences in body weight.

3. No spermatozoa were present in testes of the mice given the two higher levels of genistin.

The effects of genistin on growth and testicular development differed, both qualitatively and quantitatively from those of stilbestrol.

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# FOOD INTAKE AND UTILIZATION OF LYSINE-DEFICIENT PROTEIN BY THE CHICK IN RELATION TO THE DIGESTIBLE ENERGY CONCENTRATION OF THE DIET [1]

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THREE FIGURES

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An inverse relation between food intake and the digestible energy concentration of the food is quite well established with diets adequate in all know essentials except energy (Adolph, '47; Dansky, '52; Strominger, Brobeck and Cort, '53; Hill and Dansky, '54; Peterson, Grau and Peek, '54). It is also generally accepted that the regulation of food intake is in some way related to energy needs although the pathways by which a need for energy is translated into an impulse to eat have not been established. However, the relation of energy needs to the ad libitum intake of diets deficient in factors other than energy is less clear (Carpenter, '53). With diets moderately deficient in amino acids, the poor growth and food consumption have been variously attributed to (1) a refusal to eat (an instinctive ability to recognize the deficiency), (2) a feeling of ill-being provoked by the deficiency,

[1] The data in this paper are taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, 1954.

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(3) a response to blood and tissue levels of amino acids, and (4) a decrease in dietary palatability.

Whatever the roles of palatability and the power of the animal to recognize an amino acid-deficient diet, evidence exists that the intake of a diet moderately deficient in an amino acid is related to body size and energy needs. Harte, Travers and Sarich ('48) found no significant differences in energy intake per unit of body surface among rats fed isocaloric diets differing in protein quality although the growth rates varied widely. A similar conclusion was reached by Hegsted and Haffenreffer ('49).

Peterson, Grau and Peek ('54) observed that the substitution of cellulose for glucose in low protein diets resulted in improved growth but maximal gains could not be obtained with these low protein levels at any level of cellu flour. The improved growth was attributed to the greater protein intake resulting from a general increase in food intake in response to the lowered digestible energy concentration. The protein used in this study was a high quality fish meal which was adequate as a sole source of dietary amino acids.

The previous observations suggested a similar study with a diet slightly deficient in total protein in which the primary limitation to growth was an amino acid deficiency. If the intake of such a diet increased as a result of a decrease in digestible energy concentration, then the increased amino acid intake resulting from the increase in food intake might overcome a moderate amino acid deficiency. Such a compensation would be possible because increases in essential amino acid requirements at higher protein levels are not proportional to the increases in protein level itself although the requirements for essential amino acids do increase at higher protein levels (Grau, '48; Grau and Kamei, '50; Almquist, '52).

The amino acid-deficient diet was based upon sesame seed oil meal as the protein source. This meal supplies all amino acids except lysine in amounts adequate for chick growth. The protein level in all diets was 15%, and variations in protein quality were produced through the addition of dif-

ferent levels of crystalline L-lysine hydrochloride. Grau ('48) found that 0.68% of L-lysine permitted the maximum growth of chicks fed a diet containing 15% of sesame seed oil meal protein. In the present experiments, sesame seed oil meal at a level of 15% of crude protein was estimated to provide 0.42% of L-lysine (Block and Bolling, '51). Thus, lysine limited growth until the total level reached 0.68%.

## METHODS

Single-Comb White Leghorn chicks of both sexes were maintained on a stock laboratory diet in thermostatically controlled, electrically heated battery brooders until two weeks of age. At that time they were uniformly distributed by weight into

TABLE 1

*Composition of the basal 15% sesame-protein diet*

| INGREDIENT | | INGREDIENT | |
|---|---|---|---|
| | % | | % |
| Sesame seed oil meal [1] (45.51% or 46.0% crude protein) | 32.95 or 32.61 | Folic acid [2] | 0.0002 |
| | | Sucrose [4] | 1.00 |
| Crude soybean oil | 2.00 | Calcium carbonate | 1.75 |
| Choline chloride | 0.20 | Tricalcium phosphate | 1.8 |
| Fortified fish oil (2250 A — 300 D/gm) | 0.25 | Monosodium phosphate | 1.3 |
| | | Potassium chloride | 0.6 |
| | | Sodium chloride | 0.48 |
| Natural mixed tocopherols [5] | 0.05 | Magnesium sulfate | 0.24 |
| 2-methyl-1,4-naphthohydroquinone diacetate | 0.001 | Sodium silicate | 0.11 |
| | | Manganese sulfate | 0.015 |
| Thiamine hydrochloride | 0.0005 | Aluminum sulfate | 0.003 |
| Riboflavin | 0.0005 | Ferric citrate | 0.003 |
| Pyridoxine hydrochloride | 0.0005 | Cupric sulfate | 0.0013 |
| Nicotinic acid | 0.0015 | Zinc sulfate | 0.0013 |
| Calcium (d) pantothenate | 0.0015 | Cobalt acetate | 0.00006 |
| Biotin | 0.00001 | Vitamin $B_{12}$ [3] | 5 μg/kg |
| | | Glucose [6] | to 100% |

[1] Kindly provided by the Pacific Vegetable Oil Corporation, San Francisco, California.

[2] Kindly provided by the Lederle Laboratories, Inc., Pearl River, New York.

[3] Kindly provided by Merck and Co., Rahway, New Jersey.

[4] Sucrose was used as a carrier for water-soluble vitamins.

[5] Type IV-34 (340 mg/gm), Distillation Products, Inc., Rochester, New York.

[6] Cerelose.

groups of 10 each. Body weights and ad libitum food intakes were measured every other day for 18 days. Two similar experiments were carried out. The composition of the basal diet is given in table 1. Cellu flour [3] and L-lysine hydrochloride were added at the expense of an equal weight of glucose (wet weight).

In the discussion of the energy concentration of the diets, the concept of metabolizable energy is used although reference is made in the text to the "decrease in the digestible energy concentration of the diet" caused by the introduction of cellu flour. By lowering the digestible energy concentration, the substitution of cellu flour for glucose must also have decreased the metabolizable energy concentration. The values chosen for the calculation of metabolizable energy were 9.0 Kcal. per gram of fat, 3.8 Kcal. per gram of glucose, 4.0 Kcal. per gram of nitrogen-free extract, and 4.0 Kcal. per gram of crude protein. Cellu flour was considered to be completely indigestible.

## RESULTS

*Growth.* The effects on growth of varying levels of lysine and cellu flour are presented in figure 1.[4] In addition to the groups plotted, one lot was fed the basal ration without the addition of either lysine or cellu flour. By the 5th day, all of the latter chicks had died, primarily as a result of cannibalism. When 12% of cellu flour was added to the basal diet, 9 of 10 chicks survived although the rate of growth was poor. There was a further improvement in growth without mortality when the level of cellu flour was increased from 12% to 24%.

Increases in the lysine content of the diet resulted in more rapid growth up to 0.72% of lysine, and the presence of 12% of cellu flour caused an additional improvement in growth at each level of lysine through 0.62%. Above this level of

[3] Chicago Dietetic Supply House, Chicago, Illinois.

[4] The growth rate was calculated as the average gain per day during the experiment, divided by the average of the initial and final body weights. This calculation is useful because it allows comparison between experiments independent of small variations in body weight.

lysine, the introduction of cellu flour caused little improvement in growth although in experiment 1, growth at 0.72% of lysine was significantly greater in the presence of 12% of cellu flour.

*Food, protein and lysine intakes.* The addition of cellu flour to the diets always resulted in an increased food intake at all levels of lysine (fig. 2), and the differences in food intake in response to the variations in energy level were

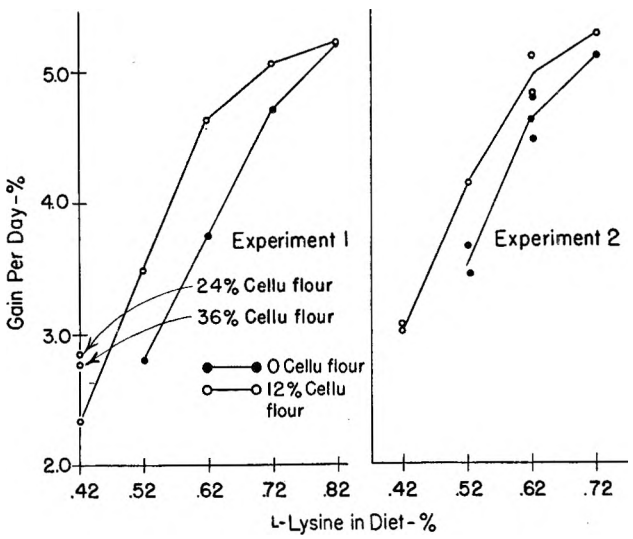


Fig. 1 Growth in relation to dietary levels of lysine and cellu flour.

apparent at the end of the second day. One-day food intakes were not measured.

In the presence of 12% of cellu flour, the food intake at a given level of lysine equalled or exceeded the intake at the next higher level of lysine in the absence of cellu flour. As food intake increased, the intake of protein and lysine likewise increased, and at any given level of lysine, the greater lysine intake occurred with the diet containing cellu flour (fig. 3).

*Energy intake.* The average estimated metabolizable energy intakes for the 18-day period are given in table 2. Until the level of lysine reached 0.82% in experiment 1 and 0.72%

in experiment 2, the energy intakes of the chicks fed the diet containing cellu flour equalled or exceeded the energy intakes from the diet without cellu flour.

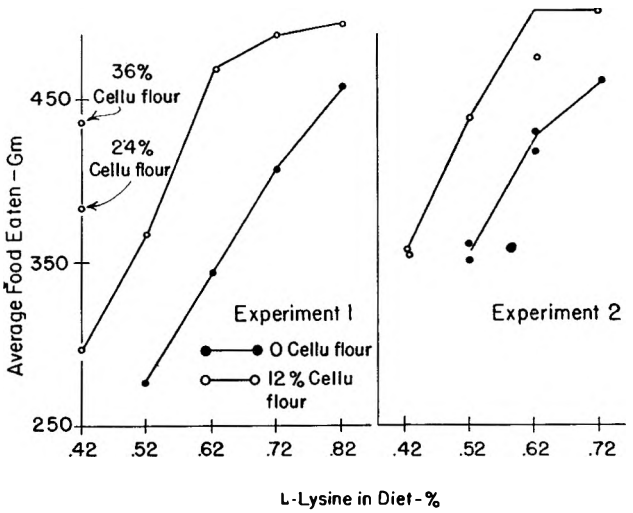


Fig. 2 Food intake in relation to dietary levels of lysine and cellu flour.

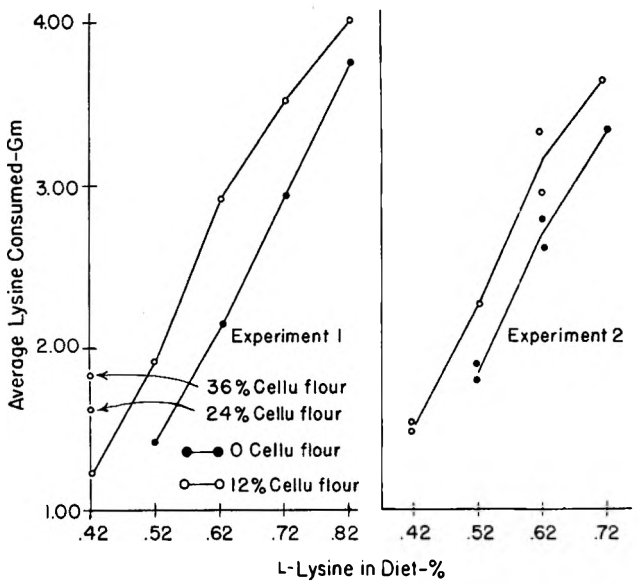


Fig. 3 Lysine intake in relation to dietary levels of lysine and cellu flour.

At 0.42% of lysine, an increase in the level of cellu flour from 12 to 24% resulted in a greater intake of metabolizable energy, but the energy intake decreased when the level of cellu flour was raised from 24 to 36%.

TABLE 2

*Feed efficiency and the efficiency of energy utilization in relation to the dietary levels of lysine and cellu flour*

| LYSINE | CELLU FLOUR | AVERAGE FINAL BODY WEIGHT | FEED EFFICIENCY | GM GAIN / Kcal. CONSUMED |
|---|---|---|---|---|
| % | % | *gm* | $\frac{gm\ gain}{gm\ feed}$ | |
| | | *Experiment 1* | | |
| 0.42 | 12 | 152 | 0.181 | 0.063 |
| 0.42 | 24 | 169 | 0.180 | 0.073 |
| 0.42 | 36 | 168 | 0.156 | 0.076 |
| 0.52 | 0 | 169 | 0.248 | 0.075 |
| 0.52 | 12 | 193 | 0.252 | 0.088 |
| 0.62 | 0 | 202 | 0.298 | 0.091 |
| 0.62 | 12 | 245 | 0.308 | 0.107 |
| 0.72 | 0 | 246 | 0.361 | 0.110 |
| 0.72 | 12 | 268 | 0.345 | 0.120 |
| 0.82 | 0 | 272 | 0.381 | 0.116 |
| 0.82 | 12 | 274 | 0.353 | 0.122 |
| | | *Experiment 2* | | |
| 0.42 | 12 | 173, 175 | 0.214, 0.219 | 0.075, 0.073 |
| 0.52 | 0 | 186, 196 | 0.252, 0.271 | 0.077, 0.083 |
| 0.52 | 12 | 217 | 0.272 | 0.095 |
| 0.62 | 0 | 234, 250 | 0.318, 0.338 | 0.097, 0.103 |
| 0.62 | 12 | 249, 266 | 0.317, 0.312 | 0.111, 0.109 |
| 0.72 | 0 | 271 | 0.377 | 0.115 |
| 0.72 | 12 | 277 | 0.354 | 0.124 |

*Feed efficiency and energy utilization.* Feed efficiency (grams gain per gram of food eaten) was increased by the introduction of 12% of cellu flour at the 0.52% lysine level (table 2), but was decreased at higher lysine levels (except at 0.62% lysine in experiment 1).[5] At all levels of lysine, how-

[5] The improvement in growth caused by the introduction of 12% of cellu flour at 0.62% of lysine was unusually great in experiment 1. This great an improvement in growth did not occur in experiment 2 or in other experiments not reported here.

ever, the reduction in the concentration of digestible carbohydrate through the substitution of cellu flour for glucose apparently produced a more efficient utilization of metabolizable food energy for weight gain. The increase was small, but was observed in every instance.

## DISCUSSION AND INTERPRETATION

*Growth.* The improved growth that resulted from the addition of cellu flour to diets moderately deficient in lysine could be explained by any one of the following hypotheses or by a combination of them:

1. Cellu flour could increase growth by altering the microbial population of the intestine.
2. Decomposition products of cellu flour might be growth stimulants, as postulated by Davis and Briggs ('47) who found later ('48a) that small amounts of levulinic acid and furfural gave significant growth responses. Peterson, Grau and Peek ('54) were unable to confirm these results with levulinic acid.
3. Cellu flour might improve digestion and absorption of food by providing a better surface for the action of digestive enzymes; in this manner, the availability of lysine and other nutrients might be improved.
4. Cellu flour might improve growth by increasing food and lysine intakes in response to the decrease in digestible energy per gram of food.

Although the effects of cellu flour suggested in the first three hypotheses may be responsible for the improved growth, the experimental results are most readily interpreted by the last hypothesis. The chicks fed the diets containing cellu flour ingested more food, hence more protein and lysine, in meeting their energy needs. With increased food intake, more lysine was available for protein synthesis, and more rapid growth was possible. It should be mentioned that the first three hypotheses, although less plausible, are not definitely eliminated by the data in this of the following paper.

A comparison of the basal, the 12%, and the 24% cellu flour diets at the 0.42% level of lysine illustrates the relation of lysine intake to growth rate (figs. 1 and 3). In the absence of cellu flour, mortality was 100%. When 12% of cellu flour was present, mortality was negligible, and the increased lysine intake permitted a slow rate of growth. With 24% of cellu flour, lysine intake again increased, and growth improved even more. The increased intake of the lysine-deficient diets indicates that (1) the chicks ate primarily to meet energy needs, (2) dietary palatability was not affected by a moderate lysine deficiency or by the introduction of cellu flour, and (3) the chicks did not instinctively recognize a lysine-deficient protein.

The introduction of 36% of cellu flour resulted in the greatest intake of lysine at the 0.42% level (fig. 3), but in this case, growth appeared limited by the bulk of the diet. Since food energy was always limiting, the additional protein and lysine could not be used for protein synthesis, but were metabolized to provide energy.

At 0.52 and 0.62% of the diet, lysine still restricted growth. Grau ('48) estimated that 0.68% of lysine is required in the diet for maximum growth with a level of 15% of protein. Until this level of lysine was reached, improved growth would be expected from an increase in lysine intake produced either by an increase in the lysine level or by increased food intake. The results verified this expectation: maximum growth occurred at 0.72% of lysine in the second experiment although, in the first experiment, the presence of 12% of cellu flour at 0.72% of lysine significantly improved growth.

The gains with the 15% sesame-protein diet supplemented with 0.3 and 0.4% of L-lysine equalled the gains observed when this type of semipurified diet contained 20% of fish meal protein. The occurrence of maximum gains at a level of 15% of protein without the addition of cellu flour differs from previous results from this laboratory in which maximum gains occurred with a 16% fish meal protein diet only after the inclusion of 12 and 24% of cellu flour (Peterson et al., '54).

These contradictory results probably reflect a lower digestibility of the sesame-protein diet, which contained 1.9% of crude fiber and 7.7% of nitrogen-free extract due to the presence of the sesame seed oil meal. Although the nitrogen-free extract was considered as digestible carbohydrate in the calculation of metabolizable energy, only a small fraction appears to have been digested by the chick (Fraps, '46). In compensation for the lower digestible energy concentration of the diet, the chicks ingested sufficient protein to make maximum gains without an additional reduction in digestible energy concentration through the introduction of cellu flour.

*Feed efficiency.* The slight increase in feed efficiency caused by the introduction of 12% of cellu flour at the 0.52% level of lysine indicated merely that body size increased more rapidly in response to the increased lysine intake than did feed intake itself. The decrease in feed efficiency caused by the presence of 12% of cellu flour in diets adequate or slightly deficient in lysine agrees with previous results (Davis and Briggs, '48b; Peterson et al., '54) and shows that gain/feed ratio is not a satisfactory measure of feed utilization with diets moderately high in cellulose.

*Energy utilization.* The most unusual observation is the apparent increase in efficiency of energy utilization for weight gain which resulted from the substitution of cellu flour for glucose at all levels of lysine. Cellu flour could not have been completely indigestible, but it is unlikely that more than 50% of the cellu flour eaten would have been digested. With a similar type of semipurified diet, Davis and Briggs ('47) reported crude fiber digestibilities of 53.9, 27.9 and 34.3%, respectively, for cellulose fed at levels of 5, 10 and 15%. Even if 27.9 or 34.3% of the energy of cellu flour were digestible food energy, the calculated efficiency of the 12% cellu flour diets would still be slightly superior.

At inadequate levels of lysine, the superior efficiency might reflect an improved nutrient balance due to the greater lysine intake. However, at 0.82% of lysine in experiment 1 and 0.72% of lysine in experiment 2, the presence of cellu flour

resulted in an equal gain with an appreciably smaller energy intake. Although the equal weight gains might represent equal energy gains, it is more likely that the equal weight gains represent smaller energy gains. This possibility was suggested by the limited studies of body composition done by Peterson et al. ('54) but their data were insufficient to allow a definite conclusion. The results of Dansky ('52) and Hill and Dansky ('54), which became available during the present study, also suggested that such equal weight gains denote unequal energy gains. These authors found that the substitution of oat hulls for corn in a natural feedstuff ration resulted in weight gains equal to or greater than the gains allowed by the basal diet, but the estimated productive energy intake and the percentage of body fat decreased as the level of oat hulls increased from 10 to 40%.

## SUMMARY

Semipurified diets containing 15% sesame protein and various levels of lysine were fed ad libitum to two-week old chicks for a period of 18 days. The substitution of 12% or more of cellu flour (wood-pulp cellulose) for an equal weight of glucose resulted in improved growth with all of the diets deficient in lysine. The improved growth was most readily attributed to the increased feed and lysine intake in response to the decrease in the digestible energy concentration of the diets caused by the substitution of cellu flour for glucose. Feed intake varied inversely with the digestible energy concentration of the diet, and energy intake appeared related to energy needs. At adequate dietary levels of lysine, the introduction of 12% of cellu flour had little effect on growth, but at all lysine levels, the presence of 12% of cellu flour reduced the estimated metabolizable feed energy required per unit of weight gain.

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# ENERGY INTAKE AND BODY COMPOSITION OF THE CHICK IN RELATION TO THE DIETARY CONCENTRATIONS OF DIGESTIBLE CARBOHYDRATE AND DIGESTIBLE FOOD ENERGY [1]

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TWO FIGURES

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Improvement in the efficiency of utilization of metabolizable food energy for weight gain by chicks was observed when moderate reductions were made in the digestible energy concentration of semipurified diets containing 15% of sesame-seed protein through the substitution of cellulose (cellu flour) for glucose (Williams and Grau, '56). In these experiments, the metabolizable energy concentration of the diets was only estimated, and cellu flour was considered completely indigestible. The equal weight gains made with an apparently smaller metabolizable energy intake might represent equal gains of energy produced by more efficient utilization of metabolizable energy. On the other hand, it is well established that equal gains of weight do not necessarily represent equal energy gains (Fraps, '43; Dansky, '52; Hill and Dansky, '54). Peterson et al. ('54) found that the percentage of body fat in chicks de-

[1] The data in this paper are taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, 1954.

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creased as the digestible energy concentration of semipurified diets was decreased through the substitution of moderate amounts of cellu flour for glucose. However, only a small number of chicks were analyzed for body composition, and the metabolizable energy concentration of the diets was only estimated.

Consequently, an experiment based on the body balance method of Swift et al. ('34) was undertaken to establish the utilization of metabolizable food energy for body energy gain by chicks fed semipurified diets containing 15% of sesame-seed protein and various levels of cellu flour.

## METHODS

The three diets used were the basal 15% sesame-seed protein diet (Williams and Grau, '56) supplemented with 0.4% of L-lysine (called the "basal diet") and the 5% and the 15% cellu flour modifications of this diet. Cellu flour [3] was substituted for an equal weight of glucose (wet weight). Preliminary experiments had shown that these diets allowed equal rates of growth. Male Single-Comb White Leghorn chicks were fed a stock diet until they were 12 days old, when they were transferred to the basal diet. At 14 days of age, 40 chicks, carefully selected according to body weight and rate of gain, were divided into 4 groups of 10 each. One group was sacrificed for carcass analyses. The other groups were divided in half since only 5 chicks were placed in each cage to avoid crowding at the later stages of the experiment. However, 10 chicks received each diet. The transfer from the stock diet to the basal diet was done so that the body composition of the chicks sacrificed at the start would be more nearly representative of the basal diet.

Body weights and food intakes were measured daily for three weeks. On alternate days, the excreta were collected, and the chicks transferred to a clean battery. In an attempt to minimize the effects of position in the battery, the groups

[3] Chicago Dietetic Supply House, Chicago, Illinois.

of chicks were shifted daily to different levels in the battery. The 6 cages used occupied the second, third and 4th levels of a 5-level battery, and the chicks on each diet occupied each level 7 times in the three-week period.

Spilled food was collected with the excreta since the calorimetric determination of metabolizable food energy would not be affected by the relatively small amount of food spilled [metabolizable energy of the food = (gross food energy eaten + gross food energy spilled) — (gross energy of the excreta + gross food energy spilled)].

Some error in food intake was introduced by the failure to measure food spillage, but this was only a small percentage of the total three-week intake. The excreta were dried for 24 hours at 70°C., weighed, and pooled. No correction was made for the loss of ammonia.

At the end of the three-week experimental period, all the chicks were killed by carbon tetrachloride inhalation. The contents of the gastrointestinal tract were removed, and the carcass and intestinal tract placed in a tared beaker. Body water was determined by drying the carcass to constant weight in a forced-draft oven at 100°C. Fat was determined from the loss in weight of the dried carcass after cold extraction with ethyl ether until the extracts were fat-free. The dried, ether-extracted carcasses were then autoclaved for 6 hours in 20% sulfuric acid, and the hydrolysates were filtered and diluted to a known volume. Carcass nitrogen was determined on aliquots of the hydrolysates by the semi-micro Kjeldahl procedure. The factor 6.25 was used to estimate carcass protein from the nitrogen.

Excreta nitrogen was determined by the semi-micro Kjeldahl method with aliquots of a digest representing 0.5 gm of excreta. The moisture content of food and excreta was obtained by drying a 2 gm sample in a vacuum oven for 48 hours at 70°C. The heats of combustion of the diets and the pooled excreta were determined by combustion in an Emerson Fuel Calorimeter.

## RESULTS AND DISCUSSION

*Growth.* Equal rates of growth were obtained with the three diets throughout the experimental period (fig. 1), and, up to 16 days, the average body weights were nearly identical. At the end of 21 days, the chicks fed the 5% and the 15% cellu

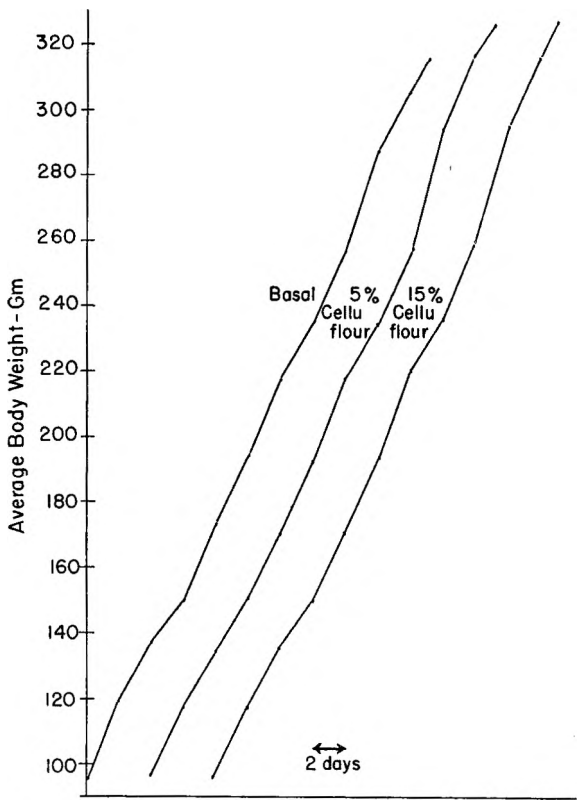


Fig. 1 Comparison of average body weights throughout the 21-day experimental period.

flour diets had gained 10 to 11 gm more than had the chicks fed the basal diet, but these differences in gain were not significant. The gains with all three diets were maximum for chicks of this age fed this type of semipurified diet although the level of protein was only 15%. The occurence of maximal gains with a diet containing 15% sesame-seed protein without

the introduction of cellu flour agrees with previous results with this type of diet (Williams and Grau, '56).

*Food intake.* The average cumulative food intakes are plotted in figure 2. Since the chicks were of similar size at all times, the comparison of average food intakes is valid. Up to 4 days, the intake of all three diets was nearly the same, and little adjustment in response to the differences in the

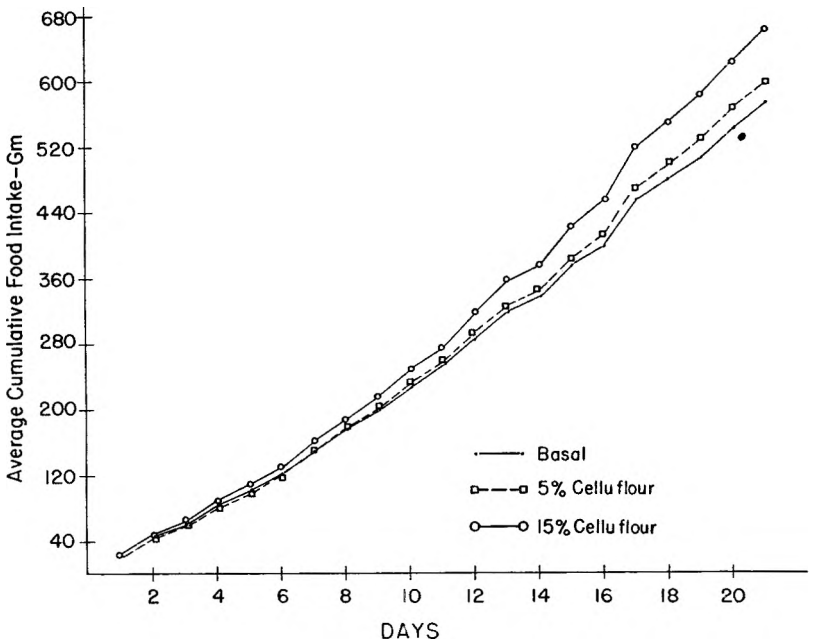


Fig. 2 Average cumulative food intakes (grams wet weight) for the 21-day experimental period.

digestible energy concentrations of the diets was apparent although the growth rates were equal. By the 5th day, the chicks fed the 15% cellu flour diet began to increase their food intake in comparison with the other two groups, and they subsequently maintained a greater intake. Little increase in the intake of the 5% cellu flour diet occurred until the 16th day. Previous experiments (Williams and Grau, '56) showed more rapid adjustments in food intake in response to greater

decreases in energy concentration. Similar results were reported by Dansky ('52), who concluded that several days might be needed to demonstrate a distinct change in food intake in response to a change in energy concentration.

TABLE 1

*Food intake and energy balance (average)*

| CATEGORY OF INTEREST | DIET | | |
|---|---|---|---|
| | Basal | 5% Cellu flour | 15% Cellu flour |
| Food intake, *gm dry wt.* | 526 | 553 | 613 |
| Average gain, *gm* | 220 | 228 | 229 |
| Gross energy of food, *Kcal./gm dry wt.* | 3.79 | 3.81 | 3.86 |
| Gross energy intake, *Kcal.* | 1994 | 2107 | 2366 |
| Excreta, *gm dry wt.* | 130 | 160 | 241 |
| Gross energy of excreta, *Kcal./gm dry wt.* | 2.85 | 3.06 | 3.30 |
| Energy excreted, *Kcal.* | 370 | 490 | 795 |
| Metabolizable energy intake, *Kcal.* | 1624 | 1617 | 1571 |
| Metabolizable energy of food, *Kcal./gm dry wt.* | 3.09 | 2.92 | 2.56 |
| Gm gain/Kcal. consumed | 0.135 | 0.141 | 0.146 |
| Nitrogen, *gm* | | | |
| consumed | 13.8 | 14.4 | 15.9 |
| excreted | 5.5 | 5.4 | 7.3 |
| retained | 8.2 | 9.0 | 8.6 |
| "Digestibility," *% of dry matter* | 75.3 | 71.1 | 60.7 |
| Organic matter consumed, *gm dry wt.* | 471 | 496 | 550 |
| Organic matter excreted, *gm dry wt.* | 89 | 117 | 192 |
| "Digestibility," *% of organic matter* | 81.1 | 76.4 | 65.1 |
| Gross energy of Cellu flour, *Kcal./gm dry wt.* | ... | 4.03 | 4.03 |
| Cellu flour consumed, *gm dry wt.* | 0 | 28 | 93 |
| Gross energy intake from Cellu flour, *Kcal.* | 0 | 113 | 374 |
| Increase over the energy content of the excreta from the basal diet, *Kcal.* | ... | 120 | 425 |

*Intake and utilization of metabolizable energy and nitrogen.* Table 1 presents the calorimetric data from which the metabolizable energy concentrations of the diets were calculated and the average intakes of metabolizable energy for the three-week period. The total intake of metabolizable energy is the

difference between the total food energy consumed and the total energy excreted per chick for the entire period.

Equal intakes of metabolizable food energy were attained with the basal and the 5% cellu flour diets, but the intake from the 15% cellu flour diet was somewhat less. The average three-week metabolizable energy intakes were 1624, 1617, and 1571 Kcal., respectively, for the basal, the 5%, and the 15% cellu flour diets. Again, the presence of cellu flour in the diet improved slightly the efficiency of utilization of metabolizable energy for weight gain (table 1). Nitrogen retention was nearly the same with all three diets and was not altered by the introduction of cellu flour. Since the nitrogen retention data indicate that equal gains of protein must have occurred, any differences in body energy gain should be reflected in the gain of body fat.

*Indigestibility of cellu flour.* The results of the present experiment confirm the assumption that cellu flour is not digested by the chick (table 1). Two different methods of approach, (1) the "digestibility" of the diet and (2) the energy content of the combined excreta, show that very little of the energy of cellu flour is available to the chick.

The "digestibility" of the diet is usually calculated from the expression 100 (A-B)/A where A is the amount of a given nutrient consumed and B is the amount of that nutrient in the combined excreta. This expression, however, when applied to chicks, actually represents the "metabolizability" of the diet and indicates the amount of food available after urine and fecal losses. If cellu flour was not digested and if it did not alter the digestibility and utilization of the diet as a whole, then the difference in digestibility between the basal and the cellu flour-containing diets should equal the level of cellu flour, i.e. 5 or 15%.

On the basis of total dry matter or total organic matter, the 5% cellu flour diet contained 4 to 5% more and the 15% cellu flour diet contained 15 to 16% more non-metabolizable material than did the basal diet. The difference of 1% between

the determined and the assumed values is within the error of the procedures used. Cellu flour at a level of 5% was no more "digestible" than at the 15% level, and the metabolizability of the remainder of the diet did not appear affected by the presence of cellu flour.

The non-metabolizability of the energy of cellu flour is shown by a comparison of the energy contents of the combined excreta. The increase in the energy content of the excreta was nearly equal to or somewhat greater than the gross energy

TABLE 2

*Effect of diet on body composition*

| CARCASS COMPONENT | BASAL DIET | | 5% CELLU FLOUR | | 15% CELLU FLOUR | |
|---|---|---|---|---|---|---|
| | *gm* | % | *gm* | % | *gm* | % |
| Empty weight [1] | 293.1 ± 22.4 [2] | | 298.9 ± 25.5 | | 299.8 ± 30.1 | |
| Water | 202.0 ± 15.8 | 69.0 ± 1.1 | 208.0 ± 16.2 | 69.6 ± 1.2 | 212.4 ± 20.1 | 70.9 ± 0.8 |
| Fat | 21.4 ± 4.5 | 7.3 ± 1.4 | 20.7 ± 4.9 | 6.9 ± 1.2 | 16.3 ± 3.4 | 5.4 ± 0.8 |
| Protein | 58.4 ± 5.0 | 19.9 ± 0.7 | 58.8 ± 5.5 | 19.7 ± 0.6 | 59.8 ± 6.3 | 20.0 ± 0.4 |

[1] Body weight minus contents of gastrointestinal tract.

[2] Mean and standard deviation.

content of the cellu flour eaten. Thus, the reduction in metabolizable energy per gram of diet caused by the introduction of cellu flour at levels of 5% and 15% was very nearly equal to the gross energy per gram of diet provided by cellu flour.

*Carcass analyses.* The results of the carcass analyses (table 2) show clearly that the equal gains of weight did not represent equal gains of energy. Although the final empty body weights did not differ significantly, the final body fat content of the chicks fed the basal and the 5% cellu flour diets was significantly greater than the final body fat content of

the chicks fed the 15% cellu flour diet ($P < 0.01$).[4] The final body water content of the chicks fed the 15% cellu flour diets was significantly greater than the body water content of the chicks fed the basal and the 5% cellu flour diets ($P < 0.01$). There were no significant differences, in the final body contents of either water or fat, between the chicks fed the basal and the 5% cellu flour diets. Equivalent gains of protein were made with all three diets as the nitrogen balance data had indicated. The gains of nitrogen calculated from the results of the carcass analyses were less than the gains calculated from the nitrogen balance data. The difference may reflect the losses of nitrogen during collection of the excreta.

These results show clearly that the utilization of metabolizable food energy for body energy gain was not increased by the introduction of cellu flour at a level of 15% although the efficiency of utilization of metabolizable energy for weight gain was increased slightly.

*The regulation of food intake.* Although the food intake and digestible energy concentration of the diets are inversely related, it remains to be explained why the chicks fed the 15% cellu flour diet did not eat sufficient food to make their metabolizable energy intake equal to that of the chicks fed the basal diet since the bulk of the 15% cellu flour diet did not restrict food intake. A similar question has been asked by Hill and Dansky ('54). Also, it may be asked why the chicks fed the 5% cellu flour diet ate sufficient food to make their intake of metabolizable food energy equal to that of the chicks on the basal diet. Conversely, it might be asked why the chicks fed the basal diet did not consume *less* food.

Even if the observed differences in metabolizable energy intakes were not significant, the differences in productive energy intakes were significant.[5] More food energy was stored

[4] The significance of the differences between groups in final body composition was tested by covariance analysis of final empty body weights and final body water, fat or protein.

[5] Produtive energy of the food = metabolizable energy of the food — heat increment of the food — energy used for maintenance.

as fat by the chicks fed the basal and the 5% cellu flour diets although equal gains of protein occurred with all three diets. The lower percentage of body fat produced by the 15% cellu flour diet could be the consequence of an initial depletion of body fat since the increases in food intake did not begin immediately after transfer to the experimental diets and since the three-week period may have been too brief to allow repletion. Yet Dansky ('52) found that similar differences in body fat were present after one week and persisted throughout an 8-week experimental period with natural feedstuff diets differing in digestible carbohydrate concentration.

The effects of the diets on feeding patterns were not noted in detail. It has been reported that infrequent feeding increases the rate of lipogenesis and decreases the heat increment of the food (Mayer, '53). Since the chicks fed the 15% cellu flour diet ate the most food (both weight and volume), they presumably ate more frequently than the other chicks. Perhaps, the smaller gain of fat by these chicks was related to their presumably more extended feeding pattern.

## SUMMARY

A 15% sesame seed protein diet, supplemented with 0.4% of L-lysine, and the two modifications of this diet produced by the substitution of 5 and 15% of cellu flour for glucose were fed ad libitum to 14-day old chicks for a period of 21 days. The metabolizable energy concentrations of the diets were determined from the heats of combustion of the diets and of the excreta collected during the three-week period.

The metabolizable energy intakes from the basal and the 5% cellu flour diets were equal, but the intake from the 15% cellu flour diet was somewhat less although all three diets allowed equal weight gains. The two former diets produced a significantly greater body fat content and a significantly lower body water content than did the 15% cellu flour diet. There were no significant differences in the final body weights or body protein contents produced by the three diets.

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# THE EFFECT OF INTERMITTENT CONSUMPTION OF CALCIUM IN RATS [1]

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It appears that animal organisms are able to utilize their nutrients more efficiently when supplied at low levels in the diet. The studies of Hegsted et al. ('52) with men on low levels of calcium intake over long periods of time indicated their daily requirement for calcium was possibly considerably lower than the generally accepted high values of the conventional dietary recommendations. The study of Henry and Kon ('53) on the relationship between calcium retention and body stores in older rats suggested considerable adaptability to varying levels of intake. Recently, Hansard and Plumlee ('54) reported the physiological behavior of labeled calcium as a function of the dietary level of this element in rats. They showed that utilization efficiency decreases with increased intake of calcium and that total retention was high with low-calcium body stores.

The present paper is a report of an investigation of the effect of a schedule of intermittent feeding on calcium utilization in rats. Different groups of animals were fed the same amount of calcium; however, except for the control group this nutrient was not consumed daily, but was made available in a periodically interrupted manner. It was hoped to learn whether the mechanism of "physiological regulation" in the body (Adolph, '43) could cope with this irregular condition: reserving more calcium during the "have" period for the "have not" period.

[1] Journal paper no. J.-2887 of the Iowa Agricultural Experiment Station, Ames. Project no. 959.

## EXPERIMENTAL

Twenty-four Sprague-Dawley male weanling rats weighing between 37 and 39 gm were allotted at random by weight to groups representing 4 calcium treatments. Each group then contained 6 animals with an avergae body weight of about 38 gm. The rats were weighed once a week. They were caged individually and maintained on equalized feeding. Feed allotments were weighed every day. Only occasionally a few rats failed to consume all of their food allowances. Fresh distilled water was always available. Cod liver oil was given orally twice each week by a medicine dropper. The animals were fed a basal diet containing 0.019% of calcium and 0.445% of phosphorus on the dry basis, and fortified with all the known B vitamins. The percentage composition of the basal diet was; cornstarch, 55.7; sucrose, 15; crude casein, 18; corn oil, 4; calcium-phosphorus-free salt mixture (Day and Stein, '38), 3; cellulose, 2; irradiated yeast, 1; and potassium phosphate, monobasic, 1.3.

The experiment was conducted for 6 weeks. During the first two weeks, the rats were fed 10 gm of the basal diet daily with the supplemental calcium furnished in the form of calcium carbonate according to the following scheme; group 1 received 20 mg daily of supplementary calcium (calculated as the element), group 2 received 40 mg on every 2nd day, group 3 received 80 mg every 4th day and group 4 received 120 mg every 6th day. The rats weighed about 100 gm after the second week on experiment. Their basal ration was increased from 10 to 15 gm daily and the supplementary calcium was increased by 50%. No further increases were made throughout the experiment.

The rats were sacrificed at the end of the experiment. Both femurs were taken from each rat as representative bone and analyzed for calcium and phosphorus. The calcium content was determined by the method of titration of the calcium oxalate with potassium permanganate. The inorganic phosphorus content was determined by the method of Fiske and Subbarow ('25).

The data of this study were analysed by the method of analysis of variance and the sum of squares for the feeding intervals were partitioned to give the mean squares for the linear, quadratic and remainder regression components (Snedecor, '46, p. 410). All expressions of statistical significance pertain to $P = 0.05$ or less.

## RESULTS AND DISCUSSION

The initial weights of the 4 groups of animals were practically identical at 38.2 to 38.3 gm. The 6-week gains for

TABLE 1

*Effect of intermittent feeding of calcium on the compositioin of the femurs of rats* [1]

| RATION GROUP | AIR-DRY WT. | ASH WT. | ASH/AIR-DRY WT. | CALCIUM | Ca/ASH | PHOSPHORUS | P/ASH |
|---|---|---|---|---|---|---|---|
| | *mg* | *mg* | *%* | *mg* | *%* | *mg* | *%* |
| 1 [2] | 571.4 | 250.5 | 43.9 | 88.0 (100) [3] | 35.1 | 54.2 (100) | 21.6 |
| 2 | 514.0 | 226.8 | 44.7 | 79.5 (90) | 33.3 | 52.7 (97) | 23.2 |
| 3 | 523.7 | 236.5 | 45.2 | 76.0 (86) | 32.2 | 51.9 (96) | 22.0 |
| 4 | 492.9 | 220.2 | 45.5 | 63.3 (72) | 27.7 | 48.2 (89) | 21.9 |
| $s_{\bar{x}}$ [4] | 3.8 | 6.4 | 0.8 | | 0.8 | | 0.6 |

[1] Values are averages for 6 rats.

[2] Group 1 received 20 mg of supplementary calcium daily; group 2 received 40 mg on every second day, group 3, 80 mg every 4th day and group 4, 120 mg every 6th day.

[3] Figures in parentheses indicate the relative values when the value for group 1 is expressed as 100.

[4] $s_{\bar{x}}$ = standard deviation.

groups 1 to 4, respectively, were 195, 188, 199 and 192 gm with a standard deviation of 4.2 gm. The body weight gains per 100 gm of ration for the 4 groups were 30.5, 29.4, 31.1 and 30.0 gm, respectively, with a standard deviation of 0.6 gm. None of the average differences in gain was statistically significant. Hence, the feeding of equivalent total quantities of calcium but at different time intervals did not affect the body weight appreciably.

Summaries of the analyses of the left and right femurs of the animal are shown in table 1. The air-dry weight and ash

content decreased in a statistically significant linear manner as the intervals between calcium feedings were lengthened. The average decrease in ash weight was approximately 11 mg between each two adjacent groups listed in the table. The percentage of ash in the bones tended to increase with increased length of interval of calcium deprivation; however, this tendency was not statistically significant.

Increasing the length of the interval between calcium feedings resulted in a marked decrease of this mineral laid down in the femurs. If the mean value of calcium content of group 1 was used as control, there was a 10 or 14% decrease, when calcium was fed on every other or every 4th day, respectively. The decrease amounted to 28% when the calcium was fed on every 6th day, in spite of the same total consumption. The average percentage of calcium in the femurs of the rats in group 1 was 35% of their ash weight, this value was lowered to 33, 32, and 28% when the total intakes of calcium were fed at 2-, 4- and 6-day intervals, respectively. This decrease of calcium in the femur ash was a statistically significant linear function of the intermittent interval of calcium feeding. The average decrease amounted to 2.3% units for each two adjacent treatment groups.

The effect on the phosphorus content of the femurs was similar to that on the calcium, but the differences between the various treatments were smaller. The proportion of phosphorus in the femur ash remained constant, however, at about 22%.

## SUMMARY

Four groups of 6 weanling rats each were fed equal amounts of a basal ration and supplementary calcium over a period of 6 weeks. The basal ration contained 0.02% of calcium and 0.45% of phosphorus. Supplementary calcium was furnished in the form of carbonate and it was given to one group daily and to the other three groups at intervals of 2, 4 or 6 days. The intermittent feeding of equal amounts of calcium carbonate did not affect the gain in body weight

appreciably. The femurs of the rats which received a daily allowance of supplementary calcium had the greatest air-dry weight, ash weight, and total calcium and phosphorus content. The average percentages of calcium in the femur ash were 35, 33, 32 and 28% for the 0-, 2-, 4- and 6-day intervals of calcium feeding, respectively. The average percentages of phosphorus in the femur ash bore no relation to the length of the interval of calcium feeding.

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# THE ASCORBIC ACID EXCRETION IN THE STOOL IN ELDERLY SUBJECTS [1]

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The excretion of ascorbic acid in the stool has been the subject of few investigations. Studies by Chinn and Farmer ('39) revealed an average excretion of 4.9 mg of ascorbic acid daily in young normal adults; similar values were reported by Martin ('41). Large variations in the intake of ascorbic acid were found to affect the fecal excretion only slightly.

No studies have as yet been reported on the ascorbic acid excretion in the stool of elderly subjects. The present investigation was undertaken with the purpose of providing such data through the determination of the ascorbic acid excretion in the feces on an ordinary diet and following a daily intake of 200 mg of ascorbic acid in tablet form.

## MATERIAL AND METHODS

The subjects included in the investigation were elderly men, who were inmates of the St. Louis Chronic Hospital. Out of a larger group of individuals 13 were selected, who were reliable and cooperative and who did not suffer from gastrointestinal disease. The ages of the men ranged between 65 and 90 with a mean of 76 years.

The ascorbic acid intake and its excretion in the stool were first studied during a one-week period, in which the individuals received the ordinary diet of the institution. The food rejected by each subject was collected, and determinations were

[1] Vitamin Studies in Middleaged and Old Individuals XIII. Funds provided by Hoffmann-La Roche, Inc., Nutley, New Jersey.

made daily of the total ascorbic acid of the diet and also of the rejected food. Collection of the feces was made immediately after each passage, and the 24-hour sample from each person treated as described below. After one week the subjects were given 200 mg of ascorbic acid by mouth daily in addition to the ordinary diet, and the food and stool collections were continued as before. It was ascertained by the division's research nurse that the individuals actually swallowed the administered ascorbic acid tablets.

The food samples representing the total daily food intake, and the individual samples of rejected food were homogenized with distilled water in a Waring blendor. The successive portions of slur from each sample were transferred to a glass flask and made up to 2500 ml with distilled water. After thorough mixing, 25 ml of the homogenate were pipetted into a stoppered cylinder, after which 25 ml of water and 50 ml of 20% trichloroacetic acid were added. The contents of the cylinder were mixed by shaking and filtered through a Whatman no. 42 filter paper.

The stool specimens were transferred immediately after passage to a glass jar containing 200 ml of freshly prepared 5% metaphosphoric acid solution and the weight of the 24-hour sample was obtained. The sample was then made up to 500 ml with distilled water and transferred to a Waring blendor for homogenization. Fifty milliliters of the homogenate were then pipetted into a stoppered cylinder and 50 ml of 20% trichloroacetic acid added. The contents of the cylinder were mixed by shaking and filtered through a Whatman no. 2 filter paper.

In the case of both the food and stool samples 2-ml aliquots of norit-treated trichloroacetic acid filtrate, diluted with 2 ml of water, were used for ascorbic acid and blank determinations, by the method of Roe and Kuether ('43).

## RESULTS

The results of the determinations of the ascorbic acid intake in the food and the ascorbic acid excretion in the stool are

presented in table 1. In the table the daily ascorbic acid measurements have been added to obtain the total values for the 7-day period on the ordinary diet, and for the 7-day period during which the diet was supplemented with 200 mg of ascorbic acid daily.

TABLE 1

*The intake of ascorbic acid and its excretion in the stool of 13 elderly men*

| SUBJECT | AGE | CONTROL PERIOD (7 days) | | | | ADMINISTRATION OF ASCORBIC ACID TABLETS (200 mg daily for 7 days) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ASCORBIC ACID INTAKE | WT. OF STOOL | ASCORBIC ACID IN STOOL | | ASCORBIC ACID IN FOOD | TOTAL INTAKE OF ASCORBIC ACID | WT. OF STOOL | ASCORBIC ACID IN STOOL | |
| | | | | Total | 100 gm | | | | Total | 100 gm |
| | *yrs.* | *mg* | *gm* | *mg* | *mg* | *mg* | *mg* | *gm* | *mg* | *mg* |
| 1 | 65 | 68 | 881 | 10.2 | 1.2 | 320 | 1720 | 760 | 11.2 | 1.5 |
| 2 | 69 | 278 | 342 | 6.8 | 2.0 | 420 | 1820 | 472 | 19.3 | 4.1 |
| 3 | 74 | 225 | 395 | 10.4 | 2.6 | 382 | 1782 | 474 | 15.7 | 3.3 |
| 4 | 65 | 192 | 765 | 12.9 | 1.7 | 350 | 1750 | 590 | 11.0 | 1.9 |
| 5 | 86 | 248 | 423 | 14.0 | 3.3 | 222 | 1622 | 430 | 12.7 | 3.0 |
| 6 | 82 | 241 | 167 | 10.4 | 6.2 | 280 | 1680 | 423 | 17.7 | 4.2 |
| 7 | 90 | 243 | 221 | 6.6 | 3.0 | 309 | 1709 | 248 | 7.6 | 3.1 |
| 8 | 80 | 193 | 322 | 3.2 | 1.0 | 260 | 1660 | 131 | 5.9 | 4.5 |
| 9 | 75 | 279 | 265 | 5.5 | 2.1 | 364 | 1764 | 366 | 4.2 | 1.2 |
| 10 | 88 | 308 | 372 | 4.5 | 1.2 | 350 | 1750 | 379 | 7.2 | 1.9 |
| 11 | 69 | 295 | 329 | 11.4 | 3.5 | 350 | 1750 | 474 | 10.3 | 2.2 |
| 12 | 78 | 292 | 100 | 1.6 | 1.6 | 272 | 1672 | 212 | 3.3 | 1.5 |
| 13 | 66 | 139 | 881 | 8.8 | 1.0 | 185 | 1585 | 350 | 8.4 | 2.4 |
| Mean | 76 | 231 | 420 | 8.2 | 2.3 | 314 | 1714 | 408 | 10.3 | 2.7 |

It will be seen from the data in the table that an average of 8.2 mg of ascorbic acid was excreted weekly in the stool on a mean ascorbic acid intake of 231 mg, and that the average weekly excretion was 10.3 mg when the ascorbic acid intake, as the result of supplementation with ascorbic acid tablets, was increased to 1714 mg. The mean ascorbic acid concentration of the wet stool was 2.3 mg % on the unsupplemented diet, and 2.7 mg % on the supplemented diet.

## DISCUSSION

The average fecal excretion of ascorbic acid on the ordinary diet was 1.2 mg daily which is somewhat lower than values reported in previous studies for young adults. In agreement with the results by Chinn and Farmer ('39) the administration of ascorbic acid in tablet form was found to have little effect on the ascorbic acid content of the stool (mean daily excretion after supplementation, 1.5 mg).

The low ascorbic acid values of the stool observed in the present investigation do not support the contention that a high ascorbic acid excretion in the feces is a factor in the production of low blood ascorbic acid values in old individuals.

## SUMMARY

Determinations were made in 13 elderly men of the ascorbic acid intake in the food and the ascorbic acid excretion in the stool. The excretion on an ordinary diet averaged 1.2 mg daily. After supplementation of the diet with 200 mg of ascorbic acid daily a mean excretion of 1.5 mg was observed. These values are somewhat lower than those reported by previous investigators for young adults.

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# SHORT-TERM FEEDING STUDIES ON ACETIN FATS

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The chief components of edible fats in the United States are triglycerides of fatty acids having chain lengths of 4 to 20 carbons. Although the predominant fatty acids in most fats contain 16 or 18 carbon atoms, considerable amounts of shorter chain fatty acids are found in some fats. Thus, butterfat may contain as much as 35% of fatty acids less than 16 carbons in length. In recent years a new type of fat has been developed in which one or two of the long-chain fatty acids of the glyceride molecule are replaced with acetic acid (Baur, '54a). Such glycerides have been referred to as acetin fats or aceto glycerides.

The most striking effect of the introduction of the acetyl group into the glyceride molecule is the reduction in melting point. This effect permits the preparation of low melting fats and oils, or acetin fats, of a high degree of saturation and of a significantly increased resistance to oxidation. Acetin fats may replace normal triglycerides in any edible fat use. Thus, edible fat products including shortenings, margarines or spreads, salad oils and frying oils can be made from acetin fats and oils (Baur, '54b; Feuge, '55).

The physical properties of the acetin fats are determined by the number of acetate residues incorporated into the molecule and the nature of the long-chain fatty acids. Since there is no standard nomenclature in this field we have selected the term monoacetin fat to refer to that fat in which one of the long-chain fatty acids of a conventional triglyceride is re-

placed by acetic acid, e.g., dioleoyl monoacetin. Similarly, a diacetin fat is one in which two of the long-chain fatty acids of a conventional triglyceride have been replaced by acetic acid, e.g., monooleoyl diacetin. For complete description, when an acetin fat is prepared from a triglyceride of mixed fatty acid composition, a term describing the type of long-chain fatty acids present must be appended. Thus, a lard monoacetin fat would be one in which lard was used as the starting material and one long-chain fatty acid of each triglyceride molecule was replaced with acetic acid.

The novel structure of these fats and their possible consumption by humans made it of interest to investigate their nutritive value by feeding experiments with laboratory animals. In these initial studies it was desired to feed these acetin fats at as high a dietary level as practicable. This was accomplished by isolating the monoacetin fat and diacetin fat and feeding these separately as essentially the sole source of fat in the diet. The results of three, short-term, high-level feeding studies are reported here. All of these studies show that acetin fats derived from the usual edible triglycerides are nutritious materials.

## PROCEDURE

All acetin fats used in these studies were prepared by the following procedure. Triglycerides consisting of fatty acids of conventional chain length were rearranged randomly with triacetin using sodium methoxide as the catalyst. The unreacted triacetin was removed by steam stripping under vacuum leaving a random mixture of triglycerides of fatty acids of conventional length, monoacetin fat, and diacetin fat. Mixtures of this type are referred to as a mixed acetin fat. The monoacetin fat and diacetin fat were isolated from the mixed acetin fat by molecular distillation. The fats were then steam deodorized at reduced pressure.

Fats hydrogenated to two different end points were used as the starting material in preparing this series of acetin fats. One was completely hydrogenated soybean oil, referred to as

TABLE 1

*Composition of fats*

| CATEGORY OF INTEREST | SERIES 1 | | | | SERIES 2 | | SERIES 3 | |
|---|---|---|---|---|---|---|---|---|
| | I.V. 80 fat | I.V. 80 diacetin fat | I.V. 1 fat | I.V. 1 diacetin fat | I.V. 80 fat | I.V. 80 diacetin fat | I.V. 80 fat | I.V. 80 monoacetin fat |
| Iodine value | 79 | 52 | 1 | 1 | 76 | 54 | 76 | 69 |
| Saponification value | 191 | 373 | 190 | 371 | 191 | 369 | 191 | 253 |
| Free fatty acid, % | 0 | 0.5 | 0.2 | 0.6 | 0.1 | 0.2 | 0.1 | 0.2 |
| Acetic acid, % | 0 | 26 | 0 | 27 | 0 | 26 | 0 | 9 |
| Trans fatty acids, % | 19 | ...[1] | 0 | ...[1] | 21 | ...[1] | 21 | ...[1] |
| Saturated acids, % | 17 | ... | 100 | ... | 22 | ... | 22 | ... |
| Oleic acid, % | 75 | ... | ... | ... | 67 | ... | 67 | ... |
| Linoleic acid, % | 7 | ... | ... | ... | 11 | ... | 11 | ... |
| Linolenic acid, % | 1 | ... | ... | ... | 0 | ... | 0 | ... |

[1] Fatty acid composition that of the corresponding conventional fat with allowance made for acetic acid content.

TABLE 2

*Percentage composition of diets*

| CONSTITUENT | SERIES 1 | | | | SERIES 2 | | | SERIES 3 | |
|---|---|---|---|---|---|---|---|---|---|
| | I.V. 80 fat | I.V. 80 diacetin fat | I.V. 1 fat | I.V. 1 diacetin fat | I.V. 80 fat | I.V. 80 fat | I.V. 80 diacetin fat | I.V. 80 fat | I.V. 80 monoacetin fat |
| | % | % | % | % | % | % | % | % | % |
| Vitamin mixture[1] | 5.9 | 5.3 | 5.9 | 5.3 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Soybean oil | 2.0 | 2.0 | 2.0 | 2.0 | .. | .. | .. | .. | .. |
| Casein | 32.1 | 28.4 | 32.1 | 28.4 | 35.0 | 35.0 | 35.0 | 27.0 | 27.0 |
| Sucrose | 28.7 | 33.1 | 28.7 | 33.1 | 4.0 | .. | .. | 47.0 | 47.0 |
| Salt mix[2] | 3.3 | 3.2 | 3.3 | 3.2 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Cellu flour | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Ethyl cellulose | .. | .. | .. | .. | .. | 4.0 | 4.0 | .. | .. |
| Fat | 25.0 | 25.0 | 25.0 | 25.0 | 50.0 | 50.0 | 50.0 | 15.0 | 15.0 |

[1] Each 5 gm contains: vitamin A, 1250 I.U.; vitamin D, 125 I.U.; $\alpha$-tocopherol, 10 mg; menadione, 0.3 mg; thiamine, 0.4 mg; riboflavin, 0.5 mg; niacin, 2.0 mg; calcium pantothenate, 2.0 mg; choline, 300 mg; para-aminobenzoic acid, 10 mg; biotin, 0.03 mg; folic acid, 0.025 mg; inositol, 200 mg; ascorbic acid, 10 mg; pyridoxine, 0.4 mg; sucrose to make 5 gm.

[2] Hubbell et al. ('37).

the I.V. (iodine value) 1 fat. Stearic acid comprises about 92% of the fatty acids in such a fat. Each of the other fats used was a 75/25 mixture of soybean oil and cottonseed oil which had been hydrogenated to an iodine value of approximately 80. Two such fats were used in these studies, one in series 1 and the other in series 2 and 3. These preparations are referred to as I.V. 80 fat. The compositions of the fats are given in table 1.

In each feeding study the performance of animals fed an acetin fat and the conventional triglyceride from which it was prepared was compared. In all, three series of experiments were carried out. In series 1, I.V. 80 fat, I.V. 80 diacetin fat, I.V. 1 fat, and I.V. 1 diacetin fat were fed at a dietary level of 25%. The compositions of the diets used are given in table 2. The protein, vitamin, and mineral levels of these diets were adjusted according to the caloric value of the diets so that the level of these three ingredients per calorie would be the same in all diets. Since the I.V. 1 fat contains none of the essential fatty acids, 2% of soybean oil was added to each of the diets in the first series of experiments. This addition was not necessary in series 2 and 3 since the diet fats contained adequate amounts of linoleic acid.

In series 2, I.V. 80 fat and I.V. 80 diacetin fat were fed at a dietary level of 50%. Incorporation of the I.V. 80 diacetin fat, which is an oil, into the basic diet resulted in a diet from which the fat separated out on standing. To overcome this tendency, ethyl cellulose was added. The I.V. 80 fat, which does not separate out of the diet, was fed in diets both with and without ethyl cellulose to determine the effect, if any, of the ethyl cellulose. The compositions of these diets are given in table 2. No attempt was made to equate these diets on the basis of their caloric contents.

In series 3, an I.V. 80 monoacetin fat and the corresponding I.V. 80 fat from which it was prepared were fed at a 15% level. The compositions of these diets also are given in table 2.

Each experimental group consisted of 10 weanling male rats of the Holtzman strain. The animals were distributed ran-

domly among groups within each experiment as to body weight and litter. Animals were housed individually in cages. Gains in body weight and food consumption were recorded on a weekly basis.

In some instances the coefficient of utilization of the dietary fat was determined by analyzing feces collected during the 5th and 6th week of the experiment for their total lipide content. The feces were dried, ground in a Wiley mill so as to pass a 60 mesh screen, saponified with alcoholic KOH, acidified, and extracted with petroleum ether. Since this procedure is likely to result in a loss of volatile fatty acids, several samples of the feces of the animals fed the acetin fats were analyzed separately for their acetic acid content. Only a trace was found. Thus, measurement of the egested long-chain fatty acids alone will give the true value for the coefficient of utilization of an acetin fat.

Where applicable, the data were analyzed statistically by an analysis of variance. Minimum significant differences were determined by the method of Tukey ('52). A confidence level of 0.05 was used in all such treatments.

## RESULTS AND DISCUSSION

The performance of the animals fed diets containing 25% of an acetin fat prepared from either partially or completely hydrogenated vegetable oil and the corresponding controls is given in series 1 of table 3. It will be noted that the gain in body weight of the animals fed an acetin fat is the same as that of animals fed a conventional triglyceride of the same long-chain fatty acid composition. The growth of the animals fed the fats containing only saturated fatty acids is inferior to that of animals fed partially hydrogenated fats. This response is in part explained by the lower coefficients of utilization of the completely hydrogenated fats. When account is taken of the portion of the fat in the diet which is not absorbed, the performance of all 4 dietary groups is in much better agreement as shown by the values for absorbed caloric efficiency.

TABLE 3

*The performance of animals fed diets containing various acetin fats. Average cumulative values per animal*

| DIETARY FAT | GAIN IN BODY WEIGHT | CALORIC CONSUMPTION | CALORIC EFFICIENCY [1] | COEFFICIENT OF UTILIZATION | ABSORBED CALORIC EFFICIENCY [2] |
|---|---|---|---|---|---|
| | *gm* | *Cal.* | | *%* | |
| | *Series 1. 25% dietary fat level. 8-week experiment* | | | | |
| I.V. 80 fat | 265 | 3490 | 7.6 | 87 | 8.0 |
| I.V. 80 diacetin fat | 262 | 3460 | 7.6 | 87 | 8.1 |
| I.V. 1 fat | 215 | 4740 | 4.5 | 9 | 7.6 |
| I.V. 1 diacetin fat | 222 | 3360 | 6.6 | 83 | 7.1 |
| Minimum significant difference | 11 | 140 | 0.8 | 5 | 0.9 |
| | *Series 2. 50% dietary fat level. 8-week experiment* | | | | |
| I.V. 80 fat | 207 | 2800 | 7.4 | 97 | .. |
| I.V. 80 fat [3] | 212 | 2870 | 7.4 | 96 | .. |
| I.V. 80 diacetin fat [3] | 224 | 3130 | 7.2 | 91 | .. |
| Minimum significant difference | NSD [4] | 270 | NSD | NSD | .. |
| | *Series 3. 15% dietary fat level. 12-week experiment* | | | | |
| I.V. 80 fat | 314 | 5230 | 6.0 | 90 | .. |
| I.V. 80 monoacetin fat | 290 | 4710 | 6.1 | 92 | .. |
| Minimum significant difference | NSD [4] | NSD | NSD | NSD | .. |

[1] $\frac{\text{Gain in body weight}}{\text{Calories eaten}} \times 100.$

[2] Caloric efficiency corrected for unabsorbed dietary fat.

[3] Diet contained ethyl cellulose.

[4] No significant differences.

The coefficients of utilization of the completely hydrogenated fats are of particular interest. The value obtained for the conventional triglyceride is of the order of magnitude that would be expected for this fat, since it is essentially all tristearin (Cheng et al., 49). The replacement in the triglyceride molecule of a portion of the stearic acid with acetic acid results in a marked increase in the amount of stearic acid that is absorbed. This high coefficient of utilization of stearic acid is of interest in the question as to whether the stearic acid content or the melting point of a fat determines its coefficient of utilization. The I.V. 1 fat which consists of about 92% stearic acid has a melting point of approximately 69°C., while the I.V. 1 diacetin fat has a melting point of 33°C. and a stearic acid content of about 62%. If the coefficient of utilization is a function of stearic acid content, not more than 40% of the I.V. 1 diacetin fat should have been absorbed. The high coefficient of utilization obtained with this fat indicates the melting point of a fat to be of more importance than the stearic acid content in determining its coefficient of utilization. However, a number of other factors, besides melting point and stearic acid content, probably play important roles in determining the extent to which a fat is digested and absorbed.

The results obtained when the I.V. 80 diacetin fat was fed at a 50% level are given in series 2 of table 3. The performance of the three groups of animals was essentially the same for all categories. The presence of ethyl cellulose in the diet did not change the growth and food consumption patterns of the animals as shown by the pair of groups fed the I.V. 80 fat. In this series, where the fats were fed at a level of 50%, a greater range in the coefficients of utilization was encountered and hence the rather large differences among groups are not significantly different. For the purposes of this experiment, the results obtained show that animals fed a diet containing 50% of an I.V. 80 diacetin fat grow as well as those fed the corresponding conventional triglyceride.

Similarly, there was a uniform response of the animals fed either the I.V. 80 monoacetin fat or the conventional fat at a 15% level as shown by the results presented in series 3 of table 3.

At the conclusion of the growth study, 5 animals of each of the groups in series 1 and 3 were housed in metabolism cages. Over a two-week period, three 24-hour collections of urine were made. These were analyzed for volume, pH, titratable acidity, total nitrogen, ammonia nitrogen, urea nitrogen, creatine, creatinine, glucose, albumin, and acetone. The animals were then sacrificed and blood samples taken. Red, white, and differential counts were made and total nitrogen, urea nitrogen, non-protein nitrogen, and plasma $CO_2$ combining power determined. In all instances the values obtained on the animals fed the acetin fat were essentially the same as those obtained on the corresponding control animals.

## SUMMARY

The nutritive value of mono- and diacetin fats prepared from completely or partially hydrogenated vegetable oils has been measured in a series of studies on weanling male rats.

The gains in body weight and food consumption of animals fed these fats at relatively high levels were essentially the same as those of animals fed the corresponding conventional triglycerides.

The levels of a number of blood and urine constituents of the rats fed the experimental and control fats were also of the same order of magnitude.

The long-chain fatty acids of a diacetin fat prepared from partially hydrogenated vegetable oils were absorbed as well as those of the conventional triglyceride from which it was prepared. On the other hand, the coefficient of utilization of stearic acid was markedly increased over that of tristearin by incorporating this fatty acid into a diacetin fat molecule.

These studies show that acetin fats derived from the usual edible triglycerides are nutritious materials.

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# THIAMINE, PYRIDOXINE AND PANTOTHENIC ACID IN THE NATURAL RESISTANCE OF THE RAT TO A CORYNEBACTERIUM INFECTION [1]

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ONE FIGURE

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This report deals with the natural resistance of the laboratory rat to spontaneous and inoculated infection by a corynebacterium (strain 197) not ordinarily pathogenic for this species.

Previous reports have shown that young pantothenate-deficient rats spontaneously develop the infection (Zucker and Zucker, '54). The corynebacterium isolated from lesions of such animals reproduces the disease when inoculated into other pantothenate-deficient rats, but does not do so in rats on a complete diet, either purified or made up of natural foodstuffs (Seronde, '54). Susceptibility increases steadily with time on the deficient diet over a period of 10 to 40 days (Seronde et al., '55). Susceptibility can be induced after a longer period on a partial pantothenate deficiency which allows continued growth (Zucker et al., '55).

[1] We gratefully acknowledge assistance from the National Vitamin Foundation, Hoffmann-LaRoche, Inc. and Red Acre Farm, Stow, Mass.

## EXPERIMENTAL

*Diets.* The various deficient diets were made up according to our previous practice; a basic diet was modified in each specific instance through the omission or diminution of the vitamin in question. Those used in the present work were: 2515, pyridoxine deficient (no added pyridoxine); 2516, partially thiamine deficient (0.04 mg thiamine HCl per 100 gm diet); 2517, pantothenate deficient (no added pantothenate); 2520, complete diet. The small allowance of thiamine in diet 2516 was necessary to ensure survivals comparable with those on the other two deficiencies.

The gross diet composition was: "vitamin free" casein,[2] 18; L-cystine, 0.2; salt mixture,[3] 3.7; cottonseed oil carrying vitamins A and E supplement,[4] 5; cellulose [5] carrying vitamins B and K supplements,[6] 1; DL-cysteine HCl (as antioxidant), 0.05; glucose [7] to 100.

*Rats.* As in previous investigations three strains (9B, 13C and 14C) developed in this laboratory were used.

*Corynebacterium 197 cultures.* All experiments were done with the same master cultures of *Corynebacterium*, and during the same period, as those of Seronde et al. ('55). The dose, as previously, was 0.15 ml of a 24-hour broth culture given intraperitoneally.

*Procedure, spontaneous infection.* In addition to our past experience with spontaneous corynebacterium infection in pantothenate-deficient rats, 5 animals were put on our panto-

[2] Labco.

[3] Salt mixture, main constituents in grams per 100 gm diet: bone ash, 2.5; NaCl, 0.38; KCl, 0.43; $MgSO_4$, 0.33; $FeSO_4$ ("dried"), 0.057; trace elements in milligrams per 100 gm diet: NaF, 2.1; ZnO, 1.2; $MnSO_4$ ("dried"), 0.72; $Cu_2O$, 0.40; $AlK(SO_4)_2 \cdot 12H_2O$, 0.33; KI, 0.18.

[4] A and E supplement, per 100 gm diet: 1,500 units vitamin A activity as carotene concentrate; 5 mg alpha tocopherol acetate.

[5] Cellu flour.

[6] B and K supplement, per 100 gm diet (complete diet 2520); thiamine HCl and pyridoxine HCl, 1 mg each; riboflavin, 2 mg; calcium pantothenate and niacin, 4 mg each; biotin, 0.02 mg; folic acid (PGA), 0.2 mg; vitamin $B_{12}$, 1.2 $\mu$g; choline chloride, 100 mg; p-aminobenzoic acid, 10 mg; inositol, 20 mg; 2-methylnaphthoquinone (Menadione), 0.5 mg.

[7] Cerelose.

TABLE 1

*Plan of inoculation experiments*

| | DEFICIENCIES AND DIET NUMBERS | | | | | | |
|---|---|---|---|---|---|---|---|
| | None 2520 | Pyridoxine 2515 | Calorie restricted 2520 | Partial thiamine 2516 | Calorie restricted 2520 | Pantothenic acid 2517 | |
| | | | | | | 9B Strain | 13C Strain |
| Number of animals | 43 | 11 | 18 | 14 | 6 | 11 | 11 |
| At 40 days on diet | | 4 killed | | 6 killed | | | |
| At 50 days on diet | 43 killed | 6 killed | 18 killed | 3 killed | 6 killed | 11 killed | |
| Spontaneous deaths (days on diet) | None | 1 at 46 days | None | 1 at 36 days<br>1 at 39 days<br>1 at 43 days<br>1 at 44 days<br>1 at 47 days | None | None | 1 at 31 days<br>3 at 32 days<br>2 at 33 days<br>2 at 37 days<br>1 at 40 days<br>2 at 41 days |

thenate-deficient diet during the course of these experiments, and three of these became infected. This was done in order to assure us of the continued presence of the organism in the animal colony. At the same time 17 rats were placed on the pyridoxine-deficient diet, and 16 on partial thiamine deficiency, and continued until they died. The starting age was 3 to 4 weeks in all three groups. Cages were interspersed with those of animals that were inoculated with Corynebacterium. There was thus insured equal opportunity for all groups to contract a spontaneous infection, providing they were susceptible.

*Procedure, inoculated animals.* The principal features of this section are outlined in table 1. Animals were placed on their respective diets at 3 to 4 weeks of age, and received an injection of *Corynebacterium* 30 days thereafter. The third and 5th columns represent groups of rats whose total caloric intake was restricted to make their growth curves imitate, respectively, those of pyridoxine and partial thiamine deficiency. Two columns were necessary for expression of the marked strain differences that exist in pantothenate deficiency. For the other deficiencies slighter strain differences may exist, but they do not appreciably affect results reported here.

All cages had ½ inch mesh bottoms. Animals that were not injected were at first kept several to a cage. As they became enfeebled by their deficiencies they were separated. The rats of the injected groups were caged individually from the start. Autopsies were done on all cases and material was taken for histological examination. The *Corynebacterium* was easily recognized in Gram stained smears, and occasional cultures were made from lesions.

## RESULTS: SPONTANEOUS INFECTION

The various pertinent data on the three deficiencies are summarized in figure 1 (growth) and in table 2.

*Pantothenic acid deficiency.* We are summarizing in this report some of the previously published observations on pantothenate deficiency, together with additional data. As

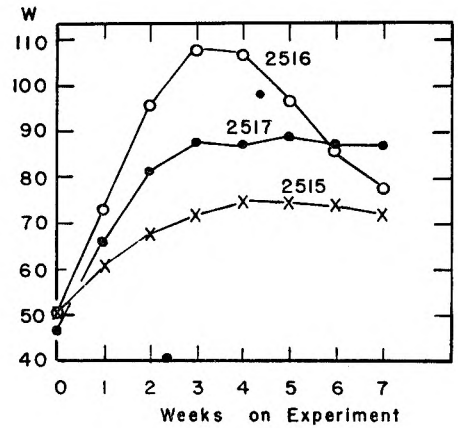


Fig. 1 Diet 2515 = pyridoxine deficiency; 2516 = partial thiamine deficiency; 2517 = pantothenate deficiency. The graph shows the average growth of rats started at three weeks of age on the three deficiencies. Each sex and strain group (6 groups in all) was averaged, and the resulting 6 means averaged to give the above curves.

TABLE 2

*Spontaneous infection with Corynebacterium 197 in deficiencies of three B factors*

| DEFICIENCY | DIET | NO. OF RATS | SURVIVAL (DAYS)[1] Started 3 wks. | SURVIVAL (DAYS)[1] Started 4 wks. | SIGNS OF DEFICIENCY (FOR GROWTH SEE FIG. 1) | CORYNEBACTERIUM INFECTION |
|---|---|---|---|---|---|---|
| Pantothenate | 2517 | 116 | 57 (21–83) | 77 (23–100) | Porphyrin on fur and whiskers<br>Lousiness<br>Adrenal hemorrhage and necrosis | Widespread |
| Pyridoxine | 2515 | 17 | 58 (45–87) | 98 (79–131) | Dermatitis of paws, lips, nose[2]<br>Muscle wasting<br>Kidney enlargement<br>Cardiac enlargement<br>Pleural and peritoneal effusion[3] | Zero incidence |
| Partial thiamine | 2516 | 16 | 54 (40–63) | 56 (40–76) | Low body temperature<br>Loss of muscular coordination<br>Muscle wasting | Zero incidence |

[1] All rats were allowed to die spontaneously.
[2] More severe and of earlier onset in the 9B strain.
[3] So far seen only in the 14C strain.

has been shown, rats placed at weaning on diet 2517 may generally be expected to develop corynebacterium disease, with an incidence of over 50% in the more susceptible 13C and 14C strains, and over 10% in the more resistant 9B strain. Once recognized, this condition has been easy to distinguish grossly from any of the other known respiratory diseases of the rat. Upon opening the chest, free serofibrinous exudate is generally encountered, and is accompanied by opaque white nodules both upon and within the tissues. Adhesions are common. Abdominal organs are involved to a variable and lesser extent by more solitary lesions, presumably of hematogenous origin.

*Pyridoxine deficiency.* In contrast to the pantothenate-deficient rats, those on pyridoxine deficiency developed outwardly, marked signs of physical deterioration, including weight loss, wasting of muscles, redness and scaling of muzzle and paws. Most of the rats showed cardiac and renal enlargement with, in a few cases, non-purulent, non-fibrous pleural and sometimes peritoneal effusion consistent with cardiac failure. No cases of corynebacterium disease were encountered. Schneider ('46) has noted examples where general undernutrition and poor physical condition have been responsible for lowered resistance to infection. In spite of their extremely poor condition, pyridoxine-deficient rats maintain their resistance to the corynebacterium infection, while the pantothenate-deficient rats, of much healthier appearance, succumb.

In connection with the cardiac findings, it is of considerable interest that Street et al. ('41) have described "large accumulations of serous fluid in the thorax, dilatation and hypertrophy of the right auricle and right ventricle, and chronic passive congestion of the liver" in dogs deprived of pyridoxine.

Some years ago we reported enlarged kidneys on diets deficient in the whole vitamin B complex (Zucker and Zucker, '46). Data published by Agnew ('49, '51) indicate that the responsible factor was pyridoxine. He, and later Olsen and

Martindale ('54), reported enlarged kidneys and hearts in pyridoxine-deficient rats. Our present data support and extend these findings and will be reported elsewhere in detail.

*Partial thiamine deficiency.* As in pyridoxine deficiency, and in contrast to the pantothenate-deficient rats, this group showed marked outward signs of physical deterioration. The animals began losing weight rapidly about three weeks after being placed on their diet. During the final week they showed muscular incoordination and lowering of body temperature. Autopsy showed marked muscle wasting. Again, no cases of corynebacterium disease were encountered, although the lungs frequently were partially atalectatic, with varying degrees of edema and occasional local hemorrhages. It is possible that these changes are attributable in part to the marked collapse of the thoracic cage and atrophy of muscles of respiration found in the thiamine-deficient rats. At any rate, these changes bore no resemblance whatever to corynebacterium infection.

In view of cardiac changes in beri-beri, and since we noted marked cardiac hypertrophy in pyridoxine deficiency, it is of interest that there was no difference in heart weight between thiamine-deficient rats and calorie controls (pair weighed).

## RESULTS: INOCULATION EXPERIMENTS

While the observation stands, that in our experience only in pantothenate deficiency do we see spontaneous corynebacterium disease, inquiry into the mechanism of this phenomenon is obviously a fundamental and long term project. Meanwhile pertinent information has been derived from studies of artificially-produced infections where the primary barrier of the animal has been bypassed. We choose the intraperitoneal route for its convenience and apparent simplicity.

The results of the inoculation experiments are summarized in table 3. Severity of infection has been expressed on an arbitrary scale which we offer as an approximate solution to this complex problem. Values were arrived at by enumerat-

ing and measuring lesions, and applying judgment of their location and microscopic appearance. Numerals entered in various positions along the scale represent how many animals were found infected to the same degree in a given diet group. The position of each numeral indicates the severity of corynebacterium infection found. Thus zero on the scale signifies absolutely no evidence of infection, past or present. Numerals in positions near zero in general stand for animals with healing or minimal lesions. Positions over 50 but below 100 correspond to severe infections in animals that were either killed before natural death could supervene, or that were overcome by the steadily developing deficiency state before the infection might have done so. One hundred was assigned to all animals that showed unchecked spread of the infection which presumably was the cause of death.

Referring to the first line of table 3, it will be noted that only one rat on the complete diet fed ad libitum had more than an inconsequential infection. This proved to be a large abscess confined to the *subcutaneous* tissue at the injection site, where an inordinate amount of the original inoculum may perhaps have been delivered. Animals on the same diet but fed in restricted amounts (line 2, table 3) do not differ materially from the first group. Of the eleven pyridoxine-deficient rats that were injected, two had overwhelming corynebacterium infections at autopsy. The table shows a number with less involvement, and one that recovered completely. Those on partial thiamine deficiency were more seriously affected, 4 animals having overwhelming infection. A 5th (rated as "55"), had several large peritoneal abscesses at the time of sacrifice, 10 days after injection. Thus far in the table, no convincing differences between strains were found worth reporting. However the last two lines of the table show the large strain difference found in pantothenate deficiency. When pantothenate deficient, strain 9B is hardly more susceptible to parental infection than are animals deprived of pyridoxine or

TABLE 3

*Autopsy results of inoculation experiments*

| Deficiency | SEVERITY OF INFECTION [1] (ARBITRARY SCALE) | | | | |
|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 |
| None<br>43 rats | 31 6 3 1 1 | 1 | | | |
| Calorie<br>24 rats | 14 3 3 | 2 1 1 | | | |
| Pyridoxine<br>11 rats | 1 3 1 | 1 | 1 | 1 | 1 |
| Partial thiamine<br>14 rats | 2 1 | 1 1 1 1 1 1 | 1 | 1 | 3 |
| Pantothenate<br>11 9B rats | 2 | 1 1 | 1 2 1 | 2 | 1 |
| Pantothenate<br>11 13C rats | | | | | 11 |

[1] Figures are numbers of rats, position of the figures moving to the right across the table represents increasing severity of infection.

thiamine. In contrast, the 13C strain has 100% fatalities from the injected organism. These animals survived only from one to 11 days.

## DISCUSSION

Resistance to infection may be regarded as a defense in depth. As such it would have many component metabolic mechanisms, operating at more or less well-defined anatomical locations. Examples of such locations include the skin, bypassed by artificial parenteral administration of organisms, the blood plasma, the interior of the phagocyte, the mesothelium, and others. The metabolic mechanisms for resistance to infection may be expected to be as vulnerable to dietary deficiencies as are any others, but selective detriment to such mechanisms through diet has not often been documented. Stoerk and Eisen ('46) first studied changes in immune body formation in rats on various vitamin deficiencies. Further progress, including his own numerous studies, has been reviewed by Axelrod ('53). This work is however concerned only with injection of antigens as such, and not with actual infection. Others (Robinson and Siegel, '44) have noted differences in the effects of nutritional deficiencies upon natural infectious disease and artificial inoculation. That such differences should exist is not surprising if one considers the primary barrier to a given organism merely as one of the several locations of defense mechanisms: Such differences in effect would suggest that the given nutrient produced its effect specifically in one defense location and not in another.

According to this general scheme, our own present findings indicate that the young rat's primary barrier to the corynebacterium depends upon an adequate intake of pantothenate. The location of this barrier is still in doubt, and its metabolic mechanism is even more obscure. Our experiments also show that neither pyridoxine nor thiamine are crucial in this primary barrier mechanism. On the other hand, when the resistance of a young rat to the corynebacterium is challenged in a different location (on the peritoneum) the resistance mech-

anisms normally operative there are found to be dependent to some extent upon adequate dietary pyridoxine and thiamine.

The complexity of the entire problem is multiplied by the probability that conditions will vary from one host species to the next. Moreover, that no two potential invading organisms need be affected in the same way is exemplified by our findings: of all the saprophytes inhabiting the rat as potential invaders (Nelson, '30), only one has so far been observed to take regular advantage of pantothenate deficiency. Our data on strain differences, illustrated in the last two lines of table 3, show that the importance of a given nutrient in natural resistance varies even within a single species. Implications of such metabolic individualism may offer an unwelcome challenge to the simpler concept of a blanket "minimum daily requirement" for every known food factor.

## SUMMARY

A corynebacterium which produces spontaneous disease in young pantothenate-deficient rats failed to do so in similar animals deficient in either pyridoxine or thiamine, or in animals whose caloric intake was severely restricted. The disease does not appear in healthy rats on a complete diet.

Injected into animals on these various regimens, this organism caused fatal infection in all pantothenate-deficient rats of one strain, and in a relatively small percentage of pyridoxine- and thiamine-deficient rats. Another strain of rats on pantothenate deficiency proved more resistant. The complete diet, even when restricted in amount, protected the inoculated animal against serious infection.

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# ANTIBODY FORMATION AND NATURAL RESISTANCE IN NUTRITIONAL DEFICIENCIES [1]

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Pyridoxine deficiency has been shown by Stoerk and Eisen ('46) and by Axelrod and coworkers ('47) to lead to loss of the ability to form antibodies (hemagglutinins). In a similar system Axelrod ('53) has studied the effect of other deficiencies. Agnew ('49) confirmed the pyridoxine findings with a killed culture of *B. typhosum (S. typhi)*. Recently Pruzansky and Axelrod ('55) also studied the response to a bacterial antigen (diphtheria toxoid), and stated: "The utilization of this antigen would also relate our studies more closely to the effects of vitamin deficiencies on resistance to infection." As has been stated (Zucker and Zucker, '54), in such experiments each host species and particular kind of antigen must be considered separately. And indeed Pruzansky and Axelrod find notable differences in the effect of the same nutritional deficiencies in response to dipththeria toxoid as against the response to red cells. However, it appears that pyridoxine deficiency has the same striking effect with a variety of antigens: sheep red cells (Stoerk and Eisen, '46; Stoerk, Eisen and John, '47), human red cells (Axelrod et

[1] We gratefully acknowledge assistance from the National Vitamin Foundation, Hoffmann-LaRoche Inc., and Red Acre Farm, Inc., Stow, Mass.

al., '47), *B. typhosum* (Agnew, '49), toxoid of *Corynebacterium diphtheriae* (Pruzansky and Axelrod, '55), and now a strain of *Corynebacterium kutscheri*.[2] We designate our particular strain as *C.* 197.

The question as to how closely related the degree of antibody response is to problems of infection depends on whether or not the sole or principal mechanism of defense involves antibody formation. There are many defense mechanisms against infection (see Raffel, '53, pp. 3 to 21, and Nungester, '51), and the warding off or recovery from infection may proceed efficiently even when there is no existing antibody level, and no capacity of the host to produce antibodies quickly.

Our data deal with agglutinin response in deficiency of thiamine, pantothenic acid and pyridoxine in response to *C.* 197, the same deficiencies whose effect on susceptibility to infection with the same *C.* 197 has been discussed in the preceding paper (Seronde et al., '56).

## EXPERIMENTAL

*Rats.* Males of the 13C strain developed in this laboratory were used exclusively.

*Diets.* The pantothenate-deficient diet 2517, pyridoxine-deficient diet 2515, partially thiamine-deficient diet 2516, and complete diet 2520, differ only in the presence or absence of the pertinent vitamine. Compositions have been given elsewhere (Seronde et al., '56). Each diet contains all other known factors essential for the rat.

*C. 197 culture and vaccine.* Details of preparation and standardization of cultures have been described elsewhere. This work was contemporary with the work of Seronde et al.

[2] *C. kutscheri* is the name given in the main listing in Bergey's Manual of Determinative Bacteriology, 6th edition, 1948. Less preferred names given there for the same type of organism are *C. pseudotuberculosis murium* and *C. murium*. A number of similar less well identified organisms are listed in the appendix. The property of particular interest to us is that the organism is the causative agent of a natural mouse disease which however has never been seen in any but experimentally conditioned rats. The rat is also highly resistant to any reasonable doses given by inoculation.

('55), and the same master cultures were used. A single batch of 48-hour broth culture, killed by the addition of 0.5% formalin and kept in the refrigerator, was used for all the vaccine and antigen preparations. On the days vaccine was required, the cells were centrifuged down, suspended in sterile saline, recentrifuged, and resuspended in sterile saline of sufficient volume to give a standard spectrophotometer reading at 700 mμ corresponding to 4.1 on the MacFarland scale (Kabat and Mayer, '48). This is about three times the optical density of the original culture.

*Procedure.* The animals were started on the various diets at three weeks of age, and caged individually in screen-bottom cages (½ in. mesh). Rats on the deficient diets were fed ad libitum, while those on a restricted intake of the complete diet received a measured amount of food daily. Each of these latter was pair-weighed to a vitamin-deficient littermate so as to match its course of growth. On the 30th, 32nd and 34th day of the experiment vaccine was given intraperitoneally. For the pantothenate- and pyridoxine-deficient groups and their controls the dosage schedule was ½, 1 and 1 ml; for the thiamine-deficient group and its controls it was ½ ml each time. After 39 days on experiment, 5 days after the last vaccine injection, blood was collected from the tail.

*Agglutination.* The serum was separated (usually without centrifuging) after standing overnight in the refrigerator. Serum dilutions were used only on the day they were made, but determinations starting out with undiluted serum were carried out over a period of up to a week after collection. Serial dilutions were made in the usual way with a twofold dilution at each step. The final solution volume was 0.4 ml (0.2 ml of serum dilution, 0.2 ml of antigen suspension) in 10 × 75 mm tubes. Because of the considerable volumetric error involved in operations on this small scale, no more than 4 successive serial dilutions were employed for the final reading.

The antigen suspension to be added to the serum dilutions was prepared just like the vaccine, except that the saline

used for washing and final resuspension contained 0.5% of formalin. The antigen-serum mixtures were kept at 37° C. for 4 hours, mixed again, refrigerated overnight and then read. The reading was based on the pattern in the bottom of the undisturbed tube. Control and over-diluted tubes showed a tight smooth button of sediment in the center of the bottom, with no streamers. A slight enlarging of the button and irregularity of the edges with short streamers was read as $\pm$; this reading was disregarded in the score but was often useful in locating the end-point range. The end-point was taken as the greatest dilution (before addition of the antigen suspension) which showed a non-uniform clumped sediment extending essentially over the bottom of the tube.

Several vaccine and many antigen preparations were used in this work, all coming from the same batch of killed culture. The reproducibility of the antigen preparations was readily checked against a single high potency serum preserved with 0.25% phenol, a procedure kindly suggested by Dr. Kabat. In all agglutination runs this standard serum was included and gave the same result in the sense of not varying by more than one dilution step. Variation in biological effectiveness of the vaccine preparations could not be controlled, although during the 4-week period covering the full-fed and restricted controls for the pantothenate and pyridoxine deficiencies, successive small lots of controls reacted with about the same agglutinin titers to the different vaccine preparations. The thiamine deficiency experiment, being run later and with a smaller dosage, had its own controls. Its results cannot be directly compared in magnitude with the others.

## RESULTS

Table 1 presents exploratory investigations on the agglutinin titers of unvaccinated animals, together with the agglutinin response to various doses of vaccine. Titers in unvaccinated animals of this age range are insignificant. It does not appear that larger doses of vaccine would lead to any

TABLE 1

*The normal agglutinin response: effect of dose*

| | | AGGLUTININ TITER | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | < 1 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
| | | Number of rats | | | | | | | | | |
| Vaccine per 100 gm rat[1] | 3 × 1.0 ml | | | | | | | | | 2 | 1 |
| | 3 × 0.45 ml | | | | | | | | | | 3 |
| | 3 × 0.15 ml | | | | | | 1 | | 1 | 1 | |
| | None | 6 | 9 | 3 | 1 | | | | | | |

[1] Stock rats aged 6 to 8 weeks, weighing 100 to 200 gm.

TABLE 2

*Agglutinin response in deficiency of pyridoxine and pantothenic acid*

| | AGGLUTININ TITER | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | < 1 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
| | Number of rats | | | | | | | | | |
| Calory deficient[1] | | | | | | | 1 | 1 | 7 | 4 |
| Pantothenate deficient | 7 | 2 | 1 | | 1 | 3 | | | | |
| Pyridoxine deficient | 7 | 3 | | | | | | | | |

[1] Pair-weighed to pantothenate-deficient rats.

Vaccine: 0.5, 1 and 1 ml. Body weight 90 to 105 gm for pantothenate-deficient rats, 60 to 75 gm for pyridoxine-deficient rats.

TABLE 3

*Agglutinin response in deficiency of thiamine*

| | AGGLUTININ TITER | |
|---|---|---|
| | 32 | 64 |
| | Number of rats | |
| Calory deficient[1] | 1 | 4 |
| Thiamine deficient (partial)[2] | 3 | 2 |

[1] Pair-weighed to thiamine-deficient rats.

[2] 0.04 mg thiamine hydrochloride per 100 gm diet. This allows survival times comparable to these on the other two deficiencies. These thiamine-deficient rats are losing weight during the vaccination period (see Seronde et al., '56, for typical growth curve).

Vaccine: 0.5, 0.5 and 0.5 ml. Body weight 100 to 115 gm at time of first injection.

greater response at this age. In adult animals higher titers have been obtained.[3]

The agglutinin titers of the pyridoxine- and pantothenate-deficient rats, and controls, are shown in table 2. Pyridoxine deficiency entirely suppressed agglutinin production, while pantothenate deficiency seriously inhibited it, without complete suppression. In table 3 the results in thiamine-deficient rats and controls are presented. In agreement with Axelrod ('53) it appears that this deficiency does not significantly depress antibody response.

The pyridoxine-deficient rats did not tolerate the vaccination very well. Contemporary unvaccinated rats (Seronde et al., '56) lived 58 days (range 45 to 87). Of the 13 pyridoxine-deficient rats started in the present work, all of which did well up to the time of vaccination, three died during the vaccination period before blood titers could be obtained, and 4 more died soon after. No deleterious effect of vaccination was noted in the other two deficiencies.

## DISCUSSION

The relative agglutinin response to vaccine of *C.* 197 in the three deficiencies is much like that recorded by Pruzansky and Axelrod ('55) for purified toxoid of *C. diphtheriae.* The absolute values are irrelevant, depending on kind and amount of antigen and other points of technique.

Our present data show in part that (a) pyridoxine deficiency totally inhibits agglutinin formation to *C.* 197, and (b) partial thiamine deficiency leaves such antibody formation intact. And yet we have found that *both these groups are equally resistant to spontaneous, naturally acquired corynebacterium infection* (Seronde et al., '56). Moreover we have found that the pyridoxine-deficient group withstands intraperitoneal inoculation of living culture better than does the thiamine-deficient group. It would thus appear that the antibody we have measured has nothing to do with the impervious-

[3] Seronde, Zucker and Zucker, unpublished data.

ness of these two groups to the natural infection; nor that it had much more effect upon the course of infection artificially induced. Considering now young pantothenate deficient rats, it would appear most doubtful that the absence or reduction in agglutinin response was responsible for their unique susceptibility both to natural and artificially induced corynebacterium disease.

It may be suggested that the (partial) thiamine deficiency makes the animals subject to inoculated infection for other reasons than the deficiency per se. It is known that in extreme thiamine deficiency body temperature drops, and also that lowered body temperature may predispose to infection. However, at the time of inoculation these rats still had normal temperatures. Only towards the very end of their lives were low temperatures noted in our thiamine-deficient rats. But under these conditions of falling body temperature, the animals in the spontaneous infection experiment showed no infection.

It may also be objected that alteration of virulence of the organism may play a role equal to that of lowered resistance of the host. In particular, pyridoxine or some metabolic product for which it is responsible might be a critical requirement for the growth or invasiveness of the organism. The low susceptibility of the pyridoxine-deficient rats would then be no obstacle to the antibody hypothesis. However, we have found that rats on the pyridoxine-deficient diet for 40 days are much more susceptible to inoculation than after the standard 30 days.[4] Significant loss of virulence of the organisms due to the pyridoxine deficiency of the host therefore seems unlikely.

Dubos states (Dubos, '54, p. 115): ". . . it is plain that the microbial diseases for which there is no explanation whatever of immunity mechanisms far outnumber those where susceptibility and resistance can be explained in terms of recognized immunological reactions — cellular or humoral." The data of this report together with those of the preceding one (Ser-

[4] Zucker, Seronde and Zucker, unpublished data.

onde et al., '56) indicate that the situation with which we are dealing belongs to that majority in which antibody formation plays at most a very minor role. This applies both to the incidence of spontaneous disease and to inoculation experiments at 7 to 8 weeks of age. We shall show elsewhere [3] that sucklings may be partially protected by passive immunity as indicated by agglutinin titer, and that adults usually have some antibody which may be protective. The well-established effects of nutritional states on antibody formation made it necessary to establish first of all to which category the relation of *Corynebacterium kutscheri* to the rat belongs as far as the nutrition experiments are concerned.

The answer as to the role of antibody formation is easily obtained. This being negative, there remains the more complex task of ascertaining which of the many biochemical determinants is of major importance. Since the pertinent experimental condition — pantothenate deficiency — is biochemical it will be natural enough for the nutrition biochemist to think of the determinants as being biochemical, especially since according to Dubos the effects of phagocytosis, inflammation, fibrin formation, etc., which are all concerned in the host-invader system can be considered in biochemical terms as well as the bodily synthesis of directly anti-microbial substances. Such substances are of varied type and often selective in their action. Examples are: lactenin, lysozyme, two basic peptides, heme compounds which are discussed by Dubos (Dubos, '54), and a lipid soluble component of tissues recently studied by Henley and Nungester ('55). Another type of anti-microbial substance reported recently is the properdin of Pillemer et al. ('54). A review by Nungester ('51) deals with some of these substances and with more general aspects of defense mechanisms.

## SUMMARY

A vaccine prepared from killed cultures of *Corynebacterium kutscheri,* strain 197, has been administered to rats with various dietary deficiencies: calories, thiamine, pyridoxine and

pantothenic acid. With deficiencies of calories or thiamine, there is no significant reduction in ability to form agglutinins. No detectable agglutinins are formed in pyridoxine deficiency. In pantothenate deficiency, some animals lose their ability to form agglutinins while this capacity is impaired in others, resulting in a mean value between those for deficiencies of thiamine and pyridoxine.

Ability to make agglutinins is entirely unrelated to the degree of resistance to the live organism shown by rats on these various regimes. Therefore it is concluded that the resistance of the normal rat, which is lost in pantothenate deficiency, does not rest upon ability to form antibodies as typified by agglutinins.

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