BONE HEALING IN LYSINE-DEFICIENT RATS

ROBERT HAGGAR, THOMAS D. KINNEY AND NATHAN KAUFMAN
Departments of Pathology, Cleveland City Hospital, and Western Reserve University School of Medicine, Cleveland, Ohio

SEVEN FIGURES

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L-Lysine has been shown to be indispensable for the growth of young rats (Osborne and Mendel, '15; Harris et al., '43; Wissler et al., '48; Kligler and Krehl, '50, '52) and for the maintenance of body weight in the adult rat (Wolf and Corley, '39; Neuberger and Webster, '45; Gillespie et al., '45). It also is necessary for the maintenance of normal weight in the dog (Rose and Rice, '39). Lysine deficiency causes a negative nitrogen balance in man within a few days (Albanese et al., '41). In 1943, Harris et al., reported cessation of growth, hypoproteinemia, atrophy of the subcutaneous fat and muscles and decrease in epiphyseal cartilage growth in young rats maintained on lysine-deficient diets. It was demonstrated by Kligler and Krehl ('50, '52) that the lack of lysine prevented normal growth but allowed limited visceral and skeletal development, apparently at the expense of fat and carbohydrate reserves. In 1948, Fischer found that the omission of lysine from synthetic nutrient media resulted in diminished growth of osteoblasts in tissue cultures.

In view of the fact that lysine is necessary for normal bone growth, the present study was undertaken to determine the effect of lysine deficiency on the healing of bone in the adult rat.

EXPERIMENTAL

One hundred and four young adult, male rats of the Sprague-Dawley strain, with an average weight of 232 gm,

were individually housed in metal, screen-bottom cages. They were weighed, fed and watered daily.

The basal lysine-free diet (table 1) consisted of zein, glucose, corn oil, salts and the known vitamins, and was enriched by additional amounts of arginine, tryptophan, histidine and valine so as to meet the minimal essential amino acid requirements (Geiger and Hagerty, '49; Kligler and Krehl, '50).

TABLE 1

Composition of basal diet (lysine-free)

Group A

COMPONENTS	
	%
Zein [1]	22.3
L-arginine	1.0
DL-valine	1.0
DL-histidine HCl	0.5
DL-tryptophan	0.2
Salt mixture [2]	4.0
Dilute haliver oil [3]	0.5
Vitamins [4]	0.0056
Corn oil	10.5
Glucose [5]	60.0

[1] Zein and amino acids purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

[2] Salt mixture as recommended by Hegsted et al. ('41).

[3] Parke-Davis haliver oil, 1 ml diluted in 500 ml corn oil.

[4] Thiamine chloride 400 μg, calcium pantothenate 1.5 mg, pyridoxine HCl 400 μg, riboflavin 800 μg and nicotinic acid 2.5 mg.

[5] For group B, glucose 58.76 and L-lysine HCl 1.24 and group C, glucose 56.28 and L-lysine HCl 3.72 so as to supply 1 and 3% respectively, of active L-lysine.

The animals were divided into 4 equal groups. Group A received the basal, lysine-free diet; group B the basal diet containing 1% lysine; and group C the basal diet containing 3% lysine. These three groups were fed ad libitum. The animals of group D, the starvation controls, were given the basal diet plus 1% lysine, but in restricted amounts, so that their weight loss paralleled that of the lysine-free group A.

After a 21-day conditioning period on these diets, each animal was anesthetized with ether and the skin of each lower

hind limb was swabbed with Zephiran solution, followed by 70% alcohol. The anterior aspect of each tibia was exposed by a sharp skin incision, approximately 1.5 cm in length. A single longitudinal saw cut was made through the anterior cortex in the midportion of each tibia by an electric saw, the rotary cutter measuring 3/8″ in diameter and 1/32″ in width. By this method all the bone defects were of the same size while the surrounding intact bone served as a splint so that no movement or deformity occurred at the operative site. The skin incisions were sutured with 4–0 nylon and covered by a single application of 4% collodion.

Two animals from each of the 4 groups were sacrificed at one and one-half-day intervals up to 9 days, at three-day intervals up to 24 days, and at 32 and 40 days, respectively. The thorax was opened and, with the heart still beating, the ascending aorta was cannulated via the left ventricle. The vascular system was perfused with 40 ml of plasma, followed by 50 ml of 10% formalin. This method, modified from that suggested by Koenig et al. ('45), results in almost instantaneous fixation of tissues in the living animal with remarkable preservation of cytologic detail. The animals then were immersed in 10% formalin for two to 4 days. The hind limbs were removed by cutting through the midshaft of the femurs. The right legs were skinned and then decalcified in a formalin-trichloracetic acid solution for three and one-half days (Hanke, '33). Transverse blocks were taken from each tibia at the operative site, sectioned in paraffin at 6 μ and stained with hematoxylin and eosin and with Masson's trichrome. The left legs were skinned and each leg stained en bloc by Lillie's silver nitrate method (Lillie, '48), decalcified, sectioned in paraffin at 6 μ and stained with Masson's trichrome. In addition, longitudinal sections were taken from the upper end of each tibia.

RESULTS

The rate and degree of healing were comparable in all 4 groups. Within 36 hours the gap in the tibia was filled by a hematoma composed of fibrin, erythrocytes and neutrophils.

From the periosteum fibroblasts were proliferating at the cut edges and chondrocytes were developing from the deep, or cambium, layer. By 4 1/2 days the hematoma was almost completely organized and newly-formed fibrous connective tissue streamed through the bone defect into the marrow

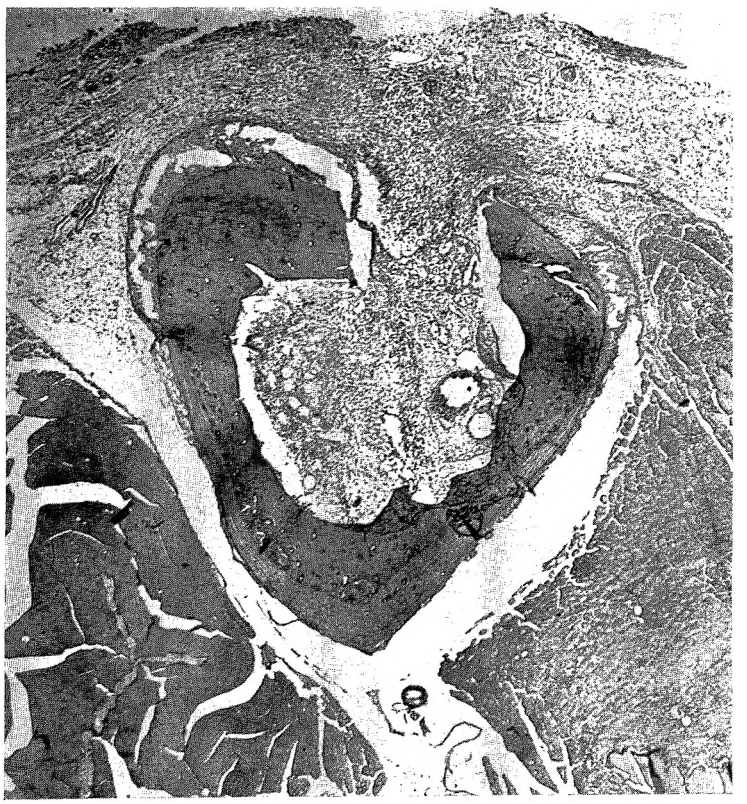

Fig. 1 Healing of defect in tibia at 4½ days — Group A (lysine deficiency) H and E × 30.

cavity (figs. 1 and 2). Spicules of sequestrated bone were being surrounded by large multinucleated foreign-body giant cells. Osteoid was beginning to appear within the fibrous callus and in the subendosteal and subperiosteal cartilaginous callus. At 7 1/2 days the fibrous callus was dense, serving as a temporary repair and also determining the manner in

which the subperiosteal osteoid fills in the defect. By the 9th day the subperiosteal osteoid had grown in from each side of the defect and turned inwards to fill the gap becoming fused with the osteoid within the marrow cavity. At the same time marginal calcification transformed the osteoid

Fig. 2 Healing of defect in tibia at 4½ days — Group B (1% lysine) H and E × 30.

into coarse, irregular, anastomosing bony trabeculae which bridged the gap. By the 12th day the subperiosteal osteoid was becomming transformed into bone and incorporated into the cortex. The bony trabeculae became thicker and fused so that by the 18th day compact bone was formed and the defect was filled. The osteoid which previously filled the marrow

cavity was resorbed and replaced by active marrow cells. Between 21 and 24 days healing was complete for all practical purposes (figs. 3 and 4). By the 32nd day all the defects contained a solid mass of bone. In this new bone the Haversian systems, instead of being arranged circumferentially,

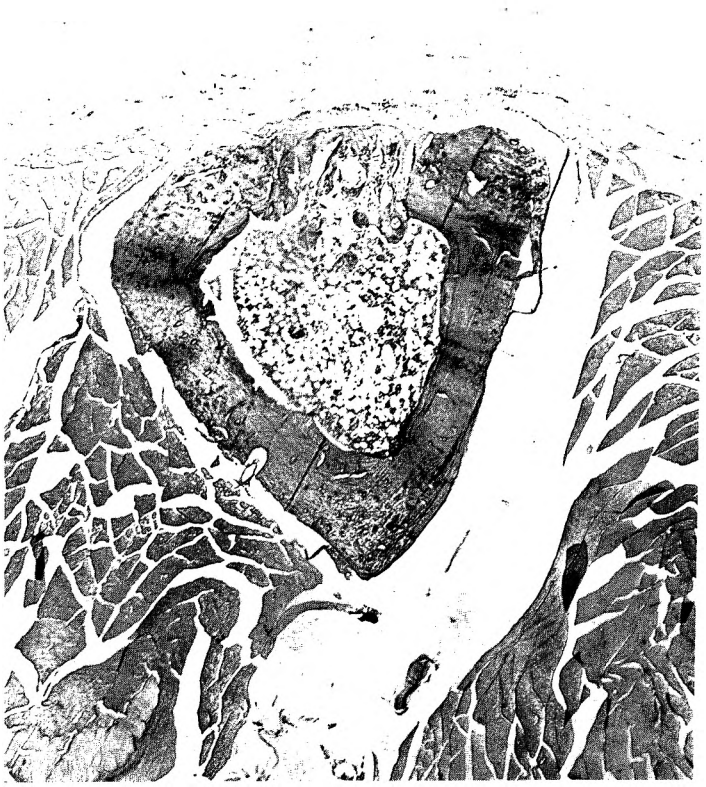

Fig. 3 Healing of defect in tibia at 24 days — Group A (lysine deficiency) H and E × 30.

passed through the site of the previous defect perpendicular to the cortex. Only in the deepest portion did the bone contour lines blend with those of the cortex.

With minor variations, the healing process proceeded at essentially the same rate and was of the same degree in all animals of the 4 dietary groups. This similarity in bone heal-

ing is in striking contrast to the differences seen in epiphyseal growth in the various dietary groups.

In groups B and C (the animals receiving 1 and 3% lysine, respectively), the epiphyseal plate at the upper end of each tibia appeared as a thick disc composed of orderly rows of

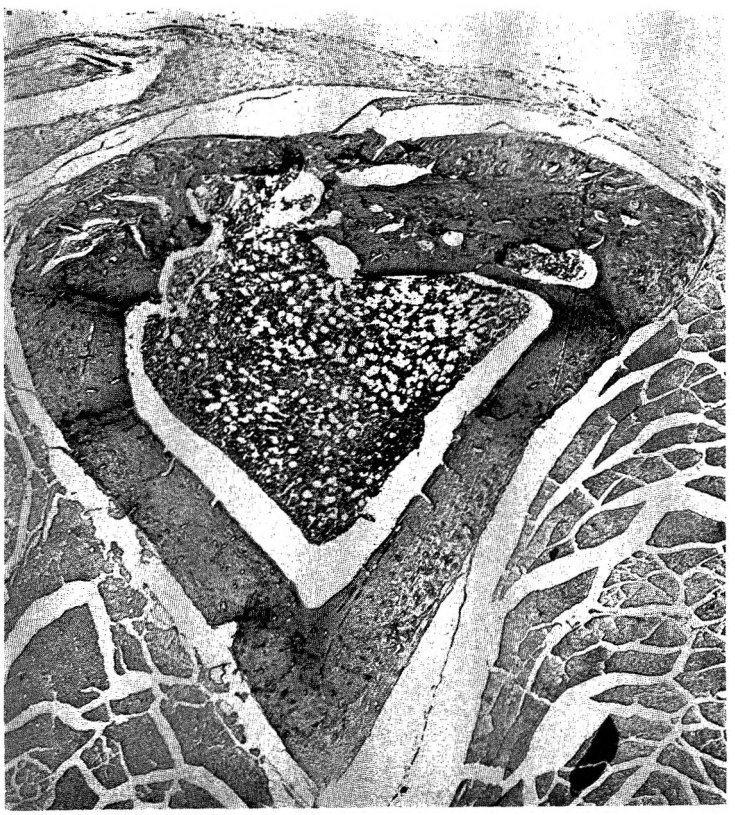

Fig. 4 Healing of defect in tibia at 24 days — Group B (1% lysine) H and E × 30.

proliferating cartilage cells with a distal row of discrete swollen chondroblasts at the zone of provisional calcification. Long, slender, bony trabeculae extended downward into the marrow cavity of the diaphysis (fig. 5). In groups A and D (the animals receiving the lysine-free diet and the starvation controls, respectively), the long bones were smaller. The

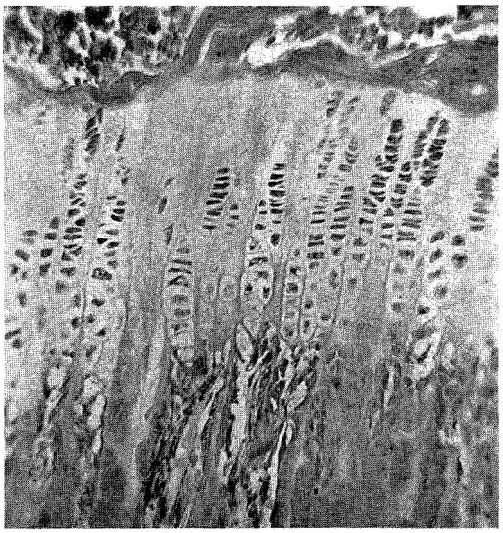

Fig. 5 Epiphysis at upper end of tibia — Group B (1% lysine) H and E × 150.

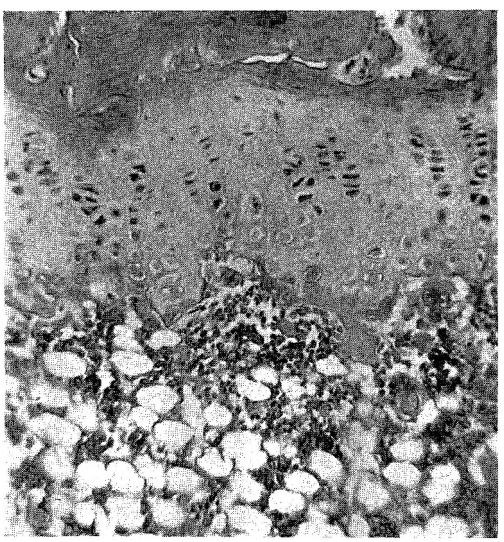

Fig. 6 Epiphysis at upper end of tibia — Group A (lysine deficiency) H and E × 150.

epiphyseal plates were somewhat narrowed due to a decrease in the actual number of proliferating cartilage cells which were no longer in orderly columns but had a tendency to become jumbled and disarrayed. In some sections the hypertrophic chondroblasts at the zone of provisional calcification were no longer discrete but closely packed and formed an almost continuous layer. The bony trabeculae were stubby, widened and irregular and, in some cases, were even completely absent, indicating failure of endochondral ossification and retardation of growth (fig. 6).

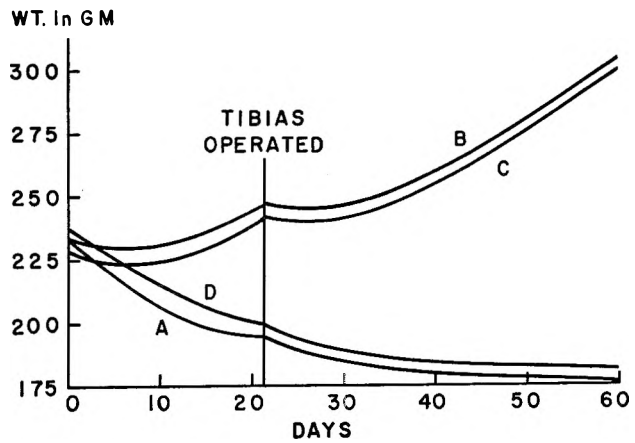

Fig. 7 Effect of lysine on weight.
Curve A — Basal, lysine-free diet.
Curve B — Basal diet containing 1% L-lysine.
Curve C — Basal diet containing 3% L-lysine.
Curve D — Starvation controls — same diet as B.

During the course of the experiment, a period of 61 days, the groups receiving the 1 and 3% lysine diets both gained weight steadily at an average rate of 1.3 gm per day. The animals receiving the lysine-free diet, and the starvation controls, lost weight steadily at an average rate of 1.0 gm per day (fig. 7).

Grossly, the lysine-deficient animals and the starvation controls exhibited considerable wasting of muscles, absence of subcutaneous fat and retarded skeletal growth. There was

a decrease in the size of the thymus, liver and testes out of proportion to the diminution in the over-all size of the animals. No significant consistent abnormalities, other than the atrophy described above, were found upon microscopic examination of the viscera.

DISCUSSION

The results described clearly show that although both lysine deficiency and restricted intake of a nutritionally adequate diet cause severe retardation of bone growth, they do not appreciably affect the healing of bone in the rat.

Zein, even when enriched with arginine, tryptophan, histidine, valine and lysine results in good but not optimum growth in adult rats. These findings are in accord with those reported by other investigators (Harris et al., '43; Geiger and Hagerty, '49; Kligler and Krehl, '50). Even though the over-all growth is not optimum in these experimental animals receiving an enriched and theoretically adequate diet, in appearance the epiphyseal growth regions are histologically indistinguishable from those that have been observed in casein-fed animals in this laboratory. Furthermore, the rate of healing in all groups is similar to that which has been described by Urist and McLean ('41) in rats receiving a complete stock diet, in that healing is complete within 24 days.

The quantity of food consumed by the animals of group A (lysine-free diet) totaled only 74% of the amount eaten by group B (1% lysine diet). This is in accord with the findings of Harris et al. ('43) who reported that lysine-deficient animals ate less. The loss of weight in animals fed lysine-deficient diets apparently is due in part to a decreased caloric intake and in part to a deficiency of lysine. Evidence for this thesis is the fact that in order for the control animals of the starvation group to lose weight at the same rate as the animals on the lysine-deficient diets, it was necessary to reduce the amount of food fed the control animals by an additional 20%.

Retarded skeletal growth in the rat has been demonstrated previously in lysine deficiency by Harris et al. ('43), in phenylalanine deficiency by Maun et al. ('45a) and Schwartz et al. ('51), in leucine deficiency by Maun et al. ('45b), in threonine deficiency by Scott and Schwartz ('53), in histidine deficiency by Maun et al. ('46) and Scott ('54) and by decreased intake of casein by Frandsen et al. ('54). It thus appears that a similar retardation of growth occurs in any of the above-mentioned specific amino acid deficiencies as it does with an insufficient intake of protein.

In kwashiorkor, which is a severe chronic protein malnutrition of children, the retarded skeletal growth appears to be identical to that occurring in the various deficiencies just described (Higginson, '54). It would be interesting to learn if there was any difference in the healing of fractures in these children. If so, it may be attributed to the fact that not only is there chronic protein malnutrition in these cases, but also a deficiency of various minerals and vitamins.

SUMMARY

Retarded epiphyseal growth results from lysine deficiency and starvation, but there is no appreciable interference with the healing of bone.

The effects of lysine deficiency are similar to those that occur with restricted food intake and include steady weight loss, retarded skeletal and visceral growth and atrophy of subcutaneous fat and muscles.

Increasing the dietary lysine from 1 to 3% has no effect on either growth or bone healing.

A method for producing uniform lesions in bone for purposes of making comparative studies of healing has been described.

LITERATURE CITED

ALBANESE, A. A., L. E. HOLT, JR., J. E. BRUMBACK, JR., M. HAYES, C. KAJDI AND D. M. WANGERIN 1941 Nitrogen balance in experimental lysine deficiency in man. Proc. Soc. Exp. Biol. Med., *48:* 728.

FISCHER, A. 1948 Amino-acid metabolism of tissue cells *in vitro*. Nature, *161:* 1008.

FRANDSEN, A. M., M. M. NELSON, E. SULON, H. BECKS AND H. M. EVANS 1954 The effects of various levels of dietary protein on skeletal growth and endochondral ossification in young rats. Anat. Rec., *119:* 247.

GEIGER, E., AND E. B. HAGERTY 1949 Growth of rats on hydrolyzed zein. Arch. Biochem., *21:* 239.

GILLESPIE, M., A. NEUBERGER AND T. A. WEBSTER 1945 Further studies on lysine deficiency in rats. Biochem. J., *39:* 203.

HARRIS, H. A., A. NEUBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Ibid., *37:* 508.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. D. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HANKE, M. T. 1933 Diet and Dental Health. The Univ. of Chicago Press, Chicago, Ill., p. 35.

HIGGINSON, J. 1954 Studies on human bone from South African Bantu subjects. II. Histopathological changes in the ribs of South African Bantu infants. Metabolism, *3:* 392.

KLIGLER, D., AND W. A. KREHL 1950 Lysine deficiency in rats. I. Studies with zein diets. J. Nutrition, *41:* 215.

———— 1952 Lysine deficiency in rats. II. Studies with amino acid diets. Ibid., *46:* 61.

KOENIG, H., R. A. GROAT AND W. F. WINDLE 1945 A physiological approach to perfusion-fixation of tissues with formalin. Stain Tech., *20:* 13.

LILLIE, R. D. 1948 Histopathologic technic. The Blakiston Co., Garden City, N. Y., p. 247.

MAUN, M. E., W. M. CAHILL AND R. M. DAVIS 1945a Morphologic studies of rats deprived of essential amino acids: I. Phenylalanine. Arch. Path., *39:* 294.

———— 1945b Morphologic studies of rats deprived of essential amino acids: II. Leucine. Ibid., *40:* 173.

———— 1946 Morphologic studies of rats deprived of essential amino acids: III. Histidine. Ibid., *41:* 25.

NEUBERGER, A., AND T. A. WEBSTER 1945 The lysine requirements of the adult rat. Biochem. J., *39:* 200.

OSBORNE, T. B., AND L. B. MENDEL 1915 The comparative nutritive value of certain proteins in growth, and the problem of the protein minimum. J. Biol. Chem., *20:* 351.

ROSE, W. C., AND E. E. RICE 1939 The significance of the amino acids in canine nutrition. Science, *90:* 186.

SCHWARTZ, C., E. B. SCOTT AND R. L. FERGUSON 1951 Histopathology of amino acid deficiencies. I. Phenylalanine. Anat. Rec., *110:* 313.

SCOTT, E. B. 1954 Histopathology of amino acid deficiencies. III. Histidine. Arch. Path., *58:* 129.

SCOTT, E. B., AND C. SCHWARTZ 1953 Histopathology of amino acid deficiencies. II. Threonine. Proc. Soc. Exp. Biol. Med., *84:* 271.

URIST, M. R., AND F. C. MCLEAN 1941 Calcification and ossification. I. Calcification in the callus in healing fractures in normal rats. J. Bone and Joint Surg., *23:* 1.

WISSLER, R. W., C. H. STEFFE, L. E. FRAZIER, R. L. WOOLRIDGE AND E. P. BENDITT 1948 Studies in amino acid utilization. III. The role of the indispensable amino acids in maintenance of the adult albino rat. J. Nutrition, *36:* 245.

WOLF, P. A., AND R. C. CORLEY 1939 Significance of amino acids for the maintenance of nitrogen balance in the adult white rat. Am. J. Physiol., *127:* 589.

EFFECT OF WATER INTAKE ON THE GROWTH RESPONSE OF POULTS AND CHICKS TO PENICILLIN [1]

S. J. SLINGER AND W. F. PEPPER
Department of Poultry Husbandry, Ontario Agricultural College, Guelph, Ontario, Canada

TWO FIGURES

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Braude and Johnson ('53) found that the addition of aureomycin to the diet of pigs caused a significant increase in urine output which could not be accounted for by the small increase in water intake. The authors suggested, among other things, that the antibiotic may have lowered the metabolic rate of the animals. Robinson et al. ('53) found that penicillin did not stimulate the growth of pair-fed pigs whose water consumption was controlled while a growth response was obtained in similar animals offered water ad libitum. Interestingly, excretion of water through urine and faeces was higer in penicillin-fed pigs than in the controls when water intake equated. Furthermore, water intake was considerably lower in the treated than in the control pigs when water was offered free choice. These results constitute evidence of greater water economy in pigs fed an antibiotic.

The present experiments were made to study the significance of the level of water intake on the growth response of poults and chicks to penicillin.

[1] This work was supported in part by a grant from Merck and Co., Inc., Rahway, N. J.

MATERIALS AND METHODS

The poult experiment involved 8 groups of 10 males and 10 females per group. The day-old Broad Breasted Bronze poults were vent-sexed, weighed individually and assigned to experimental groups on the basis of sex and weight. The effect of water intake on the growth response to the antibiotic was studied under conditions of both free choice and equated feed intake.

For the chick study 4 groups of 20 New Hampshire × Columbian Rock male chicks each were used to determine the effect of equating the water intake on the growth response to penicillin under conditions of ad libitum feeding. In previous work in our laboratory (Slinger et al., '54) no growth response to penicillin was observed in pair-fed chicks. It was therefore considered that little information could be gained by the use of pair-fed chicks for the present work. The day-old chicks were weighed individually and assigned to the experimental groups on the basis of weight. Both the poults and chicks were reared in electrically heated battery brooders with raised wire floors. Feed and water were supplied daily with similar groups of birds on the equalized feed or water intake regimes being offered the same amount of feed or water daily as was consumed by the groups on the free choice plan which consumed the least on the previous day. Water evaporation was measured daily and the amount of water given to the groups whose water intake was controlled included the amount which had evaporated from the same type of trough, in a similar location in the room, on the previous day.

Extra groups of birds were carried as spares to replace those which died in the experimental groups. These were from the same hatch of poults or chicks as the birds on experiment and were maintained on the test diets. If a bird died during the course of the trial it was replaced by one of the same sex and weight and which had been receiving the same diet. This simplified determination of the amount of feed or water to give and, since there was practically no mortality, probably introduced no appreciable error.

The composition of the poult and chick basal diets used is shown in table 1. Procaine penicillin G was included in these diets at the rate of 25 mg per kilogram for the groups receiving the antibiotic.

Records of feed consumption, water consumption and body weight were maintained daily for a 14-day period in the case

TABLE 1

Composition of basal diets

INGREDIENTS	POULT DIET	CHICK DIET
	%	%
Ground wheat	23.0	50.0
Ground yellow corn	15.0	10.0
Ground oat groats	5.0	...
Soybean oil meal (44% protein)	43.0	28.0
Stabilized animal tallow	1.0	...
Soybean oil	...	1.25
Dehydrated cereal grass	3.0	2.5
Meat meal (50% protein)	2.0	1.0
Fish meal (65% protein)	2.0	3.0
Dried buttermilk	2.0	2.0
Ground limestone	2.0	0.75
Steamed bone meal	1.5	1.25
Iodized salt	0.5	0.25
	gm/100 lb.	*gm/100 lb.*
Vitamin A oil (10,000 I.U./gm)	45.4	22.7
Dry vitamin D_3 (1,500 I.C.U./gm)	45.4	22.7
Manganese sulfate (65%)	6.0	5.7
DL-Methionine (feed grade)	11.3	11.3
Riboflavin	0.15	0.13
Niacin	0.75	...
Vitamin B_{12} supplement (3 mg/lb.)	45.4	22.7

of the poult experiment and for a 28-day period in the chick study. The birds were weighed in groups on all but the final day of each experiment when they were weighed individually.

RESULTS AND DISCUSSION

The final weight and feed efficiency data for the poult experiment are shown in table 2. The weight data were ex-

amined statistically by the method of analysis of variance. The effects due to penicillin and method of feeding, as well as the interaction between penicillin and method of feeding, proved to be highly significant ($P < 0.01$). The addition of penicillin also resulted in consistent improvement in feed efficiency. These results are in agreement with the previous findings of Slinger and Pepper ('54) and offer further evi-

TABLE 2

Effect of water intake on growth response of poults to penicillin

GROUP NO.	PENICILLIN	METHOD OF FEEDING	METHOD OF WATERING	AV. WT.[1]	RESULTS AT 14 DAYS OF AGE				
					Increase due to penicillin	Feed/gain	Feed consumption per poult	Water consumption per poult	Water/feed
				gm	%		*gm*	*ml*	
1	—	F.C.[2]	F.C.[2]	171.9		1.59	184	629	3.42
3	+	F.C.	F.C.	212.0	23.3	1.37	215	719	3.35
2	—	F.C.	Eq.[3]	164.3		1.60	174	589	3.38
4	+	F.C.	Eq.	201.5	22.6	1.38	201	589	2.93
5	—	Eq.[3]	F.C.	156.5		1.66	167	657	3.94
7	+	Eq.	F.C.	166.3	6.3	1.50	167	683	4.09
6	—	Eq.	Eq.	147.0		1.71	156	537	3.43
8	+	Eq.	Eq.	159.9	8.8	1.51	156	500	3.20

[1] Mean of 10 ♂ + 10 ♀ per group.

[2] F.C. = Free choice.

[3] Eq. = Equated as follows: Group 1 or 3 controlled feed for groups 5 and 7.
Group 2 or 4 controlled feed for groups 6 and 8.
Group 1 or 3 controlled water for groups 2 and 4.
Group 5 or 7 controlled water for groups 6 and 8.

dence that the growth response of poults to penicillin is mediated largely, though not entirely, through increased feed consumption.

Birds whose water intake was equated weighed somewhat less in all cases than similar groups receiving water on a free-choice basis. This difference proved to be highly significant statistically ($P < 0.01$). However, the growth response to penicillin did not appear to be materially altered by equating water intake with birds receiving feed either

ad libitum or on the restricted basis. Statistically, the interaction between penicillin and method of watering was not found to be significant at $P = 0.05$.

Values for feed and water consumption as well as the ratio of milliliters of water to grams of feed are also presented in table 2. No difficulty was experienced in getting the groups on the controlled-feeding regime to consume all the feed offered. However, equating the water intake proved to be somewhat of a problem in the case of poults on the controlled-feeding program. After the first 5 days the birds fed penicillin (group 8) refused to drink the amount of water consumed by the group controlling its water consumption (group 5). In an attempt to maintain equal daily water consumption for groups 6 and 8 after 5 days of age it was therefore necessary to restrict the amount of water offered these groups to a level below that indicated by the consumption of group 5. The method followed was to offer groups 6 and 8 the maximum amount of water daily which it was considered would be consumed by group 8. That this method did not prove completely successful is indicated by the water consumption figures in table 2. Obviously the penicillin-treated birds which were most severely restricted in feed had less desire for water than similar birds not fed the drug. In spite of this, the growth response due to penicillin was just as great as with pair-fed birds receiving water free choice. Similarly, with birds on the free-choice feeding regime, the addition of penicillin resulted in about the same growth response regardless of the method of watering in spite of the fact that the ratio of water to feed was considerably reduced by the antibiotic when water intake was equated.

In view of the influence of body weight on feed consumption it was considered of interest to calculate the daily feed and water consumption per unit of body weight. These data have been plotted in figure 1 for groups 1 and 3, in which case water and feed were fed free choice, and in figure 2 for groups 2 and 4, where feed was offered free choice and water was restricted.

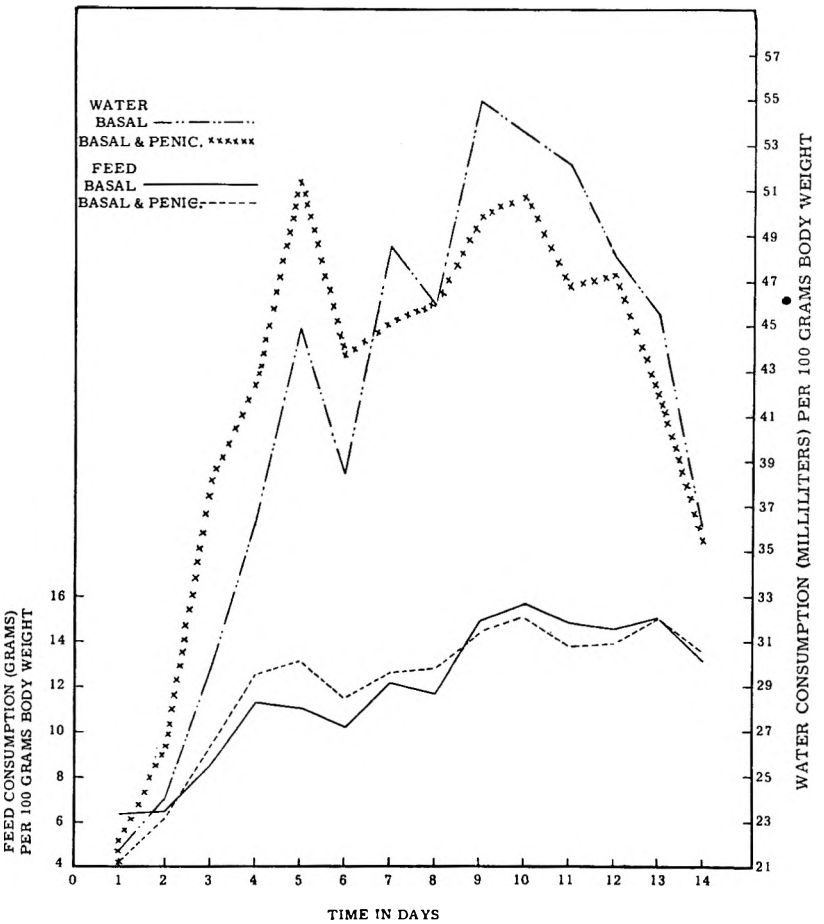

Fig. 1 Effect of penicillin on feed and water consumption per unit of body weight in poults given free access to feed and water.

It may be noted from figure 1 that penicillin caused an increase in feed and water consumption on a unit-weight basis only during the initial stages of the experiment after which there was a decrease in the treated birds as compared with the controls. The ratio of water to feed was slightly higher for the antibiotic-fed poults than the controls during the first 7 days (3.85 vs. 3.70) and somewhat lower during the last 7 days of the experiment (3.16 vs. 3.30). Since the increase

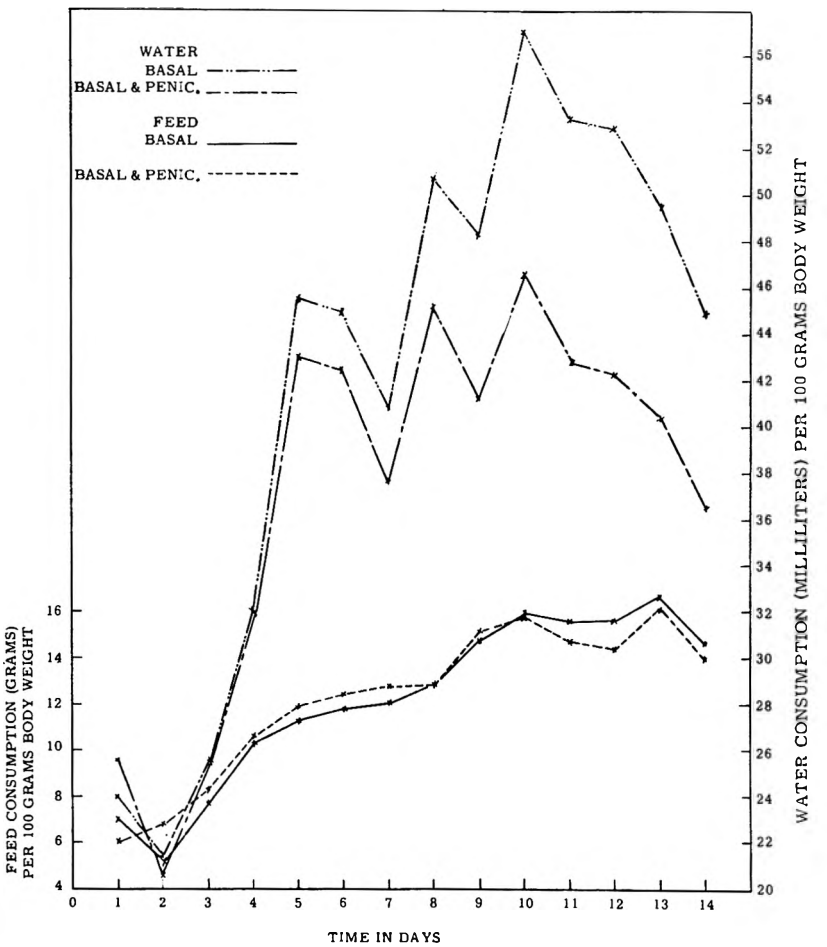

Fig. 2 Effect of penicillin on feed and water consumption per unit of body weight in poults given free access to feed but with water intake equated.

in logarithmic growth rate due to penicillin was greatest during the first week of the experiment it would appear from these data that enhanced water intake per unit of body weight may have been a factor, along with the increase in feed consumption, in mediating the growth response. On the other hand, examination of figure 2 indicates that while penicillin caused an increase in feed consumption similar to that shown in figure 1, the antibiotic at no time resulted in

an increase in water consumption, and, during most of the experiment, caused a rather marked decrease in water intake per unit of body weight. Since the growth response to penicillin was about as great under these conditions as when water consumption was increased it may be concluded that an increase in water intake is not necessary to elicit the growth response from penicillin in turkey poults.

Feed and water consumption data, on a unit weight basis, will not be presented for the groups whose feed intake was

TABLE 3

Effect of water intake on growth response of chicks to penicillin

GROUP NO.	PENICILLIN	METHOD OF WATERING	AV. WT.	RESULTS AT 28 DAYS OF AGE					
				Increase due to penicillin	Feed/gain	Feed consumption per chick	Water consumption per chick	Water consumption per 100 gm body wt.	Water/feed
			gm	*%*		*gm*	*ml*	*ml*	
1	—	Free choice	406.4		1.95	718	1316	324	1.83
3	+	Free choice	416.3	2.4	1.98	750	1321	317	1.76
2	—	Equated [1]	378.8		1.97	673	1200	317	1.78
4	+	Equated	400.6	5.4	2.00	725	1191	297	1.64

[1] Group 1 or 3 controlled water for groups 2 and 4.

equated. The relatively small growth increases due to penicillin under these circumstances are obviously not related to enhanced feed or water intake.

The results of the chick experiment at 28 days of age are shown in table 3. The use of the antibiotic resulted in only small and non-significant increases in weight and no improvement in fed efficiency. However, it is of interest that the growth response was at least as great with chicks whose water intake was restricted as when water was given ad libitum. Furthermore, penicillin caused a reduction in

both water consumption per unit of body weight and in the ratio of water to feed regardless of the method of watering. The results suggest that in the chick, as in the poult, increased water intake is not necessary to evoke the growth response from penicillin.

The chicks on the equated water regime which were fed penicillin refused, on several occasions, to consume the full amount of water indicated by the group controlling their water intake. This accounts for the slight discrepancy in absolute water consumption between groups 2 and 4. The data indicate that chicks fed penicillin had less desire for water, regardless of the system of watering, than the controls. These results are in essential agreement with those from the turkey experiment and suggest that penicillin tends to have a "sparing" effect on the water requirement of chicks and poults.

The method of equalizing water intake in these experiments involved some restriction since the birds on the controlled regime were a day behind the controls. It is of interest that in both the poult and chick work restricting the water intake caused a lowering of feed consumption and of body weight gains. This is in agreement with the observation of Crampton and Lloyd ('54) in work with rats. It is also noteworthy that turkey poults consumed considerably more water in relation to feed than did chicks. This is probably related to the difference in composition of the diets used.

SUMMARY

The effect of water intake on the growth response of turkey poults to penicillin was studied under conditions of both free choice and equated feed intake. Regardless of the method of feeding, the addition of penicillin resulted in about the same growth response in poults whose water intake was restricted as in those receiving water ad libitum. The interaction between penicillin and method of watering was not statistically significant.

A further study was made to determine the effect of equating water intake on the growth response of chicks to penicillin under conditions of free-choice feeding. While the increases in weight due to penicillin were not significant the response was at least as great with chicks whose water intake was restricted as when water was offered free choice.

In general, chicks and poults fed penicillin exhibited less desire for water than the controls. The results indicate that increased water intake is not necessary to elicit the penicillin growth response and that penicillin tends to have a "sparing" effect on the water requirement of these species.

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LITERATURE CITED

BRAUDE, R., AND B. C. JOHNSON 1953 Effect of aureomycin on nitrogen and water metabolism in growing pigs. J. Nutrition, *49:* 505.

CRAMPTON, E. W., AND L. E. LLOYD 1954 The effect of water restriction on the food intake and food efficiency of growing rats. Ibid., *54:* 221.

ROBINSON, K. L., W. E. COEY AND G. S. BURNETT 1953 The influence of procaine penicillin on the efficiency of food utilization by pigs. Chem. and Ind., *1:* 18.

SLINGER, S. J., M. M. HAUSER AND W. F. PEPPER 1954 The correlation between feed consumption and fecal flora in chicks. J. Nutrition, *52:* 75.

SLINGER, S. J., AND W. F. PEPPER 1954 Effect of penicillin on the growth and feed consumption of turkey poults. Poultry Sci., *33:* 746.

DIFFERENTIATION OF UNIDENTIFIED CHICK GROWTH FACTORS IN LIVER [1]

H. M. EDWARDS, JR., DONALD CHIN, L. C. NORRIS AND G. F. HEUSER
Agricultural Experiment Station and School of Nutrition, Cornell University, Ithaca, New York

ONE FIGURE

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Since the discovery of vitamin B_{12} by Rickes and associates ('48) and Smith ('48), numerous reports have been presented giving results which indicated the existence of other unidentified factors. The earlier reports dealing with the unidentified factors required for chick growth have been reviewed by Combs ('51).

Miller ('51), and Miller, Small, and Norris ('55) showed that the hemorrhagic and paralytic symptoms, previously reported by Carlson and associates ('49) as evidence of a deficiency of an unidentified factor, were largely due to the destruction of α-tocopheryl acetate and thiamine by the sulfite in the isolated soybean protein [2] used in the experimental diet. Miller ('51) found on washing out the sulfite from alpha-protein, however, that the poor growth preceding the development of these symptoms was caused in part at least by a deficiency of an unidentified factor(s) present in dried brewers' yeast.

[1] This work was supported in part by grants to Cornell University by The Nutrition Foundation, New York, New York; Distillers Feed Research Council, Cincinnati, Ohio; The Squibb Institute for Medical Research, New Brunswick, New Jersey; and Western Condensing Company, Appleton, Wisconsin. The studies were conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

[2] Alpha-protein, The Glidden Company, Chicago, Illinois.

Menge and associates ('52a) presented evidence of the existence of two unidentified chick-growth factors, one of which was in certain liver fractions and dried brewers' yeast, and the other of which was in dried whey and dried whey product. Menge and Combs ('52) found as a result of countercurrent distribution studies that liver concentrates contained either two forms of the same growth factor or two distinct ones. Menge et al. ('52b) described some of the properties of an unidentified chick growth factor in condensed fish solubles. Fuller, Carrick and Hauge ('52) concluded that fish solubles contained an unidentified factor required for chick growth which is different from a factor in dried whey. Dietrich, Monson and Elvehjem ('52) obtained evidence of an unknown growth factor in yeast, liver, casein and wheat bran required by the chick and the hyperthyroid rat. Wiese et al. ('53) reported that fish solubles, fish meal and dried whey contained a chick-growth factor which is not present in liver preparations. Young, Gillis and Norris ('53) found that a purified diet believed complete in known nutrients was deficient in an unidentified chick-growth factor present in alcohol-extracted peanut meal.

Novak and Hauge ('48a, b) reported the isolation of an unknown factor in distillers' dried solubles, required for rat growth. They tentatively called the factor vitamin B_{13} and described some of its properties. Manna and Hauge ('53) presented evidence which indicated a relationship between vitamin B_{13} and orotic acid and suggested that the latter is a decomposition product of the vitamin.

Schultze ('53a, b) found that rats required an unknown lactation factor, different from any of the known vitamins, which is closely associated with proteins. Ershoff ('52) reviewed his previous work and that of others showing that liver contained an unknown factor which counteracted the deleterious effects of various stress agents on rats, and presented evidence of a factor in liver distinct from any of the known B vitamins which protected rats against multiple sublethal doses of X-irradiation.

Work on unidentified chick-growth factors has been continued at this laboratory since the presentation of the reports of Carlson and associates ('49), Miller ('51) and Young, Gillis and Norris ('53). The results of some of this work are presented in this report. They provide further evidence of the existence of unknown chick-growth factors not identical with any nutrient previously reported to be required.

EXPERIMENTAL

Either purebred or crossbred day-old chicks of the American breeds were used in this work. The chicks were identified with numbered wingbands, weighed individually at the start of the experiments and weekly thereafter. They were housed in electrically heated battery brooders equipped with wire-mesh floors which prevented coprophagy. The duration of the experiments varied from 28 to 33 days. Feed and water were supplied ad libitum, and a record of feed consumption was made at the end of each week.

A purified diet was used in the investigation. The composition of the diet is given in table 1. The protein content of the diet was 20.5%. Whenever materials containing protein were included in the diet, appropriate adjustments were made in the purified protein in order to maintain a uniform protein content.

The vitamin mixture provided quantities of vitamins or vitamin activity, except vitamin B_{12}, in excess of National Research Council ('54) requirements. The amount of vitamin B_{12} was greatly in excess of that found by Norris, Miller and Yacowitz ('50) to be required for normal chick growth and prevention of mortality. The mineral mixture provided quantities of the essential mineral elements equal to or in excess of the National Research Council ('54) requirements. No values on chlorine, sodium, zinc and cobalt, however, are given in the requirement tables of the Council. The sodium content of the diet was set, therefore, at 0.24%, the amount found necessary for maximum chick growth by Small and Norris ('55). The quantity of chlorine was in excess of that

reported to be needed by Burns, Cravens and Philips ('53). Davis, Briggs and Sloan ('53) found that cobalt was not required by the chick when the diet contained sufficient vitamin B_{12}. The quantity of zinc was believed to be more than adequate.

The casein used in the basal diet was purified either by washing 5 times in an acid-salt solution at pH 2.5 followed

TABLE 1

Composition of basal diet

INGREDIENT	AMOUNT/100 GM	INGREDIENT	AMOUNT/100 GM
	gm		*mg*
Glucose [1]	64.98	Choline Cl	150.00
Purified casein	21.79	Niacin	5.00
Cellophane, ground	3.00	Calcium pantothenate	2.00
Hydrogenated fat	3.00	α-Tocopheryl acetate	2.00
Glycine	0.80	Riboflavin	1.00
L-Arginine HCl	0.625	Pyridoxine HCl	0.45
DL-Methionine	0.30	Thiamine HCl	0.40
Dicalcium phosphate	2.55	Folic acid	0.20
Limestone, ground	0.95	Menadione	0.04
Iodized salt	0.60	Biotin	0.02
K_2HPO_4	0.555		
Magnesium sulfate	0.25		
			μg
	mg	Vitamin B_{12}	0.50
$FeSO_4 \cdot 7H_2O$	54.0		
$MnSO_4 \cdot H_2O$	35.8		*I.U.*
KI	3.0	Vitamin A	445.0
$CuSO_4 \cdot 5H_2O$	1.5	Vitamin D_3	30.0
$ZnCl_2$	1.0		
$CoCl_2 \cdot 6H_2O$	0.2		

[1] Cerelose.

by three washings at pH 4.7, or by dissolving in water at pH 7.2 followed by re-precipitation at pH 4.7. The latter procedure was repeated twice. In both procedures the solution was removed by decantation. After the last decantation, the casein was pressed and the press cake dried in a forced draft oven at 60 to 70°C.

The purified casein was analyzed for essential amino acids. Glycine and cystine were determined in this laboratory and

the other essential amino acids by the Department of Biochemistry.[3] The amino acid content of the basal diet was calculated from the results of these analyses. After rectifying deficiencies of methionine and glycine, the content of the diet in essential amino acids equalled or exceeded the National Research Council requirements ('54).

TABLE 2

Nutritive adequacy of basal diet

TREATMENT	AV. WT. 28 DAYS	INCREASE OVER BASAL
	gm	*%*
None (basal diet)	283 (123)[1]	
+ double all vitamins	284 (46)	+ 0.35
+ double trace elements[2]	263 (33)	— 7.07
+ arginine and tryptophan ≅ 7.5% defatted liver[3]	311 (33)	+ 8.94
+ 5% defatted dried liver	345 (55)	+ 21.91
+ 7.5% defatted dried liver	338 (53)	+ 19.43
+ 3% fish solubles	333 (40)	+ 17.67
+ 4.5% fish solubles	348 (15)	+ 22.97
+ 5% defatted dried liver + 3% fish solubles	383 (54)	+ 35.34
+ 7.5% defatted dried liver + 3% fish solubles	395 (16)	+ 39.58
+ 7.5% defatted dried liver + 4.5% fish solubles	371 (17)	+ 31.10

[1] Numbers in parentheses indicate surviving NH, RIR × BPR, or NH × WPR chicks.

[2] Fe, I, Cu, Zn, and Co; also traces Mo and F.

[3] L-Arginine, 0.146%; L-tryptophan, 0.015%.

RESULTS AND DISCUSSION

Experiments were conducted to ascertain if the purified diet used in this investigation was complete in known nutrients. The results of the experiments are given in table 2. They showed that doubling the quantity of the known vitamins except inositol promoted no further increase in growth. Doubling the trace elements: iron, copper, iodine, zinc and cobalt, plus traces of molybdenum and fluorine also failed to increase the growth of the chicks. Adding extra arginine and tryptophan, equal to the quantity supplied by 7.5% of dried liver, and in excess of that supplied by 5% of dried

[3] These analyses were made through the courtesy of Dr. H. H. Williams.

liver plus 3% of fish solubles, improved growth slightly but not significantly. In further work, not presented here, doubling all the vitamins including inositol, increasing the quantity of the essential minerals alone and in various combinations, and raising the quantity of essential amino acids by 10% failed to improve growth.

On the other hand, when dried liver was included in the basal diet, the increase in growth amounted to approximately 21%, when fish solubles were included to about 20%, and, when both liver and fish solubles were supplied, to about 35%. The results of this work indicated, therefore, that the improved growth obtained by supplementing the purified basal diet with crude sources of unidentified factors was not due to the presence of known nutrients in these materials but rather to unidentified growth stimulating agents.

The results of the experimental work also showed that 3% of fish solubles and 5% of dried liver when fed alone were sufficient to promote maximum response from these products. Hence, the further increase in growth observed when these products were fed together indicated the existence of at least two unidentified growth factors, one present in dried liver and the other in fish solubles.

In view of the marked growth response obtained by supplementing the purified diet with dried liver, a study was made of various fractions prepared from it.[4] These included liver residue, liver concentrate, liver fraction 1 and liver fraction 2. The liver concentrate was prepared from ground fresh liver by extraction with water followed by concentration. The extracted material was designated liver residue. Liver fraction 1 was obtained by extracting the liver concentrate with 70% ethanol and subsequent concentration. The ethanol insoluble residue was called liver fraction 2. These fractions were fed to chicks according to the plan given in table 3. The results, also presented in this table, showed that liver concentrate, liver fraction 1 and liver

[4] These fractions were obtained from the Wilson Research Laboratories, Chicago, Illinois.

fraction 2 increased the growth of the chicks, while liver residue was inactive. Liver fraction 1 appeared to be much more active than liver fraction 2. At the level used, however, liver fraction 1 failed to promote as good growth as dried liver.

TABLE 3

Growth response with liver fractions

TREATMENT	AV. WT. 28 DAYS	INCREASE OVER BASAL
	gm	%
None (basal diet)	263 (19)[1]	....
+ 7.0% dried liver	347 (19)	+ 31.94
+ 6.0% liver residue	267 (19)	+ 1.52
+ 2.0% liver concentrate	322 (20)	+ 22.43
+ 1.5% liver fraction 1	332 (20)	+ 26.24
+ 1.5% liver fraction 2	302 (19)	+ 14.83

[1] Numbers in parentheses indicate surviving NH × WPR chicks.

TABLE 4

Quantity of liver fraction 1 needed for maximum response

TREATMENT	AV. WT. 32 DAYS	INCREASE OVER BASAL
	gm	%
None (basal diet)	356 (16)[1]	....
+ 1.0% liver fraction 1	380 (16)	+ 6.74
+ 1.5% liver fraction 1	410 (16)	+ 15.69
+ 2.0% liver fraction 1	428 (13)	+ 20.22
+ 2.5% liver fraction 1	398 (18)	+ 11.80
+ 3.0% liver fraction 1	407 (16)	+ 14.33
+ 7.5% defatted dried liver	405 (17)	+ 13.76

[1] Numbers in parentheses indicate surviving NH × WPR chicks.

After determining that liver fraction 1 was highly active in the unknown growth-promoting factor, a study was made to determine the quantity needed to promote maximum growth response. This was done by feeding graded levels of the fraction and comparing the responses with the response obtained by feeding 7.5% of defatted dried liver. The results of this experiment are presented in table 4. They showed that 1.5% of liver fraction 1 in the diet was enough to pro-

mote maximum growth response. The growth of the chicks fed the basal diet, however, was superior to that observed in the previous or subsequent experiments.

After completing this experiment, an attempt was made to reduce the solids content of liver fraction 1 by extracting with phenol. After extraction, the phenol was dissolved in ethyl ether and the active substance was removed by washing with water. This was repeated 5 times. The combined water washings were then concentrated under partial vacuum. After concentration, traces of phenol were removed by washing 5 more times with ethyl ether. This procedure reduced the

TABLE 5

Growth-promoting activity of phenol-soluble fraction of liver fraction 1

TREATMENT	AV. WT. 28 DAYS (Exp. 1)	AV. WT. 28–33 DAYS (Exp. 1, 2, 3)	INCREASE OVER BASAL (Exp. 1, 2, 3)
	gm	*gm*	*%*
None (basal diet)	287 (16)[1]	288 (54)	...
+ phenol-soluble fraction ≏ 2% liver fraction 1	342 (14)	348 (52)	+ 20.8
+ phenol-insoluble fraction ≏ 2% liver fraction 1	278 (16)	...	...
+ 1.5% liver fraction 1	307 (15)	319 (55)	+ 10.8

[1] Numbers in parentheses indicate surviving RIR × BPR and NH × WPR chicks.

total solids content of liver fraction 1 approximately 39% and the total ash content approximately 60%.

The phenol-soluble fraction was fed at a level equivalent to 2% of liver fraction 1 in three experiments. The phenol-insoluble fraction was fed at a level equivalent to 2% in the first experiment only. Liver fraction 1 was fed at a level of 1.5% as a positive control. The results of these experiments are presented in table 5. The results showed that the phenol-soluble fraction promoted a marked increase in growth while the phenol-insoluble fraction was inactive. The increase in growth was approximately twice as great as that obtained with 1.5% of liver fraction 1. The difference is believed to be due to the fact that the chicks used in these experiments

had smaller reserves of the liver factor at the time of hatching than those used in the previous experiment, and thus 1.5% of liver fraction 1 was not sufficient to promote maximum growth response.

After ascertaining that the phenol-soluble extract of liver fraction 1 was highly active, a quantity of the phenol-soluble fraction equivalent to a dietary level of 8% of liver fraction 1 (1090 gm) was subjected to further fractionation by means of Craig's ('44) counter-current distribution procedure. An 8-plate counter-current distribution system was used. The upper layer of the system was composed of 50% phenol and 50% n-butanol, and the lower layer was composed of distilled water containing 3% of glacial acetic acid and sufficient hydrochloric acid to bring the pH to 2.5. The two layers were brought into equilibrium by shaking. Two-liter separatory funnels were used as the counter-current tubes. After completion of the procedure, the material in the tubes was subjected to the treatment to remove phenol used in preparing the phenol-soluble extract and then concentrated to approximately 250 ml by means of the rotary vacuum distillation apparatus described by Craig, Gregory and Hausmann ('50). A second counter-current distribution was completed in the same manner as the first.

The contents of each tube from the two counter-current distributions were fed to different groups of chicks in separate experiments. Groups of chicks fed the basal ration only, liver fraction 1 and the phenol-soluble fraction equivalent to 2% liver fraction 1 were included in both experiments.

The final weights of the comparable lots of chicks were averaged and are presented in graphic form in figure 1. Calculated counter-current distribution curves and curves giving the amount of organic and inorganic solids supplied by each tube per 100 gm of diet are also presented in figure 1. The counter-current distribution curves were calculated from the growth data obtained by feeding the material in tubes 0, 4, and 6. No growth was obtained from the material in tube 6, while that promoted by the material in tubes 0 and 4 was

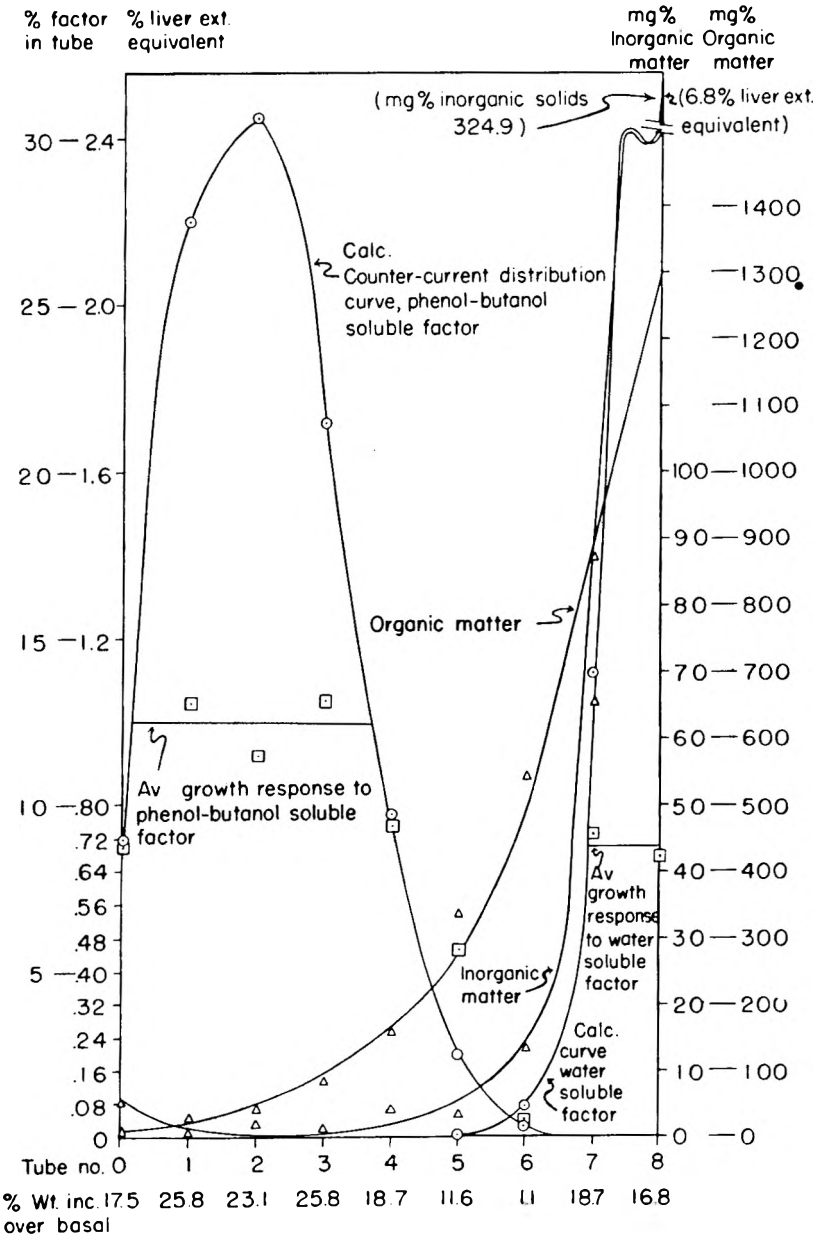

Fig. 1 Curves giving the results of studies on the fractionation of the phenol-soluble fraction of liver fraction 1.

less than maximum. Thus it was necessary to calculate counter-current distribution curves which would show little growth-promoting material in tube 6, insufficient in tubes 0 and 4 to promote maximum growth, and enough in tubes 1, 2, and 3 and tubes 7 and 8 to promote highest response from the material in the tubes.

The results showed that the original phenol extract of liver fraction 1 contained at least two growth-promoting factors, neither of which according to previous work appeared to be identical with the fish solubles factor. One of these, according to the calculations, appeared to be 74% soluble in the phenol-butanol layer, and the other 98% soluble in the water layer. The factor in the phenol-butanol layer was present in sufficient amounts in tubes 1, 2, and 3 to promote maximum growth response from it under the experimental conditions. Thus, when the growth responses were plotted, the curve showed a plateau rather than the characteristic peak of a typical counter-current distribution curve. In spite of this, the growth responses obtained with the chicks were found to fit reasonably well the calculated counter-current distribution curve for the phenol-butanol soluble factor.

Tubes 7 and 8 also contained more of the water-soluble factor than was required for maximum growth response. When this is taken into consideration, these responses fitted approximately the calculated curve for the water-soluble factor.

The quantity of organic matter supplied by tube 1, which amounted to 13.1 mg per 100 gm of diet, represented a concentration of the more phenol-butanol-soluble factor of approximately 130 times. The material in this tube and in tubes 2 and 3, which contained somewhat more organic matter, therefore seemed suitable for more detailed counter-current distribution study.

Practically all of the mineral matter, presumably because of high solubility in water, was found in tubes 7 and 8. These two tubes proved to be the only ones containing significant amounts of the water-soluble factor. In view of recent find-

ings by Morrison, Scott, and Norris ('55), it seems possible that the growth response obtained from these tubes was not due to an organic factor but rather to a mineral or minerals not previously reported to be required by the chick. The close correlation between the calculated counter-current distribution curve for the water soluble factor and the curve for the inorganic matter provided by each tube per 100 gm of diet, and the lack of correlation with the curve for the organic matter supports this hypothesis. The more phenol-butanol soluble factor on the other hand appeared to be organic in nature, as the amount of ash per 100 gm of diet supplied by tube 1 was 1.09 mg, while the quantities supplied by tubes 7 and 8 were 88.4 and 324.9 mg respectively.

In the first counter-current distribution study, the phenol soluble fraction promoted a considerably higher rate of growth than the fractions in tubes 1, 2, and 3, but the combined average responses from the fractions in these tubes and tubes 7 and 8 were nearly equal to that obtained with the phenol soluble fraction. This provided further evidence that the factor present in tubes 1, 2, and 3 was distinct from the factor present in tubes 7 and 8.

The response from the phenol soluble fraction used in the second counter-current distribution study was out of line for reasons which are not clear. In the last 5 days of the experiment, 5 chicks of this lot made little or no gain and the average increase in weight of all chicks (296 gm) was slightly less than the response obtained with liver fraction 1 (313 gm), in contrast to the consistently higher growth results of earlier experiments. Thus, evidence of the additive effect of the factor in tubes 1, 2, and 3, and that in tubes 7 and 8, observed in the first counter-current distribution study, was not obtained in the second one.

The increase in weight over the basal lot promoted by 0.22% of liver fraction 1 equivalent of the phenol-butanol soluble factor was 28 gm, that promoted by 0.77% of liver fraction 1 equivalent was 47 gm and that promoted by 1.75% of liver fraction 1 equivalent, a quantity believed just suffi-

cient to promote top response, was 66 gm. The growth responses obtained with the phenol-butanol soluble factor, therefore, were not proportional to the quantity of the factor supplied the chicks. This suggests the possibility that the phenol-butanol soluble factor exerts a sparing effect upon at least one of the other unknown chick growth factors, but does not completely replace it.

The work by Morrison, Scott and Norris ('55) showed that one or more organic factors were required for normal growth as well as the unidentified growth promoting mineral(s). In all probability the phenol-butanol soluble factor is at least one of these organic factors. Whether or not the phenol-butanol soluble factor is a complex cannot be determined without resorting to a counter-current distribution system consisting of more plates than were used in this investigation.

SUMMARY

Experiments have been completed the results of which showed that chicks require for normal growth two factors present in liver not identical with any nutrient previously reported to be needed. Both factors were found to be present in a 70% ethanol extract of the concentrated water-soluble material of liver. The results of counter-current distribution indicated that one of the factors is organic in nature and the other probably inorganic. Evidence was also obtained of the presence of a third chick growth factor in fish solubles.

ACKNOWLEDGMENT

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LITERATURE CITED

Burns, C. H., W. W. Cravens and P. H. Philips 1953 The sodium and potassium requirements of the chick and their interrelationships. J. Nutrition, *50:* 317.

Carlson, C. W., R. F. Miller, H. T. Peeler, L. C. Norris and G. F. Heuser 1949 The complex nature of the animal protein factor. Poultry Sci., *28:* 750.

COMBS, G. F. 1951 Unidentified factors required for chick growth. Reports, 9th World's Poultry Congress, *2:* 35.

CRAIG, L. C. 1944 Identification of small amounts of organic compounds by distribution studies. II. Separation by counter-current distribution. J. Biol. Chem., *155:* 519.

CRAIG, L. C., J. D. GREGORY AND W. HAUSMANN 1950 Versatile laboratory concentration device. Anal. Chem., *22:* 1462.

DAVIS, R. L., G. M. BRIGGS AND H. J. SLOAN 1953 Effect of cobalt in diet of the chick. Proc. Soc. Exp. Biol. and Med., *82:* 175.

DIETRICH, L. S., W. J. MONSON AND C. A. ELVEHJEM 1952 Studies on an unidentified growth factor for chicks and hyperthyroid rats fed purified rations. Arch. Biochem. Biophys., *38:* 91.

ERSHOFF, B. H. 1952 Increased survival of liver-fed rats administered multiple sublethal doses of X-irradiation. J. Nutrition, *47:* 289.

FULLER, H. L., C. W. CARRICK AND S. M. HAUGE 1952 A comparison of B_{12}, fish solubles and whey in the growth of chicks. Poultry Sci., *31:* 473.

MANNA, L., AND S. M. HAUGE 1953 A possible relationship of vitamin B_{13} to orotic acid. J. Biol. Chem., *202:* 91.

MENGE, H., G. F. COMBS, PENG-TUNG HSU AND M. S. SHORB 1952a Unidentified growth factors required by chicks and poults. I. Studies with chicks using purified diets. Poultry Sci., *31:* 237.

MENGE, H., AND G. F. COMBS 1952 Unidentified growth factors required by chicks and poults. II. Fractionation of a faetor in liver. Ibid., *31:* 994.

MENGE, H., C. A. DENTON, R. J. LILLIE AND H. R. BIRD 1952b Some properties of an unidentified chick growth factor in condensed fish solubles. Ibid., *31:* 927.

MILLER, R. F. 1951 Differentiation of unidentified factors required by the chick. Ph.D. thesis, Cornell University.

MILLER, R. F., G. SMALL AND L. C. NORRIS 1955 Studies on the effect of sodium bisulfite on the stability of vitamin E. J. Nutrition, *55:* 81.

MORRISON, A. B., M. L. SCOTT AND L. C. NORRIS 1955 Evidence for an unidentified mineral required by the chick. Poultry Sci., *34:* 738.

NATIONAL RESEARCH COUNCIL 1954 Recommended nutrient requirements for domestic animals. I. Recommended nutrient requirements for poultry. Report of the Committee on Animal Nutrition, Washington, D. C.

NORRIS, L. C., R. F. MILLER AND H. YACOWITZ 1950 Studies on the vitamin B_{12} requirements of poultry. Proc. Cornell Nutr. Conf. for Feed Mfgrs. pg. 53. Also, unpublished results, Cornell University Agr. Exp. Sta.

NOVAK, A. F., AND S. M. HAUGE 1948a Some properties of an unidentified growth factor in distillers' dried solubles. J. Biol. Chem., *174:* 235.

———— 1948b Isolation of the unidentified growth factor (vitamin B_{13}) in distillers' dried solubles. Ibid., *174:* 647.

RICKES, E. L., N. G. BRINK, F. R. KONIUSZY, T. R. WOOD AND K. FOLKERS 1948 Crystalline vitamin B_{12}. Science, *107:* 396.

SCHULTZE, M. O. 1953a Nutritional value of plant materials. VIII. Lactation failure of rats fed diets containing purified soybean proteins. J. Nutrition, *49:* 231.

———— 1953b Nutritional value of plant materials. IX. The effect of various supplements on lactation failure of rats fed diets containing purified soybean proteins. Ibid., *49:* 245.

SMALL, G., AND L. C. NORRIS 1955 Unpublished results. Cornell University Agr. Exp. Sta.

SMITH, E. L. 1948 Purification of anti-pernicious anemia factors from liver. Nature (London), *161:* 638.

WIESE, A. C., C. F. PETERSON, A. R. PAPPENHAGEN AND H. D. WYCOFF 1953 Chick growth factor present in fish meal and fish solubles. Fed. Proc., *12:* 433.

YOUNG, R. J., M. B. GILLIS AND L. C. NORRIS 1953 An unidentified factor in peanut meal required by the chick. J. Nutrition, *50:* 291.

EFFECT OF DRIED WHEY ON GROWTH, EGG PRODUCTION AND HATCHABILITY

A. L. KURNICK, B. L. REID, R. L. SVACHA AND J. R. COUCH

Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas Agricultural and Mechanical College System, College Station

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Prior to the isolation of vitamin B_{12} evidence was accumulated on the existence of an unidentified hatchability and growth factor in dried whey (Bethke et al., '33; Nestler et al., '36; Hill et al., '44; Hill, '48). It has been possible to evaluate the activity of dried whey and other unidentified factor supplements since the isolation of vitamin B_{12} (Rickes et al., '48; Lillie et al., '48; Ott et al., '48; Nichol et al., '48).

Menge et al. ('49) obtained better growth with chicks on a purified diet in the presence of vitamin B_{12}, when Wilson's liver fraction "L" and dried whey were added to the basal diet. The results indicated that at least one factor was required for optimum growth in addition to the known vitamins. Subsequent studies by other workers indicated that whey contained a factor or factors required for growth of chicks (Reed et al., '51; Arscott and Combs, '50; Couch et al., '51; Combs et al., '54). Norris et al. ('53) reported that the factors found in fish solubles and in dried whey was different from that found in liver preparations and dried brewers' yeast. Dried whey has been reported to stimulate egg production, and to a lesser extent hatchability (Couch et al., '50).

The present study was designed to determine the effect of dried whey on the growth, egg production, and hatchability.

MATERIALS AND METHODS

Experiment 1 Three hundred and fifty Single-Comb White Leghorn sexed females, from dams maintained on the Agricultural and Mechanical College of Texas Poultry Farm, were hatched on February 22, 1954, and placed on battery brooders with raised wire floors. The chicks were previously wing-banded and vaccinated intraocularly with live-virus Newcastle vaccine. A practical diet consisting of 56% ground yellow corn, 40% soybean oil meal (44% protein), 2% dicalcium phosphate, 1.5% ground oyster shell, 0.5% salt supplemented with the following per kilogram of diet: 5 mg riboflavin, 11 mg calcium pantothenate, 28 mg niacin, 880 mg choline chloride, 6.6 μg vitamin B_{12}, 4,400 I.U. vitamin A, 1320 I.C.U. vitamin D_3, 5.5 mg bacitracin, 2.2 mg penicillin, 13.2 mg vitamin K, and 0.176 gm $MnSO_4$ was fed to all groups for the first 67 days of the study.

At 67 days of age, the chicks were individually weighed and distributed into two groups of 163 birds each. At that time the chicks were dubbed, debeaked, and vaccinated against Newcastle disease and Fowl Pox.

The chicks in group 1 were fed a purified diet consisting of 65% sucrose, 24% Drackett assay protein,[1] 3% soybean oil, and 8% mineral mixture. The mineral mix supplied the following amounts per 100 grams of diet: 1.865 gm $CaCO_3$, 3.283 gm $CaHP_4$, 0.95 gm NaCl, 0.114 gm $MnSO_4 \cdot 7H_2O$, 3.925 mg KI, 0.893 gm K_2HPO_4, 0.450 gm $MgSO_4 \cdot 7H_2O$, 0.158 gm $FeC_6H_5O_7 \cdot 6H_2O$, 1.675 mg $CuSO_4 \cdot 5H_2O$, 1.375 mg $ZnCl_2$, and 0.050 mg $CoCl_2 \cdot 6H_2O$. Vitamins in milligrams per kilogram of diet were added as follows: thiamine hydrochloride 4, riboflavin 6, calcium pantothenate 15, niacin 100, pyridoxine hydrochloride 4, para-amino-benzoic acid 20, menadione 0.5, alpha tocopheryl acetate 24, pteroylglutamic acid 2, biotin 0.2, inositol 1,000, choline chloride 2,000 and vitamin B_{12} 0.03. Each kilogram of diet also contained 3,800 I.C.U. vitamin D_3, 10,000 I.U. vitamin A, 7.5 gm methionine, 4 gm glycine and

[1] The Drackett Co., Cincinnati, Ohio.

33 mg penicillin. Group 2 was fed the basal diet in which 4% dried whey was substituted for an equivalent amount of sucrose.

Prior to sexual maturity (143 days of age) the pullets in each group were weighed and placed in individual laying cages with raised wire floors. The management and experimental procedures with regard to the care and handling of the birds, insemination, incubation and embryonic mortality records were the same as those previously reported by Couch et al. ('50). The experimental diets were altered to increase the calcium and phosphorus levels to 2.5 and 1.0%, respectively, to meet the requirements for egg production.

At the close of a 12-week period the pullets remaining in the unsupplemented group in the first study were distributed on the basis of egg production and hatchability into two groups, 1 and 2 respectively. Group 1 was continued on the basal diet and group 2 was fed the basal diet supplemented with 4% of dried whey for a period of 4 weeks.

Experiment 2. One hundred and thirty-five pullet chicks hatched on September 18, 1954 were used in this study. The birds were raised on the practical diet to 86 days of age and then placed on the experimental purified diets; one group was fed the purified basal diet unsupplemented and the other group was fed the ration supplemented with dried whey. Management of the birds was the same as has been reported for the previous study with the exception that the birds were placed in individual laying cages at 132 days of age.

RESULTS AND DISCUSSION

Dried whey was found to contain an unidentified factor required by pullets for optimum growth and development (table 1, experiments 1 and 2). At 143 days of age, the average weight of the pullets (table 1) fed the whey supplement in experiment 1 was found to be 68.5 gm more than that of the pullets fed the unsupplemented diet. These differences in weight were found to be significant at the 0.01 level of probability.

The results obtained with the fall-hatched pullets (table 1, experiment 2) showed an increase in growth upon the addition of dried whey to the diet during the period from 12 weeks to sexual maturity. The birds fed the dried whey-supplemented diet in this experiment exhibited a 129-gram increase in growth; this difference was found to be significant at the 0.01 level of probability.

Studies of the "whey chick growth factor" have been extensively reviewed in the literature (Reed et al., '51; Arscott and Combs, '50; Combs et al., '54). Most studies have measured the effect of dried whey by means of 4-, 8- or

TABLE 1

Effect of dried whey on growth of pullet chicks

GROUP	SUPPLEMENTS TO BASAL DIET	EXPERIMENT 1		EXPERIMENT 2	
		No. birds	Av. wt.[1] at 143 days	No. birds	Av. wt.[2] at 132 days
1	None	144	1568	56	1230
2	4% Dried whey	144	1636[3]	52	1359[3]

[1] The average weights of the two groups of this experiment were 848 and 843 gm, respectively, when placed on the experimental diets at 67 days of age.

[2] The average weights of the two groups of this experiment were 953 and 968 gm, respectively, when placed on the experimental diets at 86 days of age.

[3] The differences in weights due to dried whey were found to be significant at the 0.01 level of probability.

10-week chick-growth assays. In these experiments it was found that the beneficial effect of dried whey on the growth of the chicks extended to 20 weeks of age.

In the first study the age at sexual maturity was found to be affected by the addition of the dried whey to the diet of pullets at 67 days of age (table 2). The percentage of the pullets in egg production at 150 days of age for groups 1 and 2 was 15.9 and 22.2, respectively. At every 5-day interval thereafter, the pullets in the group fed the supplemented diet (group 2, table 2) exhibited a higher percentage of birds in egg production than those in the unsupplemented group (group 1, table 2). At the end of 175 days 94.8% of

the pullets in the whey-supplemented group had reached sexual maturity as evidenced by the egg production record, whereas only 62.2% of the pullets in the basal group had reached a similar state of maturity.

An unidentified factor in dried whey was found to be required for normal egg production in hens fed a purified diet.

TABLE 2

Effect of dried whey on the sexual maturity of single-comb White Leghorn pullets reared on a purified soybean-protein diet from 12 to 22 weeks of age

Experiment 1

GROUP NO.	SUPPLEMENTS TO BASAL DIET	PER CENT OF PULLETS REACHING SEXUAL MATURITY					
		Days after hatching					
		150	155	160	165	170	175
1	None	15.9	30.1	38.5	50.2	58.5	62.2
2	4% Dried whey	22.2	35.4	52.6	62.7	81.4	94.8

TABLE 3

Effect of dried whey in a purified growing diet on resultant egg production and hatchability of single-comb White Leghorn pullets

GROUP NO.	SUPPLEMENTS TO BASAL DIET	NUMBER OF BIRDS	% EGG PRODUCTION			% HATCHABILITY (6–12 wk.)
			0–4 wk.	5–8 wk.	9–12 wk.	
		Experiment 1				
1	None	144	13.7	21.6	30.8	34.8
2	4% Dried whey	144	18.8	35.9	59.9	44.2
		Experiment 2				
1	None	56	4.5	23.4	33.1	...
2	4% Dried whey	52	5.2	38.2	51.8	...

During each 4-week interval the egg production of hens fed the dried whey supplement was higher than that of birds fed the basal diet in both studies (table 3). The low levels of egg production in all groups of both experiments during the first 4 weeks of the experimental period were due to the low intensity of egg production during the early part of the laying period. As pullets in the supplemented and unsupple-

mented groups of both experiments approached the maximum level of egg production, the difference in production between the two groups became more apparent. During the last 4-week interval the egg production of the hens fed the whey supplement was 29.1 and 18.7% higher for the two experiments, respectively, than of hens fed the basal diet. The results are in agreement with those of Couch et al. ('50) in that dried whey produced an increase in egg production.

Evidence obtained in the present investigation points to the fact that dried whey is an inadequate source of hatchability factors. The hatchability of eggs from hens fed the

TABLE 4

The effect of adding 4% dried whey on egg production and hatchability after single-comb White Leghorn pullets have been reared (12–22 weeks) on a purified soybean protein-sucrose diet and have been in production for 12 weeks

Experiment 1

GROUP NO.	SUPPLEMENTS TO BASAL DIET	NUMBER OF BIRDS	% EGG PRODUCTION (13–16 weeks)[1]	% HATCHABILITY (13–16 weeks)
1	None	45	32.6	28.8
2	4% Dried whey	45	47.3	40.1

[1] The basal group of experiment 1 was divided into two groups of equal egg production and hatchability.

basal diet and the whey-supplemented diet in experiment 1 was below normal (34.8% and 44.2%, respectively, table 3). The slight difference (9.5%) in hatchability of eggs from hens in group 2 over those in group 1 in this experiment may be due either to the accelerated rate of egg production of hens receiving the supplement (Byerly et al., '33; Funk, '34) or as postulated by Couch et al. ('50) whey may stimulate the intestinal synthesis of a factor required for hatchability.

Dried whey increased egg production when substituted for sucrose in the basal diet after hens were in production for 12 weeks (table 4). The egg production of the hens in group

1, fed the unsupplemented diet, remained relatively the same (table 3), while the egg production of those in group 2 increased by 14.7%, which may be attributed to the addition of dried whey to the diet.

A slight effect of dried whey on hatchability was also observed during the course of this study. The level of hatchability of fertile eggs from hens in both groups (groups 1 and 2, table 4) was below normal which points to the need for the liver "L" hatchability factor (Couch et al., '50).

SUMMARY

Two experiments were conducted with a total of 486 pullets.

Evidence was presented for the presence of an unidentified factor(s) required by pullets for growth and egg production.

Dried whey was found to be a good source of the growth and egg production factor(s) at the level used in these experiments.

Dried whey failed to support normal hatchability and therefore, may be considered as an inadequate source of hatchability factors under the conditions of these studies.

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LITERATURE CITED

ARSCOTT, G. H., AND G. F. COMBS 1950 Effect on growth of various crude supplements added to corn-soybean meal chick rations adequate in vitamin B_{12}. Poultry Sci., *29:* 746.

BETHKE, R. M., P. R. RECORD AND D. C. KENNARD 1933 Relation of the vitamin G complex to hatchability and nutritive value of eggs. Ibid., *12:* 332.

BYERLY, T. C., H. W. TITUS AND N. R. ELLIS 1933 Production and hatchability of eggs as affected by different kinds and varieties of protein in the diet of laying hens. J. Agr. Res., *46:* 1.

COMBS, G. F., G. H. ARSCOTT AND H. L. JONES 1954 Unidentified growth factors required by chicks and poults. 3. Chick studies involving practical-type rations. Poultry Sci., *33:* 71.

COUCH, J. R., O. OLCESE, B. G. SANDERS AND J. V. HALICK 1950 Vitamin B_{12}, APF concentrates, dried whey, fish solubles and liver fraction "L" in the nutrition of the mature fowl. J. Nutrition, *42:* 473.

COUCH, J. R., J. R. REED, R. L. ATKINSON AND B. E. WELCH 1951 Distillers grain solubles and dried whey with an APF concentrate in broiler feeds. Proc. 6th Distillers Feed Conference, p. 47.

FUNK, E. M. 1934 Factors influencing hatchability in the domestic fowl. Mo. Agr. Expt. Sta. Bull. 341.

HILL, F. W. 1948 The multiple nature of the deficiency of unidentified nutrients in crude all-vegetable protein chick starter rations. Poultry Sci., *27:* 536.

HILL, F. W., M. L. SCOTT, L. C. NORRIS AND G. F. HEUSER 1944 Deficiency of unidentified vitamins in practical chick rations. Ibid., *23:* 253.

LILLIE, R. J., C. A. DENTON AND H. R. BIRD 1948 Relation of vitamin B_{12} to the growth factor present in cow manure. J. Biol. Chem., *176:* 1477.

MENGE, H., G. F. COMBS AND M. S. SHORB 1949 Evidence for an unidentified chick factor in the presence of vitamin B_{12}. Poultry Sci., *28:* 775.

NESTLER, R. B., T. C. BYERLY, N. R. ELLIS AND H. W. TITUS 1946 A new factor, not vitamin G, necessary for hatchability. Ibid., *15:* 67.

NICHOL, C. A., A. R. ROBBLEE, W. W. CRAVENS AND C. A. ELVEHJEM 1948 Distribution of an unidentified chick growth factor. Ibid., *27:* 438.

NORRIS, L. C., H. M. EDWARDS, JR. AND D. CHIN 1953 The problems of unidentified vitamins. Proc. of the 1953 Cornell Nutrition Conference for Feed Manufacturers, p. 81.

OTT, W. H., E. L. RICKES AND T. R. WOOD 1948 Activity of crystalline vitamin B_{12} for chick growth. J. Biol. Chem., *174:* 1047.

REED, J. R., JR., R. L. ATKINSON AND J. R. COUCH 1951 Dried whey as a source of unidentified factors for the growing chick. J. Nutrition, *43:* 501.

RICKES, E. L., N. G. BRINK, F. R. KONIUSZY, T. R. WOOD AND K. FOLKERS 1948 Crystalline vitamin B_{12}. Science, *107:* 396.

THE INFLUENCE ON THE GROWTH AND PROGENY OF THE GUINEA PIG RESULTING FROM ORAL ADMINISTRATION OF AUREOMYCIN (CHLORTETRACYCLINE) AND PENICILLIN [1]

C. GORDON HEWES [2]
Department of Anatomy, Dental School, University of Maryland, Baltimore

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Stimulation of the growth of many animals occurs as a result of continued oral or parenteral administration of aureomycin and penicillin. According to Sewell and Glasscock ('51) growth-promoting effects of antibiotics vary with the species of animal, providing the basal diet is complete. The mode of action of the drugs in producing growth, however, has not been definitely established, although studies show the antibiotics to have vitamin-sparing effects (Lih and Baumann, '51; Sauberlich, '52; and Monson et al., '52). Following oral administration aureomycin and penicillin are readily absorbed into the general circulation and are found in the liver, kidney, placenta, spleen, lung, cerebrospinal fluid and other body fluids in measurable concentrations. Reports indicate that there is stimulation of growth, also, in the progeny of certain animals which have received antibiotics during gestation. In particular, Slinger et al. ('52) found that progeny from hens, which were fed a regular diet plus

[1] From a thesis submitted to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address: Department of Anatomy, College of Medical Evangelists, Loma Linda, California.

penicillin, grew more rapidly than those from hens fed the same diet without the antibiotic.

Apparently, phagocytic tests (Hewes and Shay, '55) and toxic and anaphylactic reactions are the only studies that have been made of antibiotic effects on the guinea pig. No information is available as to whether or not these drugs, when used as dietary supplements, promote growth in this animal. The present investigation was designed to study the possibility of stimulation of growth, initiation of anatomical changes, and influence on progeny of the guinea pig resulting from daily oral administration of low levels of aureomycin and penicillin.

METHODS AND MATERIALS

Experiment 1

Male and female 6-week-old guinea pigs, of the English breed, were used. Aqueous solutions of auroemycin [3] and penicillin [4] were administered orally throughout the experimental period. Aureomycin in the form of crystalline hydrochloride and penicillin in the form of crystalline G sodium were used. The animals were fed ad libitum a complete ration [5] except for Vitamin C, which was adequately supplied by the addition of 500 mg of ascorbic acid to each liter of drinking water. The commercial ration contained no antibiotics or other similar drugs.

Male and female animals were separated and kept in raised screen floor cages. The animals were arranged in three groups each: aureomycin-fed, penicillin-fed, and control. Twelve male and 12 female animals comprised each group. The weight of each animal was recorded and weights were taken weekly thereafter. To govern the drug intake accurately, the antibiotics were administered orally by means

[3] Aureomycin was generously supplied by Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, New York.

[4] Penicillin was supplied through the kindness of Dr. Lyon P. Strean of Merck and Company, Rahway, New Jersey.

[5] Purina rabbit chow checkers.

of a 0.5 ml calibrated pipette, each animal receiving an aqueous solution of 0.55 mg of the drug per day in addition to the regular diet. This dosage was continued for one week, and, because of toxic effects, the dose was administered on alternate days throughout the remainder of the experiment.

At the end of 9 weeks 7 female animals of each group were bred by boars of the same group. The male controls, which had not been used for breeding, were sacrificed for histological studies. The liver, kidney, heart, and spleen were removed, freed of fat, weighed, and placed in 10% formalin. The left inferior extremity was removed, and the foot disarticulated at the ankle joint. The muscles of the thigh and leg were detached from the femur, tibia, and fibula, and the bones were cleaned and weighed. Measurements of the lengths of the tibias were recorded. All tissues were placed in 10% formalin.

The average gestation period of the guinea pig is 10 weeks. Within 24 hours after the sows gave birth to their litters, the weight of each offspring was recorded. Within 48 hours after the birth of each experimental animal, 0.1 mg of the specified drug in aqueous solution was administered orally. This dose was given daily for 6 weeks. After three weeks the young were weaned. The control animals were fed only the usual diet of rabbit chow with ascorbic acid added to their drinking water. The weights of the young animals were recorded weekly.

Experiment 2

To determine the effects of different levels of supplementary aureomycin on growth and structure, 12 male weanling guinea pigs 6 weeks old were arranged in two groups of 6 animals each, so that each group weighed approximately the same. Each animal in the first group was given 0.3 mg of aureomycin and each in the second group was given 0.5 mg of the drug per day. Aqueous solutions of the drug were administered orally for a period of 9 weeks and the weight of each animal was taken weekly throughout the experimental period. At the end of the experiment the animals in the two groups were

sacrificed. In order to determine true growth and structural changes, tissues of these animals were compared with those of the male controls of experiment 1. The same procedure was followed in the removal of tissues as in the first experiment.

RESULTS AND DISCUSSION

The groups of guinea pigs in experiment 1, from which animals for breeding were taken, showed toxic effects as a result of the daily dose of 0.55 mg of aureomycin and penicillin. Eleven of the 24 penicillin-fed and two of the 24 aureomycin-fed animals died. No deaths resulted from the administration of the drugs on alternate days. Heilman ('48) reported that aureomycin and penicillin have a cumulative toxic effect on guinea pigs, whereas they are relatively non-toxic for most other animals. The 0.55 mg dose was used in the present experiment, because it was proportional to the small quantities of antibiotics usually mixed with the feed for larger animals.

TABLE 1

Comparative average birth weights of first-litter progeny of antibiotic-fed and control guinea pigs

GROUP	AVERAGE WEIGHT
	gm
Aureomycin-fed	97.5
Penicillin-fed	94.0
Control	92.0

The average birth weights of groups of first-litter offspring of aureomycin-fed, penicillin-fed, and control guinea pigs are listed in table 1. Slightly higher average birth weights were noted in the progeny of the aureomycin-fed and penicillin-fed animals than in the progeny of the control animals. However, no apparent weight increase was noted in the experimental animals over the controls at the end of 6 weeks of daily administration of the low level of 0.1 mg of the antibiotics. The number of animals in each group

varied from 11 to 15. The increased birth weights of the progeny of the experimental guinea pigs confirms the report of Lillie and Bird ('52) that aureomycin in the maternal diet seems to have a stimulating carry-over effect on chicks. No evidence was apparent that antibiotic feeding influenced the number of offspring in each litter. Mirone ('53) substantiates this observation in experiments with mice by reporting no differences in the number of animals per litter from antibiotic-fed and control animals. Uram et al. ('55) made a similar observation with rats which were given terramycin- and streptomycin-supplemented diets.

In experiment 2 the average weight gains of the groups of guinea pigs, which were fed 0.3 mg and 0.5 mg of aureomycin,

TABLE 2

Comparative average weight gains of groups of aureomycin-fed and control guinea pigs

GROUP	AVERAGE WEIGHT AT		AVERAGE WEIGHT GAIN	PER CENT GAIN
	6 weeks	15 weeks		
	gm	*gm*	*gm*	
Fed 0.3 mg aureomycin	273.1	606.3	333.2 [1]	122.0
Fed 0.5 mg aureomycin	276.9	610.8	333.9 [1]	120.6
Control	259.1	537.8	278.7	107.6

[1] Indicates significance ($P = < 0.05$).

respectively, and the control group are compared in table 2. The groups which were given different dosages of aureomycin showed a significant average body weight gain over the control group. Little difference was evident between the weight gains of animals given the two different doses. Many theories have been advanced to explain why the continued administration of low levels of antibiotics increase body weight in animals, but the mode of action has not been satisfactorily determined.

The effects of the antibiotics, administered as supplements to the regular ration, on the organ and tissue weights in this experiment varied considerably. A comparison of the average weights of organs and tissues of aureomycin-fed

animals with those of controls is shown in table 3. The weights of the liver, tibia and muscles of the thigh and leg of the experimental animals were greater than those of the controls, the weights of the tibias being significantly higher. In respect to the heart and spleen weights, those of the experimental animals were significantly below those of the controls. Possibly, aureomycin affects the growth of these organs. Such antibiotics as chloromycetin and streptomycin in high con-

TABLE 3

Comparative average weights of organs and tissues of aureomycin-fed and control guinea pigs

GROUP	LIVER	KIDNEY	HEART	SPLEEN	TIBIA	MUSCLES (Thigh, leg)
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Fed 0.3 mg aureomycin	24.2	4.3	1.5 [1]	0.5 [1]	1.4 [1]	20.3
Fed 0.5 mg aureomycin	24.1	4.3	1.5 [1]	0.4 [1]	1.5 [1]	20.5
Control	19.8	4.4	1.9	0.9	1.1	18.0
	Relation to body weight					
	%	%	%	%	%	%
Fed 0.3 mg aureomycin	4.00	0.70	0.25	0.08	0.20	3.30
Fed 0.5 mg aureomycin	3.90	0.70	0.24	0.07	0.20	3.30
Control	3.60	0.80	0.30	0.16	0.20	3.20

[1] Indicates significance ($P = < 0.05$).

centrations have been found by Fusillo et al. ('52) to inhibit the growth of a 9-day-old chick embryo heart. Uniformity of organ weights in the experimental animals was found regardless of whether the dose of aureomycin was 0.3 or 0.5 mg.

A study of sections of the organs and tissues of antibiotic-fed and control animals revealed no outstanding differences. Skeletal growth, represented by measurements of the lengths of the tibias, was greater in the groups of experimental animals than in the controls (table 4). Such structural growth

was pointed out by Quimby ('48) when he used the length of the femur in rats as an indicator in inanition and refeeding studies.

Further studies are needed to determine the factors that are responsible for these effects, which follow the use of antibiotics as supplements to a normal diet.

TABLE 4

Relative average lengths of tibias of aureomycin-fed and control guinea pigs

GROUP	LENGTH OF TIBIA
	mm
Fed 0.3 mg aureomycin	45.9
Fed 0.5 mg aureomycin	46.1
Control	44.2

SUMMARY

Aqueous solutions of aureomycin and penicillin as dietary supplements were administered orally to groups of guinea pigs to study some of the effects of these antibiotics on the animals. First-litter progeny of animals treated with antibiotics showed a slight increase in mean birth weight over that of first-litter progeny of control animals. However, this increase was not apparent after 6 weeks of administration of the low level of 0.1 mg of the drugs to the young. No other effects on reproduction were observed.

Male guinea pigs fed 0.3 and 0.5 mg of aureomycin per day showed a significant average weight gain over the controls after a period of 9 weeks. Of the organs and tissues removed for study, only the tibias of the experimental animals weighed significantly more than those of the controls. The heart and spleen weighed significantly less in the antibiotic-fed animals than in the controls. Sections of these organs and tissues indicated no apparent histological differences between experimental and control animals. Increased structural growth was noted in the greater length of the tibia in the aureomycin-fed animals than in the controls.

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LITERATURE CITED

FUSILLO, M. H., J. F. METZGER AND D. M. KUHNS 1952 Effect of chloromycetin and streptomycin on embryonic tissue growth in *in vitro* tissue culture. Proc. Soc. Exp. Biol. Med., *79:* 376.

HEILMAN, F. R. 1948 Aureomycin in the treatment of experimental relapsing fever and leptospirosis icterohaemorrhagica (Weil's disease). Proc. Staff Meet. Mayo Clinic, *23:* 569.

HEWES, C. G., AND D. E. SHAY 1955 Antibody formation in the guinea pig as a result of oral administration of chlortetracycline and penicillin. Antibiotics and Chemotherapy, *5:* 101.

LIH, HWA, AND C. A. BAUMANN 1951 Effects of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavin, or pantothenic acid. J. Nutrition, *45:* 143.

LILLIE, R. J., AND H. R. BIRD 1952 Effect of antibiotic supplements upon hatchability and upon growth and viability of progeny. Poultry Sci., *31:* 513.

MIRONE, L. 1953 Effect of aureomycin and terramycin on growth, reproduction, and blood count in mice. Antibiotics and Chemotherapy, *3:* 600.

MONSON, W. J., L. S. DIETRICH AND C. A. ELVEHJEM 1952 The effect of different carbohydrates and antibiotics on the growth of chicks and the storage of vitamins. J. Nutrition, *46:* 411.

QUIMBY, F. H. 1948 Organ weights of rats receiving hormone supplements during recovery from chronic starvation. Endocrinology, *42:* 263.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirements of the rat. J. Nutrition, *46:* 99.

SEWELL, A. M. P., AND R. S. GLASSCOCK 1951 A comparison of aureomycin, streptomycin, penicillin, and an aureomycin-vitamin B-12 feed supplement for the pig. Arch. of Biochem., *30:* 269.

SLINGER, S. J., A. E. FERGUSON AND J. D. MCCONACHIE 1952 A carry-over effect of penicillin from dam to progeny. Poultry Sci., *31:* 172.

URAM, J. A., C. E. FRENCH, G. P. BARRON AND R. W. SWIFT 1955 The effect of high levels of terramycin or streptomycin on growth, reproduction, and lactation of the rat. J. Nutrition, *55:* 481.

ASCORBIC ACID NUTRITURE IN THE HUMAN[1]

II. CONTENT OF ASCORBIC ACID IN THE WHITE CELLS AND SERA OF SUBJECTS RECEIVING CONTROLLED LOW INTAKES OF THE VITAMIN

BETTY F. STEELE, RACHEL L. LINER, ZAIDA H. PIERCE AND H. H. WILLIAMS

The Departments of Food and Nutrition, and Biochemistry and Nutrition, and The School of Nutrition, Cornell University, Ithaca, New York

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Lowry and his coworkers ('46) have reported the white cell ascorbic acid content for subjects given 8 or 23 mg of ascorbic acid daily for 8 months. Neither amount maintained the starting white cell ascorbic acid content and they found no difference between the white cell ascorbic acid content of the two groups at the end of this time. Studies on ascorbic acid deprivation in man by the Vitamin C Sub-Committee of the Medical Research Council of England ('48, '53) indicated that 10 mg of ascorbic acid daily would not maintain white cell ascorbic acid content in normally nourished subjects, but there was some indication that it would cause an increase in white cell ascorbic acid content in subjects who were scorbutic. Davey et al. ('52) have reported that 25 mg of ascorbic acid per day failed to maintain ascorbic acid concentration in 4 subjects in good nutriture at the beginning of the experiment.

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A previous report from this laboratory was concerned with the ascorbic acid content of sera and white cells of human subjects ingesting a diet containing 7 mg or less of vitamin C for 78 days (Steele et al., '52). Under these conditions neither white cell nor serum ascorbic acid content was maintained at the starting level. The present study is concerned with the amount of ascorbic acid needed to cause an increase in white cell ascorbic acid concentration while the subjects were under the stress of partial depletion of the vitamin.

PROCEDURE

Thirteen adults (10 men and three women) were given a diet (Steele et al., '52) low in ascorbic acid for 78 days. Each of the subjects received approximately 10 mg of ascorbic acid per day from the food alone during the first 38 to 42 days and became partially depleted of the vitamin. After the depletion period, the diet for each subject was controlled so that he received a fixed amount of ascorbic acid each day from the food [2] plus a solution of pure ascorbic acid to drink with his noon meal. For 14 days enough solution was ingested so that the total daily intake of ascorbic acid was 20 mg; the amount was increased later so that the intake was 30 and finally 40 mg.

Blood from a finger puncture was withdrawn at frequent intervals for analysis of white cell and serum ascorbic acid content. During the supplementation period these intervals were three to 4 days.

METHODS

Ascorbic acid in serum and white cells was determined by the methods of Bessey and Lowry and described by György ('50) and outlined in the Northeastern Regional Publication No. 5 ('51). The method of Roe and Oesterling described by György ('50) was used to determine the total ascorbic acid content of the diet.

[2] To help maintain a constant ascorbic acid intake, Starlac, kindly supplied by the Borden Company, was substituted for the boiled milk used earlier.

RESULTS

The values for ascorbic acid in the white cells and sera during ascorbic acid depletion and supplementation are given in tables 1 and 2.

After 38 to 42 days of the depletion diet which contained 10 mg or less of ascorbic acid, the white cell ascorbic acid content had fallen to 39 to 78% of the pre-diet level (table 1). All but one of the subjects had a pre-supplement white cell ascorbic acid content of less than 20 mg % when considered on the basis of average values of duplicate analysis from each of the last two days on which samples were taken before supplementation. This average was thought to be more reliable as a base line for comparison with the values obtained during the supplemented periods. A daily intake of 20 mg of ascorbic acid at this level of depletion allowed either maintenance of white cell ascorbic acid or an increase over the pre-supplement amount in all the subjects except J. P. Subjects E. B., Y. B., H. K., B. MC., B. M., E. W., J. W. and B. W. were able to maintain this increase so that the average of their white cell ascorbic acid content during the 14 days of the 20-mg intake was greater than their pre-supplement average. The differences between the pre-supplement average for ascorbic acid content of the white cells and the average during the 20-mg intake were not significant (table 3) for the group according to Student's "t" test for paired data (Snedecor, '46). When the intake was raised to 30 mg daily, H. K., E. W. and B. F. had white cell ascorbic acid contents that were increased over the average found with the 20-mg intake. The majority of the subjects had some decrease in white cell ascorbic acid content on the 30-mg intake (table 1), but again the differences were not significant (table 3). All of the subjects showed some increase in white cell ascorbic acid content when the intake of the vitamin was increased to 40 mg per day. In 9 instances the increases appear to be definite, while in 4 (E. B., E. W., J. W. and J. P.) these increases were slight and probably well within the experimental error of the method. Differences between the averages for the ascorbic acid con-

TABLE 1

Content of ascorbic acid in the white cells of subjects receiving low intakes of the vitamin for varying periods of time

(all values expressed in mg %)

DAILY INTAKE OF ASCORBIC ACID	NUMBER OF DAYS OF INTAKE	SUBJECT												
		E.B.	Y.B.	H.K.	B.MC.	B.M.	E.W.	J.W.	B.F.	S.F.	F.P.	J.P.	B.W.	A.W.
Unknown	Pre-diet	19.0 [1]	24.2	18.5	33.7	27.1	21.1	28.2	23.9	32.3	38.6	35.3	45.6	33.6
10 mg or less	14	20.0	24.7	27.0	22.0	27.0	14.7	25.6	20.2	28.9	33.0	35.0	28.8	23.5
	28	12.5	15.7	15.7	14.8	19.2	12.2	18.4	18.0	23.8	19.8	18.0	20.6	16.3
	35	10.5	11.9	12.5	[2]	[2]	11.1	9.1	16.3	18.9	24.0	16.0	20.0	21.1
	38	10.2	12.4	14.4	14.4	14.5	8.0	11.6	16.2	17.5	19.3	23.6	18.0	17.0
	42								15.9	18.7	17.8	23.1	17.7	20.0
Pre-supplement av.[3]		10.4	12.2	13.5	14.4	14.5	9.6	10.4	16.1	18.1	18.6	23.4	17.9	18.5
20 mg	4	12.9	14.3	13.4	16.3	16.6	12.3	14.9	17.2	14.7	19.2	19.0	19.2	19.7
	8	12.4	14.2	13.6	[2]	16.2	13.1	14.8	16.8	18.1	21.1	22.3	19.5	18.7
	11	11.8	15.0	15.2	18.0	16.0	11.0	14.2	13.3	15.4	18.3	16.2	16.5	14.5
	14	12.1	12.2	13.0	17.2	14.1	11.5	12.9	15.7	19.4	16.1	18.0	17.0	17.0
Average of period		12.3	13.9	13.8	17.2	15.7	12.0	14.2	15.8	16.9	18.7	18.9	18.1	17.5
30 mg	4	11.2	12.0	14.9	11.1	14.2	12.0	12.9	17.6	18.6	12.7	20.8	16.2	18.7
	7	13.2	14.4	14.1	12.2	16.0	14.3	13.0	16.9	15.5	17.2	15.5	14.6	16.6
	11	10.0	11.0	12.7	12.9	13.4	11.9	15.4	17.5	16.0	20.2	17.6	18.9	15.5
	14	11.3	13.5	15.6	13.2	16.1	12.8	13.7						
Average of period		11.4	12.7	14.3	12.4	14.9	12.8	13.8	17.3	16.7	16.7	18.0	16.6	16.9
40 mg	4	10.9	17.0	17.1	15.0	16.3	12.5	15.6	21.5	15.4	17.8	17.8	19.7	17.9
	7	11.7	12.9	17.5	15.7	16.8	14.2	14.3	20.1	18.5	20.6	21.0	21.4	19.7
	11	12.3	18.5	19.0			12.3	14.0	21.4	18.0	18.5	17.0	20.1	20.3
Average of period		11.6	16.1	17.9	15.4	16.6	13.0	14.6	21.0	17.3	19.0	18.6	20.4	19.3

[1] White cell ascorbic acid content determined on day denoted in 2nd column on the left.

[2] Value discarded for analytical reason.

[3] The pre-supplemented average is an average of the last two values for an individual except in the cases of B.MC. and B.M. where values on the 38th day only were used.

TABLE 2

Content of ascorbic acid in the sera of subjects receiving low intakes of the vitamin for varying periods of time
(all values expressed in mg %)

DAILY INTAKE OF ASCORBIC ACID	NUMBER OF DAYS OF INTAKE	SUBJECT											
		E.B.	Y.B.	H.K.	B.MC.	B.M.	E.W.	J.W.	B.F.	S.F.	F.P.	J.P.	B.W.	A.W.
Unknown	Pre-diet	0.49[1]	0.78	0.46	0.97	0.82	0.35	1.32	1.03	1.05	1.10	1.48	0.88	1.22
10 mg or less	14	0.46	0.36	0.32	0.62	0.48	0.33	0.54	0.49	0.56	0.65	0.71	0.51	0.51
	28	0.25	0.33	0.21	0.38	0.29	0.18	0.32	0.29	0.30	0.32	0.38	0.26	0.33
	35	0.22	0.24	0.16	0.30	0.26	0.24	0.34	0.27	0.25	0.30	0.30	0.27	0.39
	38	0.19	0.17	0.16	0.23	0.21	0.23	0.24	0.34	0.20	0.30	0.27	0.22	0.26
	42								0.16	0.16	0.12	0.23	0.19	0.17
Pre-supplement av.[2]		0.21	0.21	0.16	0.27	0.24	0.24	0.29	0.25	0.18	0.21	0.25	0.21	0.22
20 mg	4	0.15	0.20	0.14	0.22	0.18	0.13	0.17	0.27	0.20	0.28	0.28	0.23	0.26
	8	0.21	0.19	0.20	0.27	0.19	0.22	0.23	0.19	0.17	0.23	0.23	0.28	0.22
	11	0.16	0.20	0.15	0.22	0.17	0.15	0.23	0.19	0.16	0.19	0.18	0.21	0.19
	14	0.15	0.17	0.15	0.19	0.14	0.14	0.18	0.17	0.14	0.16	0.19	0.17	0.19
Average of period		0.17	0.19	0.16	0.23	0.17	0.16	0.20	0.21	0.17	0.22	0.22	0.22	0.22
30 mg	4	0.15	0.14	0.14	0.19	0.15	0.12	0.18	0.30	0.17	0.19	0.18	0.22	0.21
	7	0.18	0.20	0.14	0.23	0.19	0.18	0.18	0.26	0.13	0.15	0.19	0.20	0.24
	11	0.21	0.16	0.16	0.25	0.12	0.12	0.14	0.20	0.16	0.17	0.18	0.23	0.20
	14	0.16	0.16	0.14	0.23	0.17	0.17	0.21						
Average of period		0.18	0.17	0.15	0.23	0.16	0.15	0.18	0.25	0.15	0.17	0.18	0.22	0.22
40 mg	4	0.18	0.20	0.20	0.29	0.19	0.17	0.20	0.25	0.19	0.20	0.20	0.21	0.20
	7	0.19	0.25	0.21	0.24	0.20	0.20	0.21	0.27	0.17	0.20	0.22	0.27	0.22
	11	0.21	0.23	0.19			0.23	0.21	0.33	0.22	0.22	0.23	0.26	0.22
Average of period		0.19	0.23	0.20	0.27	0.20	0.20	0.21	0.28	0.19	0.21	0.22	0.25	0.22

[1] Serum ascorbic acid content determined on day denoted in 2nd column on the left.
[2] The pre-supplement average is an average of the last two values for an individual.

tent of the white cells for the periods with intakes of 30 mg and 40 mg of the vitamin were highly significant (table 3).

Serum ascorbic acid content dropped rapidly from the pre-diet amount until it was approximately 0.2 mg % for each subject by the time the depletion period was ended (table 2). The content continued to show some decline during the 20-mg-intake period for most of the subjects. Differences

TABLE 3

Comparisons of responses to different intake levels of ascorbic acid

COMPARISONS	MEAN DIFFERENCE	STANDARD ERROR OF MEAN DIFFERENCE	"t" [1]
White cell ascorbic acid content			
Pre-supplement vs. 20 mg av.	— 0.446	0.574	0.78
20 mg av. vs. 30 mg av.	+ 0.654	0.334	1.96
40 mg av. vs. 30 mg av.	+ 2.023	0.379	5.33 [2]
Serum ascorbic acid content			
Pre-supplement vs. 20 mg av.	+ 0.0285	0.00901	3.16 [2]
20 mg av. vs. 30 mg av.	+ 0.0092	0.00674	1.37
40 mg av. vs. 30 mg av.	+ 0.0346	0.00474	7.30 [2]

[1] Student's "t" test for paired data.

[2] $P < .01$.

between the averages of the serum ascorbic acid content for the 20-mg and the 30-mg periods were not significant (table 3). Raising the intake of ascorbic acid to 40 mg daily caused some increase in serum ascorbic acid for the majority of the subjects. Differences between the averages of the serum ascorbic acid content for the 30-mg and the 40-mg periods were highly significant (table 3).

DISCUSSION

When 13 subjects, who were partially depleted of ascorbic acid, were given 40 mg per day of the vitamin for 7 to 11 days, the ascorbic acid content of the white cells and sera showed a significant increase over the amount found when the subjects were given an intake of 30 mg for 11 to 14

days. It should be emphasized that these periods of intake of varying levels of ascorbic acid were extremely short and it is not known whether the 40-mg intake would cause a continued increase in the white cell and serum ascorbic acid, or would maintain the blood values at the levels reached in this experiment.

Some of the subjects had an increase in white cell ascorbic acid content over that of the pre-supplement amount when their intake was 20 mg per day for 14 days. These subjects, except for one, were in lower nutriture with respect to ascorbic acid when the supplementation was begun than were the subjects who had no increase. Davey et al. ('52) had found that 25 mg of ascorbic acid per day for 50 days was not adequate to maintain white cell ascorbic acid content in subjects in good nutriture. Our subjects were partially depleted of the vitamin. The differences in these findings might indicate that the nutriture of the subject would have some influence over the daily amount of ascorbic acid necessary to maintain or increase ascorbic acid content of white cells. Work by Van Eekelen ('36) has given some evidence that the rate of usage of ascorbic acid in man varies directly with the blood level, and the experiment by the English workers (Vitamin C Sub-Committee of the Medical Research Council of England, '48, '53) indicated that amounts of ascorbic acid as low as 10 and 20 mg per day caused some increase in white cell ascorbic acid content when the subjects were depleted of the vitamin. It is planned to test this postulation of effect of initial nutriture further in studies of human subjects.

SUMMARY

Thirteen adults (10 men and three women) were given a diet low in ascorbic acid for 78 days. After 38 to 42 days, when the subjects had been partially depleted of the vitamin, they were given enough pure ascorbic acid so each subject had in succession, three levels of intake, 20, 30 and 40 mg. The ascorbic acid content of their white cells and sera was determined at frequent intervals.

When the intake of ascorbic acid was 40 mg per day for 7 to 11 days, the ascorbic acid content of the white cells and sera showed a significant increase over that amount found when the subjects were given an intake of 30 mg for 11 to 14 days. There was some evidence that level of ascorbic acid nutriture affected the response to the varying amounts of the vitamin.

LITERATURE CITED

DAVEY, B. L., M. WU AND C. A. STORVICK 1952 Daily determination of plasma, serum and white cell-platelet ascorbic acid in relationship to the excretion of ascorbic and homogentisic acids by adults maintained on a controlled diet. J. Nutrition, *47:* 341.

GYÖRGY, P. 1950 Vitamin Methods. I. Academic Press, Inc., New York, N. Y.

LOWRY, O., O. BESSEY, M. J. BROCK AND J. A. LOPEZ 1946 The interrelationship of dietary, serum, white blood cell, and total body ascorbic acid. J. Biol. Chem., *166:* 111.

NORTHEAST REGIONAL PUBLICATION No. 5 1951 Cooperative nutritional status studies in the northeast region. I. Techniques. Cornell Univ. Agr. Exp. Station Memoir 307.

SNEDECOR, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames.

STEELE, B. F., C. HSU, Z. H. PIERCE AND H. H. WILLIAMS 1952 Ascorbic acid nutriture in the human. I. Tyrosine metabolism and blood levels of ascorbic acid during ascorbic acid depletion and repletion. J. Nutrition, *48:* 49.

VAN EEKELEN, M. 1936 On the amount of ascorbic acid in blood and urine. The daily human requirements for ascorbic acid. Biochem. J., *30:* 2291.

VITAMIN C SUB-COMMITTEE: MEDICAL RESEARCH COUNCIL 1948 Vitamin C requirement of human adults. Experimental study of vitamin C deprivation in man. Lancet, *i,* 853.

———— 1953 Vitamin C requirements of human adults. Special Report Series No. 280. London: Her Majesty's Stationery Office.

THE DIGESTION OF ACETYLATED MONOGLYCERIDES AND OF TRIGLYCERIDES [1]

DAVID C. HERTING, STANLEY R. AMES, MELPOMENI KOUKIDES AND PHILIP L. HARRIS

Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Company
Rochester, New York

ONE FIGURE

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INTRODUCTION

Distilled acetylated monoglycerides are fats with considerable potential use in the food field as additives and as edible coatings. The nutritive value of diacetylated monoglycerides has been shown (Mattson et al., '55) to correspond to that of natural triglycerides composed of similar long-chain fatty acids. Robbins and Ambrose ('55) have found that the rate of absorption of acetostearins and acetooleins by the rat equalled or exceeded that of partially hydrogenated shortening.[2] These workers also found that the coefficients of digestibility for acetostearins and acetooleins were 80 to 85 and 99%, respectively. Ambrose and Robbins ('55) reported that dietary levels of 20% of acetoolein or 10% of acetostearin did not interfere with growth or food utilization by the rat for periods over one year, although a level of 20% of acetostearin inhibited growth slightly.

Except for a preliminary report from this laboratory (Ames et al., '55) no data have appeared concerning the mode of

[1] Communication no. 224, Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N. Y.

[2] Crisco.

digestion of the acetylated monoglycerides. This report summarizes the results of a comprehensive investigation of the digestive products of the distilled acetylated monoglycerides as well as of triglycerides of corresponding fatty acid composition.

EXPERIMENTAL

Adult albino rats of the Sprague-Dawley strain were housed individually in hanging wire screen cages and were fed a

TABLE 1

Composition of diets

FAT SOURCE	FAT [1]	GLUCOSE [2]	VITAMIN-TEST CASEIN [3]	SALTS [4]
	%	%	%	%
Cottonseed oil	22.1	45.6	26.7	5.6
Cottonseed oil -B [5]	22.1	48.9	20.0	5.0
Lard	22.1	45.6	26.7	5.6
Hydrogenated lard	22.1	45.6	26.7	5.6
Distilled fully acetylated mixed saturated and unsaturated monoglycerides from lard [6]	33.0	39.2	23.0	4.8
Diacetylated saturated monoglycerides (distilled fully acetylated monoglycerides from hydrogenated lard)	33.2	39.1	22.9	4.8
Distilled partially acetylated monoglycerides from hydrogenated lard [7]	30.0	41.0	24.0	5.0
Distilled partially acetylated monoglycerides from hydrogenated lard [7] -B [5]	30.0	41.0	20.0	5.0

[1] Calculated to contain fatty acid residue equivalent to that of 30% MYVACET Type 5-00.

[2] Cerelose.

[3] General Biochemicals, Inc.

[4] The Pharmacopeia of the United States ('50).

[5] These diets also contained 4% of B-vitaminized casein which supplied the following vitamins per 1000 gm diet: B_1-HCl, 20 mg; B_2, 20 mg; B_6, 20 mg; Ca pantothenate, 60 mg; niacin, 100 mg; choline, 2.0 gm; inositol, 1.0 gm; 2-Me-1,4-naphthoquinone, 10 mg; *p*-aminobenzoic acid, 200 mg; biotin, 0.4 mg; folic acid, 4.0 mg; B_{12}, 0.04 mg.

[6] MYVACET distilled acetylated monoglycerides, Type 9-40.

[7] MYVACET distilled acetylated monoglycerides, Type 5-00.

stock diet composed of natural ingredients prior to the experiment. The test diets contained glucose, vitamin-test casein, fat, and salts, and were relatively similar in composition except for the source and amount of fat (table 1). The proportion of fat used supplied a level of about 21.5% fatty acids in the diet. The compositions and other physical characteristics of these fats are listed in table 2. Melting points were determined by the capillary method, and iodine values were obtained by the Wijs technique (A.O.A.C., '50).

All groups were given a test diet and water ad libitum for at least 5 days before sacrificing. Feces which were voided during the first three days on the experimental diet were discarded. Thereafter, feces were collected daily, brushed free of hair and food, and stored at — 20°C. until analyzed. At the completion of the experiment, the rats were killed with chloroform. Hemostats were placed immediately at the cardiac and pyloric sphincters and the ileocecal junction, and the entire gastrointestinal tract was removed. The contents of the stomachs were removed, ground with anhydrous Na_2SO_4, pooled, extracted with three 500-ml portions of anhydrous diethyl ether, and filtered. The residue was then extracted with three 500-ml portions of ether acidified to pH 3 with 25% HCl in ethanol. Feces and the contents of the cecum-colon were treated similarly. The contents of the small intestines were flushed with diethyl ether into a flask containing anhydrous Na_2SO_4, and the ether was evaporated. The Na_2SO_4-residue was ground and then was extracted as described above. All extracts were washed with distilled water to remove HCl and other water-soluble substances, dried with anhydrous Na_2SO_4, filtered, evaporated under a stream of nitrogen on the steam bath, and weighed. Previous studies had indicated that this procedure resulted in complete extraction of lipids.

Samples of the ether-soluble lipids were analyzed in a 50-tube Craig counter-current distribution apparatus, using Skellysolve B and aqueous methanol (15% water by volume) for the solvent system (Kuhrt et al., '52). The Craig ap-

TABLE 2

Characteristics of the various fats

FAT	ESTIMATED COMPOSITION [1]	MELTING POINT [2]	IODINE NUMBER [2]	FREE ACID CONTENT [3]
		C.		%
Cottonseed oil	Mono-S-di-U-triglyceride — about 3/4 Tri-U-triglyceride — about 1/4	— 4° to + 4°	~ 110	0.5
Lard	Mono-S-di-U-triglyceride — about 3/5 Mono-U-di-S-triglyceride — about 3/10 Tri-S- and Tri-U-triglyceride — about 1/10	~ 30°	~ 55	1.1
Hydrogenated lard	Tri-S-triglyceride	62°–64°	0	0.4
MYVACET Type 9-40	1,2-diacetylated-3-monoglyceride (U or S) — about 2/3 1,3-diacetylated-2-monoglyceride (U or S) — about 1/3	— 5° to + 5°	~ 40	0.4
Diacetylated saturated monoglyceride (Experimental research sample)	1,2-diacetylated-3-monoglyceride (S) — about 2/3 1,3-diacetylated-2-monoglyceride (S) — about 1/3	29°–32°	0	0.8
MYVACET Type 5-00	Monoacetylated monoglyceride (mainly 1-acetylated-3-monoglyceride [S]) — about 1/2 Monoglyceride (S) (mainly 1-monoglyceride) — about 1/5 Diacetylated monoglyceride (S) (cf. above sample) — about 3/10	42°–44°	0	0.8

[1] Suggested by Dr. G. Brokaw, Distillation Development Laboratory, D.P.I., except for lard and cottonseed oil which have been obtained from Hilditch ('49). U = unsaturated fatty acid; S = saturated fatty acid.

[2] Courtesy of Distillation Development Laboratory.

[3] Maximum, since calculated on basis that all free acid is long-chain fatty acid.

paratus was equipped with an electrical robot drive to provide automatic operation (Perry and Weber, '54). Infrared spectra were obtained for the lipid in the various peak tubes. All samples were analyzed for free fatty acid by a slight modification of the method described by the A.O.A.C. ('50). A weighed homogeneous sample of fat was dissolved in a 100-ml portion of ethanol which had been made just pink to phenolphthalein with 0.1 N NaOH. The free fatty acid was titrated with 0.1 N or 0.05 N NaOH.

RESULTS

Formation and excretion of fatty acids. Since fatty acid is formed during the first step in fat digestion, a complete investigation was carried out of the fatty acid content of the ether extracts and of the acidified ether extracts. The results are tabulated in table 3.

The high levels of fatty acid found in the stomach after feeding fully hydrogenated lard have been reported previously (Herting and Ames, '55). Similar results were obtained with two other saturated fats, the distilled partially acetylated monoglycerides from hydrogenated lard [3] and the distilled diacetylated monoglycerides from hydrogenated lard. Little gastric digestion of the unsaturated triglycerides occurred, confirming the findings of Mattson et al. ('52). When both saturated and unsaturated molecules were present, as with the distilled, diacetylated, mixed saturated and unsaturated monoglycerides from lard [4], the levels of fatty acid were intermediate between the high values for saturated fats and the low values for unsaturated fats. As would be expected, the amount of lipid in the acidified-ether extracts from the stomach was usually low.

The average levels of total fatty acid in the lipid recovered from the small intestines varied from 55.2 to 82.7% and were higher with the saturated fats. However, some overlapping of ranges occurred. These levels were higher than those

[3] MYVACET Distilled Acetylated Monoglycerides, Type 5-00.
[4] MYVACET Distilled Acetylated Monoglycerides, Type 9-40.

TABLE 3

Fatty acid content of lipids recovered from the stomach, the small intestine, and the cecum-colon

FAT IN DIET	RATS AND SEX	AVERAGE RAT WEIGHT	STOMACH					SMALL INTESTINE					CECUM-COLON				
			Ether		Ether-HCl			Ether		Ether-HCl			Ether		Ether-HCl		
			Total lipid [1]	Free fatty acid	Total lipid [1]	Fatty acid from soaps	Total fatty acid [2]	Total lipid [1]	Free fatty acid	Total lipid [1]	Fatty acid from soaps	Total fatty acid [2]	Total lipid [1]	Free fatty acid	Total lipid [1]	Fatty acid from soaps	Total fatty acid [2]
		gm	*mg*	*mg*	*mg*	*mg*	%	*mg*	*mg*	*mg*	*mg*	%	*mg*	*mg*	*mg*	*mg*	%
Cottonseed oil	2 M	342	182	24	11	2	13.5	92	25	22	(22)	41.2	120	47	104	79	56.2
Cottonseed oil -B	3 M	412	724	30	8	1	4.2	306	220	71	44	70.0	248	83	352	317	66.7
	3 M	464	409	21	5	1	5.3	202	100	45	40	56.7	274	132	468	456	79.2
							7.7					56.0					67.4
Lard	3 M	378	952	47	22	8	5.6	58	36	31	23	66.3	123	47	378	348	78.8
	2 M	399	47	5	12	2	11.9	112	57	29	23	56.7	151	65	367	336	77.4
							8.8					61.5					78.1
Hydrogenated lard	6 M	339	1224	772	321	297	69.2	625	399	500	469	77.2	1988	1048	4113	3817	79.7
	3 M+F	406	1838	664	366	106	39.6	611	330	415	354	66.7	1767	587	1856	1657	61.9
	3 M+F	390	1686	673	344	158	40.9	869	567	483	451	75.3	1576	679	1784	1707	71.0
							49.9					73.1					70.9
MYVACET Type 9-40	6 M	378	3397	900	1124	615	33.5	403	211	136	118	61.0	290	164	1687	1618	90.1
	3 M	337	1115	191	16	5	17.3	175	81	40	25	49.3	155	56	426	403	79.0
							25.4					55.2					84.6
Diacetylated saturated monoglyceride	6 M	350	2940	1835	535	494	67.0	721	370	511	474	68.5	1006	542	2583	2389	81.7
	3 M	328	1366	624	4	3	45.8	...	...	...	...	...	....	...	....	....	...
							56.4										
MYVACET Type 5-00	6 M	361	2652	1345	194	166	53.1	632	432	358	339	77.9	1433	817	3370	3097	81.5
	3 M	334	937	510	11	7	54.5	...	...	...	...	...	....	...	....	....	...
	3 M	338	1270	616	7	3	48.5	...	...	...	...	...	....	...	....	....	...
MYVACET Type 5-00 -B	3 M+F	(300)	3614	1456	76	74	41.5	484	369	404	385	84.9	1060	691	1850	1783	85.0
	3 M+F	(300)	464	237	4	4	51.5	190	142	295	272	85.4	554	381	1459	1459	91.4
							49.8					82.7					86.0

[1] Does not include any water-soluble materials.

[2] Based on total lipid from extractions with ether and acidified ether.

found previously by Borgström ('52a, b, '54a) and Desnuelle and Constantin ('52) within three hours after giving oil by stomach tube. The lipids from the cecum-colon presented the same general picture as those from the small intestine, with a general trend toward higher levels of total fatty acid and a much higher proportion of soaps.

The fecal data are presented separately in table 4, since fecal lipid was not always obtained from the same rats as the gastrointestinal lipid. On all diets the levels of total fecal fatty acids declined from those found in the cecum-colon. The

TABLE 4

Fatty acid content of lipids recovered from the feces [1]

FAT IN DIET	ETHER SOLUBLE		ETHER-HCl SOLUBLE		TOTAL FATTY ACIDS [3]
	Total lipid [2]	Free fatty acid	Total lipid [2]	Fatty acid from soaps	
	mg	*mg*	*mg*	*mg*	*%*
Cottonseed oil	289	97	98	86	47.3
Lard	146	19	227	209	61.1
Hydrogenated lard	3527	1569	2010	1487	55.2
MYVACET Type 9-40	145	40	270	246	68.9
Diacetylated saturated monoglyceride	2252	1401	721	650	69.0
MYVACET Type 5-00	1979	1405	935	870	78.1

[1] Collected from 6 rats and calculated as excretion per day per 6 rats.

[2] Does not include any water-soluble substances.

[3] Based on total lipid in extractions with ether and acidified ether.

amounts of lipid recovered from the feces, as well as from the small intestine and cecum-colon (table 3), were consistently lower when lard, cottonseed oil, or MYVACET Type 9–40 was the source of fat than when the fully saturated fats were fed. This would be expected from the well-known rapid absorption and high digestibility coefficient of most unsaturated fats (Deuel, '54).

The differences in the fatty acid content of stomach lipid after feeding saturated and unsaturated fats have been studied further with the aid of pylorus-ligated adult male rats, prepared essentially as described by Madden et al. ('51).

After a preliminary period of three to 7 days on either the diet containing cottonseed oil or that containing hydrogenated lard (table 1), food was removed and the rats were given a 10% sucrose solution for 20 to 24 hours. The pylorus was ligated under ether anesthesia, and three or 4 ml of an emulsion of the appropriate fat in 10% gelatin solution was given by stomach tube. These emulsions were prepared by high speed dispersion of liquid fat in hot gelatin solution and were maintained near 40°C. during use. After 4 hours the stomachs were removed, and the contents were ground with anhydrous Na_2SO_4. The lipid was extracted, weighed, and analyzed for fatty acid as described above.

TABLE 5

Free fatty acid content of fat recovered from stomachs of pylorus-ligated rats

FAT GIVEN	GROUP	NUMBER OF RATS	WEIGHT OF FAT GIVEN	WEIGHT OF FAT RECOVERED FROM STOMACHS [1]	TOTAL FREE FATTY ACID
			mg	*mg* ± σ	% ± *S.E.*
Cottonseed	A	6	374	410 ± 31	6.9 ± 0.6
oil	B	6	835	745 ± 41	5.5 ± 0.9
Hydrogenated	C	4	399	428 ± 26	5.6 ± 1.0
lard	D	6	868	876 ± 31	3.4 ± 0.9

[1] Four values were discarded because coprophagy had occurred. The stomach contents from these rats were gray, pasty, and foul-smelling. The discarded values were as follows: group A, 471 mg; group C, about 493 and 518 mg; and group D, 941 mg.

Values for several rats were discarded because coprophagy had occurred. The ether and acidified-ether extractable lipids were combined since the latter were negligible. Under these conditions, very little lipolysis occurred with either cottonseed oil or hydrogenated lard (table 5), but the amount of free fatty acid which was found in the stomach of the pylorus-ligated rat after feeding cottonseed oil was similar to that found previously with the intact rat.

Our previous communication (Herting and Ames, '55) suggested two explanations for the effect of saturation on gastric lipolysis. A gastric lipase may be much more active when

the substrate is a fully saturated glyceride or the high levels of fatty acid may result from some physiological effect of the saturated fats such as a decreased emptying time of the stomach. The results with the pylorus-ligated rat seem to favor the latter explanation although Tidwell and Cameron ('42) have reported that the gastric emptying time for saturated triglycerides is less than that for unsaturated triglycerides. However, the existence of a specific lipase has not been eliminated, since the enzyme may have been inactivated under the conditions used in the present experiment.

Separation of individual lipids. Craig distributions of the ether-soluble lipids supplied a more detailed picture of the lipid composition. Typical curves are given in figure 1. The

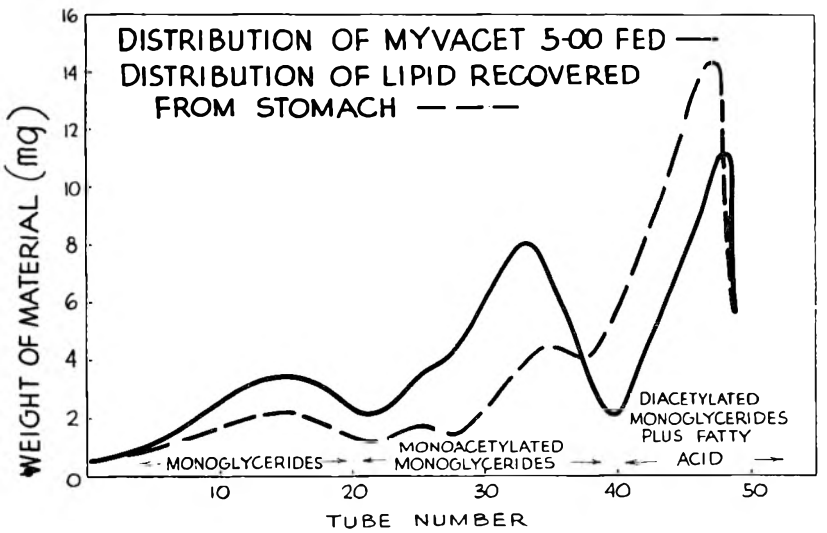

Fig. 1 Separation of lipid samples by the Craig apparatus.

material in the various peaks was identified by comparison with the peak locations of known materials and by infrared analyses. Under these conditions monoglycerides peak between tubes 6 and 19 (Perry and Brokaw, '55), monoacetylated monoglycerides between tubes 21 and 36, and fatty acids,

diacetylated monoglycerides, diglycerides and triglycerides between tubes 39 and 49. The exact location of a peak is dependent to a large extent on the degree of unsaturation of the fat, on the length of the carbon chains, and on the isomer content (Perry and Brokaw, '55).

Material that appeared in the first two tubes, which included any traces of water-soluble substances, was designated the "pre-monoglyceride" fraction. The amount of monoglycerides or of monoacetylated monoglycerides was determined by extrapolating the sides of its characteristic peak to zero weight. The free fatty acid was measured by titration. Since a 50-tube Craig apparatus is inadequate to resolve the mixture of lipids found in the least polar peak with this solvent system, the difference between the input weight and the sum of the above fractions was designated "di- and triglyceride". After these values had been determined, the fatty acids obtained from soaps by the acidified ether extractions were included in the calculations (table 6).

As expected from the work of Mattson et al. ('52) with unsaturated triglycerides, only small amounts of monoglyceride, 2 to 8%, were found in the stomach samples, except with the diet containing MYVACET Type 5–00. This fat contained appreciable quantities (23.8%) of monoglycerides, and a level of 12% was found in the stomach lipid. The intestinal levels of monoglycerides (5 to 11%) compare favorably with those reported by Desnuelle and Constantin ('52), Borgström ('54a), and Mattson et al. ('52, '54), who did not recover soaps. However, they are lower than those reported by Frazer and Sammons ('45) and Borgström ('52a, b). All these workers gave unsaturated fats by stomach tube, and the time of lipid recovery varied.

Although the monoglyceride fractions have not been analyzed chemically, infrared absorption spectra indicated that the intestinal monoglycerides from all fats consisted of predominantly or totally the l-isomer and were predominantly saturated except those found after feeding cottonseed oil. The fecal monoglycerides were saturated l-isomers in all

TABLE 6

Composition of original fat and of fat recovered from digestive sites

FAT IN DIET	SITE OF LIPID	TOTAL LIPID [1]	DISTRIBUTION OF LIPID					
			Free fatty acid [2]	Fatty acid from soaps	Mono-glyceride	Mono-acetylated mono-glyceride	Di- and tri-glyceride [3]	Other [4]
		mg	%	%	%	%	%	%
Cottonseed oil	Original	. . .	0.5	0	1.4	. . .	97.6	0.5
	Stomach	1339	6	0	8	. . .	82	4
	Small intestine	738	47	14	11	. . .	18	10
	Cecum-colon	1566	17	54	7	. . .	15	7
	Feces	387	25	22	15	. . .	33	5
Hydrogenated lard	Original	. .	0.4	0	1.5	. . .	98.1	0
	Stomach	1545	50	19	2	. . .	27	2
	Small intestine	1125	35	42	6	. . .	14	3
	Cecum-colon	6101	17	63	2	. . .	13	5
	Feces	5537	28	27	6	. . .	29	10
MYVACET Type 5-00	Original	. . .	0.8	0	23.8	47.0	28.1	0.3
	Stomach	2846	17	6	12	20	13	2
	Small intestine	990	44	34	8	3	9	2
	Cecum-colon	4803	17	64	6	1	6	6
	Feces	2914	48	30	8	6	4	4
Diacetylated saturated monoglyceride	Original	. . .	0.8	0	4.1	8.7	86.0	0.4
	Stomach	3475	53	14	5	4	21	3
	Small intestine	1232	30	38	5	2	21	4
	Cecum-colon	3589	15	67	4	3	5	6
	Feces	2973	47	22	12	5	11	3
MYVACET Type 9-40	Original	. . .	0.4	0	3.2	15.6	80.5	0.3
	Stomach	4521	20	14	3	10	41	12
	Small intestine	539	39	22	6	1	28	4
	Cecum-colon	1977	8	82	0	1	5	4
	Feces	415	10	59	1	1	23	6

[1] For feces, total lipid calculated as excretion per day per 6 rats.
[2] Free fatty acid was determined by titration.
[3] "Di- and triglyceride" portion was determined by difference between other fractions and 100%.
[4] Includes "pre-monoglyceride" fraction as well as lipid extracted by acidified ether which was not soap.

series which were studied. With MYVACET Type 9–40, the monoglyceride fraction was small and not analyzed. The absorption spectra of monoglycerides have been reported previously by Kuhrt et al. ('52).[5] Our observations regarding intestinal monoglycerides differ from the *in vivo* results of Mattson et al. ('52) and the *in vitro* results of Borgström ('54b), who concluded that 2-monoglycerides are formed in much greater amounts than 1-monoglycerides during intestinal digestion. Although our extraction procedure does not appear to differ greatly from that of Mattson et al. ('52), conversion of the 2-isomer to the 1-isomer may have occurred during the removal of solvent from our samples, particularly those obtained after feeding cottonseed oil or hydrogenated lard. With the acetylated monoglycerides, predominantly 1-monoglycerides would be expected from the configurations of the original fat (table 2).

Monoacetylated monoglycerides, whether fed or formed in the gastrointestinal tract, apparently were hydrolyzed fairly rapidly by lipases, as judged by the small amounts recovered. Similarly, diglycerides arising during the lipolysis of the hydrogenated lard in the stomach seemed to be hydrolyzed rapidly since infrared analysis of this sample indicated no diglyceride. The same technique indicated appearance of diglycerides in the other samples from this diet. Since absorption is negligible in the stomach, these results suggest that complete splitting of a given molecule of saturated fat is accomplished before another molecule is started. This mechanism has been proposed by Balls et al. ('37–'38) on the basis of the *in vitro* action of pancreatic lipase on tristearin.

The "di- and triglyceride" fractions decreased considerably in the stomach samples from the diets containing hydrogenated lard and the acetylated monoglycerides but never

[5] Mr. W. P. Blum, who has carried out these infrared analyses, has discovered a very specific difference between the spectra of crystals of the 1- and 2-isomers. The 1-monoglycerides absorb strongly at a wavelength of 10.08 μ whereas the 2-monoglycerides absorb strongly at 10.19 μ. Mixtures absorb at both wavelengths.

disappeared completely at any time during digestion. In most instances the relative amount of "di- and triglyceride" increased in the fecal sample. Absorption of fatty acid from or resynthesis of glycerides in the colon may have occurred.

DISCUSSION

In spite of extensive studies on the fate of dietary fat, the mode of digestion of unsaturated and saturated fats under approximately normal dietary conditions had not heretofore been determined. Most previous investigations of fat utilization in the rat have dealt either with the overall aspect by comparing dietary intake with fecal output and expressing the result as the coefficient of digestibility or with the composition of the lipids recovered from the intestinal tract after giving a fat by stomach tube to a fasting rat. The former technique is valuable in measuring the absorption of a fat but can only describe an overall result. The latter procedure does not regard the impact of dietary constituents other than fat on the digestion of the fat itself. The importance of other factors is illustrated by the work of Desnuelle and Constantin ('52), who found that administering calcium as well as fat to rats increased the rate of intestinal lipolysis. The inclusion of fat in a normal diet should permit a more accurate picture of the digestive processes, recognizing that the residual lipids reflect the equilibrium resulting from lipolysis, re-esterification and absorption.

In general, our results with cottonseed oil, an unsaturated triglyceride, are similar to those of previous studies with fat administration by stomach tube. Little lipolysis occurred in the stomach, but the lipid recovered from the small intestine contained large amounts of fatty acid and lesser amounts of mono-, di, and triglycerides. In fact, the levels of fatty acid were higher than might have been predicted from previous results. The components of the lipid recovered from the cecum-colon differed little from those of the small intestine but the fecal lipid was characterized by an increase in the "di- and triglyceride" fraction. The digestion of fully

saturated fats heretofore has been neglected except for work by Mattson et al. ('54) with tricaprin. Our results with hydrogenated lard indicate that a greater gastric lipolysis distinguishes the digestion of these fats from that of triglycerides containing unsaturated fatty acids.

The digestion of an acetylated monoglyceride is analogous to that of a corresponding triglyceride. When mono- and diacetylated monoglycerides containing only saturated fatty acids were fed, high levels of fatty acid and relatively low levels of intermediate lipolytic products were recovered from all sites of collection. A mixture of saturated and of unsaturated acetylated monoglycerides (MYVACET Type 9–40) was digested in a similar fashion except for a lower level of free fatty acid in the stomach.

The fate of acetic acid, glycerol, and glycerol esters of acetic acid, which appear during the lipolysis of the acetylated monoglycerides, has been extensively studied. Deuel and coworkers found that triacetin was a source of liver glycogen ('37) and that triacetin was absorbed more rapidly from the gastrointestinal tract in three hours than any other fat tested ('40). Cox ('33) found that 55% of triacetin in the diet produced good growth and appeared to be converted into longer chain, unsaturated fatty acids. McManus et al. ('43) fed triacetin equal in caloric value to 15% glucose and concluded that the triacetin was utilized with a degree of efficiency equal to that of glucose. When the acetic acid was given as the sodium salt, Deuel et al. ('41) indicated that it was absorbed less than many other acids in the free or salt form. However, the recovery of only the intestinal contents raises the question of differential retention rates of the various acids by the stomach. Garner and Roberts ('54) found that the maximum level of dietary sodium acetate which could be tolerated by rats appeared to be about 13%. However, the sodium increment probably also played a role in determining this tolerance. In view of these results and of the metabolic importance and ubiquity of acetic acid and glycerol, there

seems to be no doubt that these portions of the acetylated monoglycerides would be utilized efficiently.

Although the fully saturated fats were split rapidly, the relatively large amounts of fecal lipid indicate that the digestive products of these fats are utilized less efficiently than those of the unsaturated fats. Cheng et al. ('49) found low coefficients of digestibility of 10.6 and 12.8 for tristearin and tripalmitin, respectively, when these fats were fed at levels of 15% in the diet. However, the digestibility of the saturated acetylated monoglycerides is apparently much higher since Robbins and Ambrose ('55) have reported that the coefficient of digestibility for acetostearin is 80 to 85%. The behavior of the acetylated monoglycerides may resemble that of butterfat and coconut oil. Deuel ('54) has suggested that the relatively high initial rate of absorption of butterfat may result from rapid utilization of the short-chain glycerides. Coconut oil, though containing about 85% of fully saturated triglycerides (Hilditch, '40) has a digestibility coefficient of 93 to 99 even when fully hydrogenated (Evans and Lepkovsky, '32; Hoagland and Snider, '43a), although the former workers may not have recovered all soaps. These results indicate a rapid utilization of the relatively short-chain fatty acids which are predominant in coconut oil. In addition, the work of Barnes et al. ('44) indicated that the presence of short-chain acids may even enhance the utilization of long-chain saturated fatty acids. These workers showed that incorporation of hydrogenated fats into butter or lard gave no apparent alteration in the digestibility of "hardened" butterfat but decreased that of "hardened" lard.

Studies by Cheng et al. ('49) with lard of varying degrees of hydrogenation have shown that decreasing the unsaturated fatty acid in the dietary fat decreases the utilization of the fat. However, very little has been done to determine whether the digestion of saturated fats in a normal diet is influenced by the large amount of unsaturated fats present. Hoagland and Snider ('43b) found that the digestibility of small amounts of tristearin dissolved in olive oil was very

low. Carver and co-workers ('54) reported that the absorption of hydrogenated tallow by the chick was not enhanced by the presence of partially unsaturated fats. On the other hand, the data presented by Mattil and Higgins ('45) indicated that the addition of unsaturated fat to the diet increased the digestibility of tristearin relative to that reported by Cheng and co-workers ('49) for tristearin alone. Our data provide further evidence for such an effect. MYVACET Type 9–40, which contains about 40% fully saturated glycerides depending on the fatty acid composition of the lard from which it is derived, disappeared in the intestinal tract to almost the same extent as cottonseed oil or lard. These results confirm in essence the coefficient of digestibility of 99% reported by Robbins and Ambrose ('55) for acetooleins, of which one was MYVACET Type 9–40.

SUMMARY

The mode of digestion of cottonseed oil, lard, hydrogenated lard, and distilled acetylated glycerides has been studied by analyzing the lipid recovered from the stomach, small intestine, cecum-colon, and feces after feeding diets containing the various fats to adult rats.

The digestion of cottonseed oil and lard followed the pattern which might be predicted for partially unsaturated triglycerides. Little lipolysis occurred in the stomach, but extensive lipolysis occurred in the small intestine, with the resulting products being absorbed rapidly.

The digestion of a characteristic saturated triglyceride, fully hydrogenated lard, was similar to that of unsaturated triglycerides except for a greater degree of gastric lipolysis. This increased lipolysis appeared to reflect some physiological effect of the fat. In contrast to the unsaturated triglycerides, a large amount of fecal lipid was found, indicating a relatively poor absorption coefficient for the hydrogenated lard.

The digestion of the distilled acetylated saturated monoglycerides prepared from hydrogenated lard coincided with

that of hydrogenated lard. The digestion of distilled, acetylated, mixed saturated and unsaturated monoglycerides prepared from lard was intermediate between those for the saturated and unsaturated triglycerides. Analogous to that of other fats, the absorption of the digestive products of the distilled acetylated monoglycerides depended largely on their fatty acid composition.

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LITERATURE CITED

AMBROSE, A. M., AND D. J. ROBBINS 1955 Toxicological studies on acetoglycerides. Fed. Proc., *14:* 314.

AMES, S. R., D. C. HERTING, M. KOUKIDES AND P. L. HARRIS 1955 Digestion of acetylated monoglycerides and triglycerides. Ibid., *14:* 173.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official Methods of Analysis, 7th ed.

BALLS, A. K., M. B. MATLACK AND I. W. TUCKER 1937–38 The hydrolysis of glycerides by crude pancreas lipase. J. Biol. Chem., *122:* 125.

BARNES, R. H., M. F. PRIMROSE AND G. O. BURR 1944 The influence of the protein content of the diet upon fat digestibility. J. Nutrition, *27:* 179

BORGSTRÖM, B. 1952a Investigation on lipid separation methods. Separation of cholesterol esters, glycerides, and free fatty acids. Acta Physiol. Scand., *25:* 111.

———— 1952b On the action of pancreatic lipase on triglycerides in vivo and in vitro. Ibid., *25:* 328.

———— 1954a The formation of new glyceride-ester bonds during digestion of glycerides in the lumen of the small intestine of the rat. Arch. Biochem. Biophys., *49:* 268.

———— 1954b On the mechanism of pancreatic lipolysis of glycerides. Biochim. et Biophys. Acta, *13:* 491.

CARVER, D. S., E. E. RICE, R. E. GRAY AND P. E. MONE 1954 The utilization of fats of different melting points added to broiler feeds. Poultry Sci., *33:* 1048.

CHENG, A. L. S., M. G. MOREHOUSE AND H. J. DEUEL, JR. 1949 The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. J. Nutrition, *37:* 237.

COX, W. M., JR. 1933 The nutritive value of pure fatty acid esters. J. Biol. Chem., *103:* 777.

DESNUELLE, P., AND M. J. CONSTANTIN 1952 Formation of partial glycerides during lipolysis of triglycerides in the intestine. Biochim. et Biophys. Acta, *9:* 531.

DEUEL, H. J., JR. 1954 Nutritional significance of the fats. In "Progress in the chemistry of fats and other lipids." Vol. 2. Academic Press, New York, p. 99.

DEUEL, H. J., JR., J. S. BUTTS, H. BLUNDEN, C. H. CUTLER AND L. KNOTT 1937 Studies on ketosis. IX. Glycogen formation from various purified and natural fats. J. Biol. Chem., *117:* 119.

DEUEL, H. J., JR., AND L. HALLMAN 1940 The rate of absorption of synthetic triglycerides in the rat. J. Nutrition, *20:* 227.

DEUEL, H. J., JR., L. HALLMAN AND A. REIFMAN 1941 The rate of absorption of various fatty acids by the rat. Ibid., *21:* 373.

EVANS, H. M., AND S. LEPKOVSKY 1932 The sparing action of fat on vitamin B. II. The role played by the melting point and the degree of unsaturation of various fats. J. Biol. Chem., *96:* 165.

FRAZER, A. C., AND H. G. SAMMONS 1945 The formation of mono- and diglycerides during the hydrolysis of triglyceride by pancreatic lipase. Biochem. J., *39:* 122.

GARNER, R. J., AND R. ROBERTS 1954 Effect of acetate on the fasting blood-sugar level of the rat. Nature, *174:* 1194.

HERTING, D. C., AND S. R. AMES 1955 On the gastric lipolysis of fat. Arch. Biochem. Biophys., *55:* 295.

HILDITCH, T. P. 1940 The Chemical Constitution of Natural Fats. John Wiley and Sons, Inc., New York, p. 162.

———— 1949 The Industrial Chemistry of the Fats and Waxes. 3rd ed. Bailliere, Tindall and Cox, London.

HOAGLAND, R., AND G. G. SNIDER 1943a Digestibility of some animal and vegetable fats. J. Nutrition, *25:* 295.

———— 1943b Digestibility of certain higher saturated fatty acids and triglycerides. Ibid., *26:* 219.

KUHRT, N. H., E. A. WELCH, W. P. BLUM, E. S. PERRY AND W. H. WEBER 1952 The isolation of monoglycerides from lard and from bread. J. Am. Oil Chem. Soc., *29:* 261.

MADDEN, R., H. RAMSBURG AND J. HUNDLEY 1951 A critical study of the Shay rat. Gastroenterology, *18:* 119.

MATTIL, K. F., AND J. W. HIGGINS 1945 The relationship of glyceride structure to fat digestibility. I. Synthetic glycerides of stearic and oleic acids. J. Nutrition, *29:* 255.

MATTSON, F. H., F. J. BAUR AND L. W. BECK 1955 Nutritive value of acetin fats. Fed. Proc., *14:* 443.

MATTSON, F. H., J. H. BENEDICT AND L. W. BECK 1954 Composition of intestinal lumen lipides following the feeding of triglycerides, partial glycerides, or free fatty acids. J. Nutrition, *52:* 575.

MATTSON, F. H., J. H. BENEDICT, J. B. MARTIN AND L. W. BECK 1952 Intermediates formed during the digestion of triglycerides. Ibid., *48:* 335.

MCMANUS, T. B., C. B. BENDER AND O. F. GARRETT 1943 A comparison of acetic acid, fed in the form of triacetin, with glucose as a nutrient in feeds. J. Dairy Sci., *26:* 13.

PERRY, E. S., AND G. Y. BROKAW 1955 The countercurrent distribution of pure monoglycerides and some related substances. J. Am. Oil Chem. Soc., *32:* 191.

PERRY, E. S., AND W. H. WEBER 1954 Automatic drive for countercurrent distribution apparatus. Anal. Chem., *26:* 498.

ROBBINS, D. J., AND A. M. AMBROSE 1955 Comparative rate of absorption and digestion of acetoglycerides. Fed. Proc., *14:* 382.

"THE PHARMACOPEIA OF THE UNITED STATES OF AMERICA" 1950 Mack Publishing Company, Easton, Pa., p. 789.

TIDWELL, H. C., AND E. S. CAMERON 1942 Relation between the chemical structure of fats and their ability to produce gastric inhibition. Bull. Johns Hopkins Hosp., *70:* 362.

CELLULOLYTIC-FACTOR ACTIVITY OF CERTAIN SHORT-CHAIN FATTY ACIDS FOR RUMEN MICROORGANISMS IN VITRO [1,2]

ORVILLE G. BENTLEY, RONALD R. JOHNSON, T. V. HERSHBERGER, J. H. CLINE AND A. L. MOXON

Department of Animal Science, Ohio Agricultural Experiment Station, Wooster

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TWO FIGURES

Unidentified nutritional factor(s) required by rumen microorganisms *in vitro* have been described by several investigators (Hungate, '50; Burroughs et al., '50; Huhtanen et al., '52; Doetsch et al., '52; Bentley et al., '53; Garner et al., '54). Ruf et al. ('53), Bentley et al. ('54b) and McNeill et al. ('54) found that a heat stable substance(s) could be concentrated by adsorption from various natural materials, including rumen juice. The microbial response to these factors as shown by increased cellulose digestion and volatile fatty acid production also appears to involve certain B-vitamins, particularly biotin, para aminobenzoic acid (PABA), vitamin B_{12}, vitamin B_6, and pantothenic acid (Hall et al., '53; Bentley et al., '54a).

In a preliminary report, Bentley et al. ('54c) showed that certain short-chain fatty acids had cellulolytic-factor activity for rumen microorganisms. Likewise, Bryant and Doetsch ('55) found that *Bacteroides succinogenes,* previously isolated from rumen contents, required a combination of a branched-

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[2] We gratefully acknowledge the support, in part, of the studies reported herein by a Grant-in-Aid from Swift and Company, Chicago, Illinois.

chain (iso-butyric or iso-valeric) and a straight-chain acid (valeric or caproic) for growth in a purified medium. The results to be presented herein describe in detail the cellulolytic-factor activity of short-chain fatty acids and also their relationship to non-protein nitrogen utilization.

EXPERIMENTAL

Inoculum. Rumen juice which was expressed from ingesta obtained through a permanent ruminal fistula in a steer fed a good-quality alfalfa hay was used as a source of the inoculum. The filtered juice (cheesecloth) was passed through a Sharples supercentrifuge and the microflora and other suspended material collected on a celluloid liner placed in the centrifuge cylinder. The sediment from one liter of juice was suspended with the aid of a loose fitting Potter-Elvehjem homogenizer in 200 ml of phosphate buffer (pH 7.0) which contained 0.01% cysteine-HCl. Eight milliliters of this suspension were used to inoculate the *in vitro* fermentation flasks.

Assay method. The composition of the basal medium was as follows in grams per 100 ml: cellulose, 1.0; urea, 0.168; glucose, 0.1; disodium hydrogen phosphate, 0.113; sodium dehydrogen phosphate, 0.109; potassium chloride, 0.043; magnesium carbonate, 0.004; calcium chloride, 0.004; sodium chloride, 0.043; sodium sulfate, 0.015; sodium carbonate, 0.2; ferric chloride ($6H_2O$), 0.044. Para-aminobenzoic acid (50 μg/100 ml) and biotin (20 μg/100 ml) were also added. Adjustments in the pH (6.7 to 6.9) when indicated by pH determinations were made by the addition of a saturated sodium carbonate solution. Carbon dioxide bubbled through the flask contents served to establish anaerobiosis and to agitate the suspension.

At the end of the 30-hour fermentation period, the volume was adjusted to 100 ml and the disappearance of cellulose determined on duplicate 10 ml samples of the reaction mixture. Cellulose was determined by a procedure developed in this laboratory (Hershberger et al., '55) based on a

modification of the Crampton-Maynard method (Crampton and Maynard, '38).

The increase in 10% trichloroacetic acid (TCA) insoluble nitrogen was used as an indication of microbial protein synthesis.

Distillation of volatile fractions from rumen juice. Distillates from centrifuged rumen juice acidified to pH 2 to 2.5 with 85% phosphoric acid were prepared in a Kjeldahl apparatus. After the initial distillation, the flask and contents were cooled, diluted with water and redistilled. Three such distillations were carried out in an effort to remove all of the volatile components. Similar distillations were made from rumen juice adjusted to pH 10 to 11 with sodium hydroxide.

Chromatographic separation of fatty acids. Volatile fatty acids were separated using the method of Moyle et al. ('48) with slight modification. Samples of rumen liquor or distillates (5 to 6 ml) mixed with 10 gm of dry silicic acid were added as a chloroform slurry to a buffered (pH 7.25) silicic acid column (Harper, '53). The chromatogram was then developed using successive portions of one, 5, 15, 30 and 80% butanol in chloroform, equilibrated with water.

RESULTS

The results summarized in figure 1 show the cellulolytic response of rumen microorganisms *in vitro*. As has been previously reported from our laboratory, the addition of rumen juice (Bentley et al., '54b) or the distillate from acidified rumen juice (Bentley et al., '54c) increased the rate of cellulose digestion about three-fold. Further, it was found that the distillates from alkaline rumen juice (pH 10 to 11) were inactive.

The behavior of the active components in the distillates suggested that a volatile fatty acid might be involved. Short-chain fatty acids (up to 10 carbons in length) were added to the medium at levels of from two to 10 mg per 100 ml of reaction mixture. As can be seen in figure 1, valeric or

caproic acid increased the rate of cellulose digestion almost three-fold while iso-butyric or iso-valeric acid were about two-thirds as effective as the straight-chain acids. Acetic, propionic and butyric acids, which are produced in relatively large amounts by rumen microorganisms, and formic acid were ineffective, as were the C_7 to C_{10} acids tested. The acids were added to the medium and the pH was adjusted.

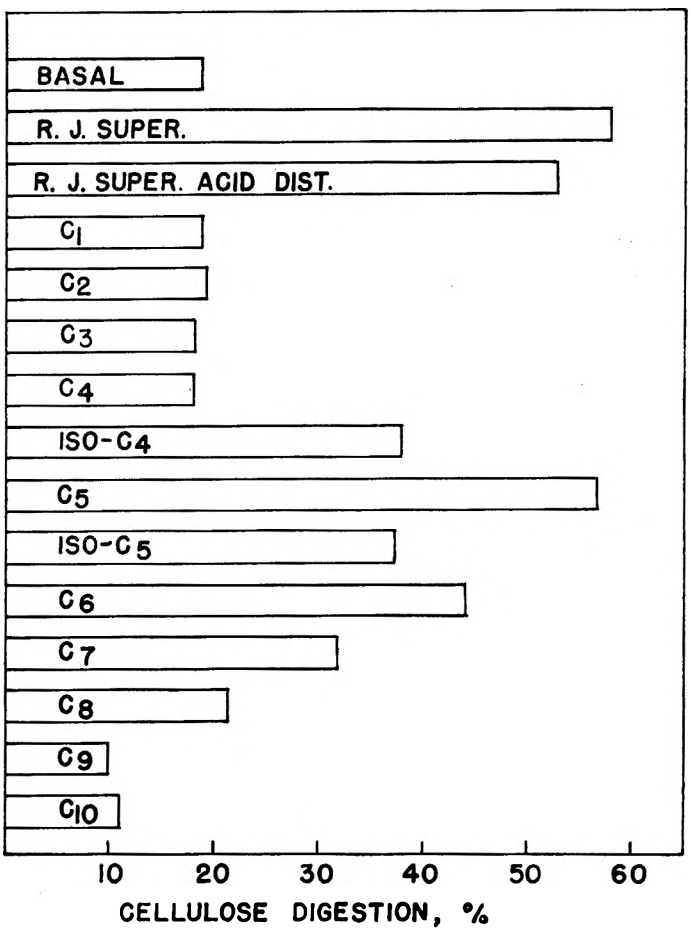

Fig. 1 The cellulolytic response of rumen microorganisms to additions of rumen juice supernatant, a distillate prepared from acidified rumen juice supernatant, and various straight- and branched-chain fatty acids to the fermentation medium. Ten milliliters of rumen juice, the distillate from 20 ml of rumen juice, and 10 mg fatty acids were added per flask.

The response in cellulose digestion to different levels of valeric acid is shown in table 1. These results together with the cellulose digestion data in table 2 indicate that a maximum response was elicited by 10 mg of valeric acid per flask. The addition of more acid (20 to 50 mg) appeared to depress the rate of cellulose digestion.

Besides increasing cellulose digestion, valeric acid affected the rate of urea nitrogen utilization as indicated by the increase in TCA-insoluble protein in the fermentation flasks (see table 2). The presence of more protein would suggest

TABLE 1

The effect of various levels of valeric acid on cellulose digestion

MEDIUM	NUMBER OF EXPERIMENTS	AVERAGE CELLULOSE DIGESTION
		%
Basal	5	19.4 ± 2.9 [1]
Basal + valeric acid, 2 mg/flask	1	34.0
Basal + valeric acid, 4 mg/flask	2	43.5 ± 3.0
Basal + valeric acid, 10 mg/flask	5	58.2 ± 9.4
Basal + valeric acid, 20 mg/flask	1	43.0
Basal + valeric acid, 50 mg/flask	1	38.0
Basal + centrifuged rumen juice, 10% [2]	5	57.8 ± 5.4

[1] Standard deviation from the mean.

[2] Since centrifuged rumen juice gave maximum growth-factor activity, it was used as a positive control in each experiment.

TABLE 2

Valeric acid and the increase in trichloracetic acid (TCA)-insoluble nitrogen

MEDIUM	[1] TCA-N/100 ML	CELLULOSE DIGESTION
	mg	%
Basal	4.7	5.4
Basal + 1 mg valeric acid/flask	6.7	16.8
Basal + 2 mg valeric acid/flask	9.0	33.8
Basal + 3 mg valeric acid/flask	9.5	39.4
Basal + 5 mg valeric acid/flask	11.0	57.6
Basal + 8 mg valeric acid/flask	10.5	59.3
Basal + 10 mg valeric acid/flask	10.7	63.2

[1] TCA-insoluble protein nitrogen per flask.

that bacterial numbers had increased as a result of adding valeric acid.

Studies carried out by Hall et al. ('53) and Bentley et al. ('53) had indicated that under certain conditions B-vitamins exert an effect on the rate of cellulose digestion *in vitro*. Bentley et al. ('54b) found that the cellulolytic response to various fractions prepared from rumen juice was greater in the presence of biotin, PABA, and vitamin B_{12}.

The addition of valeric acid to the basal medium without biotin and PABA gave no response in cellulose digestion

TABLE 3

Biotin, PABA, and valeric acid and cellulose digestion

ADDITION TO MEDIUM [1]	CELLULOSE DIGESTED	
	Experiment A	Experiment B
	%	%
Biotin and PABA	21.6	22.8
Valeric acid, 10 mg/flask (V.A.)	19.3	31.9
V. A. + biotin	58.0	44.3
V. A. + PABA	27.3	36.4
V. A. + biotin + PABA	61.3	56.9
V.A. + biotin + PABA + vitamin B_{12}	54.6	...
Centrifuged rumen juice	62.5	61.4

[1] Basal medium used in these experiments the same as described under "Experimental" except that the biotin and PABA were omitted.

(see table 3). However, valeric acid with either of the vitamins alone or a combination of them was stimulatory. The effect of the two vitamins was additive.

The short-chain fatty acids in rumen juice were separated chromatographically using a silicic acid column. A typical separation of the fatty acids is shown in figure 2. Rumen juice was found to contain from 0.21 to 0.35 mg of valeric acid and a trace to 0.08 mg of caproic acid per milliliter (see table 4). It is apparent that the distillation procedure used failed to remove all of the volatile fatty acids from the rumen juice.

TABLE 4

Short-chain fatty acid content of rumen juice

ACID	DISTRIBUTION OF ACIDS [1]							
	Rumen juice				Acid distillate from rumen juice			
	Hours after feeding [2]				Hours after feeding			
	0	3	6	9	0	3	6	9
Caproic	0.04	0.08	Trace	Trace	Trace	0.02	Trace	Trace
Valeric	0.21	0.35	0.23	0.22	0.18	0.26	0.10	0.07
Butyric		0.78				0.67		
Propionic		1.54				1.33		
Acetic		3.98				3.73		

[1] Expressed in milligrams per milliliter.

[2] Sample of rumen contents taken at the indicated time intervals after feeding the animal hay.

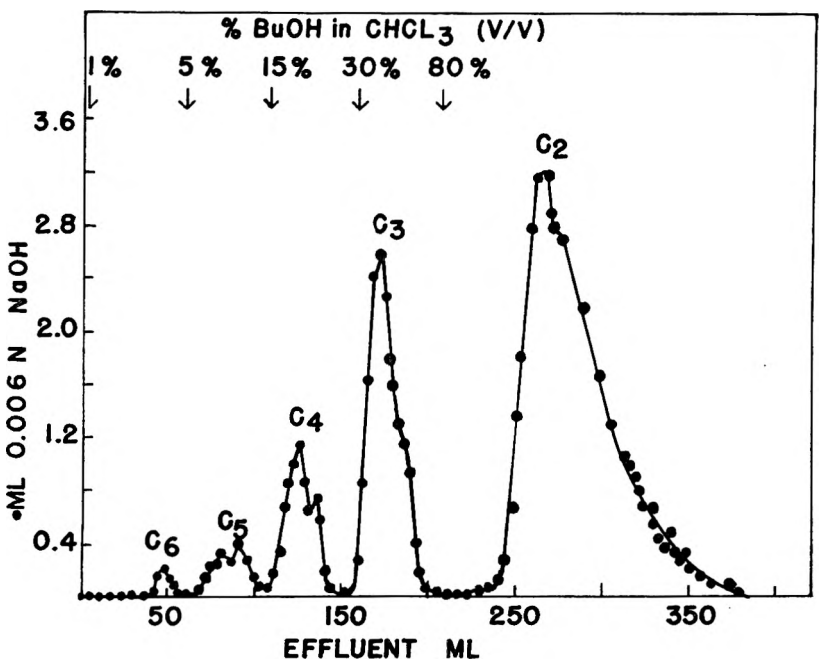

Fig. 2 The distribution of C_2 to C_6 fatty acids in 5 ml of rumen juice supernatant. By this procedure, 97.8% of the titratable acidity was recovered. The isoacids, if any were present, were not separated by this method. With mixtures of pure (commercial) acids, quantitative recovery of acetic and propionic acids was obtained; however the recovery of the next three acids was somewhat less, *e.g.*, butyric, 94.7%; valeric, 84.8%; and caproic, 82.8%.

These analytical findings are in agreement with those of Annison ('54) and Annison and Pennington ('54) in that only trace amounts of caproic acid are present in rumen juice. Thus it appears that valeric acid would be the most likely source of short-chain fatty acid activity in the rumen of the intact animal under natural conditions. Likewise, rumen juice contains all of the B-vitamins.

TABLE 5

Effect of distillation on factor activity of natural materials

ADDITIONS TO BASAL MEDIUM	CELLULOSE DIGESTION	
	Experiment C	Experiment D
	%	%
None	18.2	20.5
Dist. from acidified alfalfa meal ext.	29.6	31.6
Dist. from acidified yeast ext.	20.5	...
Dist. from acidified dried distillers' solubles	20.5	19.3
Yeast ext.	62.5	...
Residue from distillers' solubles after acid distillation	56.9	56.9
Centrifuged rumen juice, 10%	61.4	60.3

Ruf et al. ('53), Bentley et al. ('54b), and McNeill et al. ('54) have reported that various natural materials contained cellulolytic-factor activity for rumen microorganisms. Attempts to separate the active components by distillation from acidified water extracts of alfalfa meal, yeast extract, or dried distillers fermentation solubles or suspensions of these materials were unsuccessful. Further autoclaving of these materials in 1.0 N HCl for 12 hours did not affect the release of a volatile growth-stimulating factor. The data are presented in table 5.

DISCUSSION

These results suggest that 5- and 6-carbon acids, either branched or straight chain, are important in the metabolism of a mixed rumen microorganism population as grown *in vitro*. The report by Bryant and Doetsch ('55) with *B. succinogenes* further substantiates this conclusion. Since one

of the major functions of the rumen microflora is the degradation of cellulose from roughages to acetic, propionic and butyric acids, it is probably not surprising that these acids were found to be inactive. However, these acids may be involved in the formation of the 5- and 6-carbon acids. Gray et al. ('51a) found that carboxyl-labelled radioacetate incubated *in vitro* with rumen contents gave rise to radiobutyrate, radiovalerate, and radiopropionate. In similar experiments radiopropionate gave rise to radiovalerate and a trace of radioacetate. It thus appears possible that the 5- and 6-carbon acids may take part in metabolic systems involving the normal end products of carbohydrate digestion in the rumen, namely, short-chain fatty acids.

Since Elsden ('46) first investigated the volatile fatty acids in rumen juice, Gray et al. ('51b) have also demonstrated the presence of iso-butyric, valeric, caproic and heptanoic acids. Annison ('54) made a thorough investigation of the distribution of branched- and straight-chain fatty acids using the gas-liquid partition chromatography method. These workers found valeric acid to be present in amounts comparable to those reported herein (table 4) while caproic acid was present in trace amounts. Besides in rumen juice, 5-carbon acids, both straight and branched, have been found in other products from ruminant animals, such as beef tallow and wool fat (Hansen and McInnes, '54).

El-Shazly ('52) found that both straight and branched short-chain fatty acids were among the degradation products formed by rumen microorganisms from proteins and amino acids. A possible relationship of El-Shazly's findings to the microbial-stimulating action of volatile acids is that certain amino acids, particularly valine and proline, stimulate cellulose digestion by rumen microorganisms *in vitro*. A preliminary report of this observation has appeared (Bentley et al., '54c) and more work on this phase is now in progress. Further, if these factors influence amino acid metabolism or synthesis or both, it becomes of interest, particularly since the utilization of urea nitrogen by the rumen microflora for

amino acid synthesis (Loosli et al., '49) is of practical importance in livestock feeding.

Nevertheless, there still appear to be additional rumen microbial factors in natural feedstuffs and in whole, uncentrifuged rumen juice. A combination of valeric acid, biotin and PABA will duplicate the cellulolytic response obtained from yeast extract, rumen juice, etc., yet a non-volatile substance is present in these natural materials which is also active *in vitro* for rumen organisms. This relationship is also being further investigated. Hall et al. ('54) have found that partial hydrolysates of proteins stimulate the rate of cellulose digestion. This observation would likewise appear to be related to the type of response reported herein.

SUMMARY

Studies on the cellulolytic-factor activity of rumen juice for rumen microorganisms *in vitro* indicated that the volatile fatty acid fraction contained the activity. Results of *in vitro* experiments using commercially available volatile fatty acids showed that valeric and caproic acids and to a lesser extent iso-butyric and iso-valeric acids markedly increased the rate of cellulose digestion and ammonia utilization. Analytical results showed that the activity of the volatile fatty acid fraction of rumen juice was primarily due to valeric acid.

Biotin and para-amino benzoic acid were also required by the microflora for maximum cellulose digestion.

This vitamin short-chain fatty acid combination duplicated but was not identical to the microbial cellulolytic factor(s) previously found to be present in certain natural feedstuffs fed to cattle and sheep.

ACKNOWLEDGMENTS

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LITERATURE CITED

ANNISON, E. F. 1954 Some observations on volatile fatty acids in the sheep's rumen. Biochem. J., *57:* 400.

ANNISON, E. F., AND R. J. PENNINGTON 1954 The metabolism of short-chain fatty acids in the sheep. 3. Formic, n-valeric and some branched-chain acids. Ibid., *57:* 685.

BENTLEY, O. G., R. R. JOHNSON AND A. L. MOXON 1954a Factors influencing cellulose digestion by rumen microorganisms *in vitro*. Fed. Proc., *13:* 182.

BENTLEY, O. G., R. R. JOHNSON, S. VANECKO AND C. H. HUNT 1954b Studies on factors needed by rumen microorganisms for cellulose digestion *in vitro*. J. Animal Sci., *13:* 581.

BENTLEY, O. G., A. LEHMKUHL, R. R. JOHNSON, T. V. HERSHBERGER AND A. L. MOXON 1954c The "cellulolytic factor" activity of certain short chained fatty acids. J. Am. Chem. Soc., *76:* 5000.

BENTLEY, O. G., S. VANECKO, C. H. HUNT AND A. L. MOXON 1953 Nutritional requirements of rumen microorganisms for cellulose digestion *in vitro*. J. Animal Sci., *12:* 908.

BRYANT, M. P., AND R. N. DOETSCH 1955 Factors necessary for growth of *Bacteroides succinogenes* in the volatile acid fraction of rumen fluid. J. Dairy Sci., *38:* 340.

BURROUGHS, W., H. G. HEADLEY, R. M. BETHKE AND P. GERLAUGH 1950 Cellulose digestion in good and poor quality roughages using an artificial rumen. J. Animal Sci., *9:* 513.

CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition, *15:* 383.

DOETSCH, R. N., R. Q. ROBINSON AND J. C. SHAW 1952 Techniques employed in cultural investigations of the bacteriology of bovine rumen contents. J. Animal Sci., *11:* 536.

ELSDEN, S. R. 1946 The application of the silica gel partition chromatogram to the estimation of volatile fatty acids. Biochem. J., *40:* 252.

EL-SHAZLY, K. 1952 Degradation of proteins in the rumen of the sheep. 2. The action of rumen microorganisms on amino acids. Ibid., *51:* 647.

GARNER, G. B., M. E. MUHRER AND W. H. PFANDER 1954 Rumen microorganism growth regulation factor(s) found in extracts of rumen contents and in some ruminant feeds. J. Animal Sci., *13:* 983.

GRAY, F. V., A. F. PILGRIM, H. J. RODDA AND R. A. WELLER 1951a Volatile fatty acids in the rumen of the sheep. Nature, *167:* 954.

———— 1951b Fermentation in the rumen of the sheep. IV. Nature and origin of the volatile fatty acids in the rumen of the sheep. J. Exp. Biol., *29:* 57.

HALL, G., E. W. CHENG AND W. BURROUGHS 1953 B-vitamins and other factors stimulatory to cellulose digestion by washed suspensions of rumen microorganisms. J. Animal Sci., *12:* 918.

HALL, G., E. W. CHENG, W. H. HALE AND W. BURROUGHS 1954 Chemical and enzymatic preparations of protein hydrolysates stimulatory to cellulose digestion by rumen microorganisms. Ibid., *13:* 985.

HANSEN, R. P., AND A. G. MCINNES 1954 Volatile acids of ox perinephric fat. Nature, *173:* 1093.

HARPER, W. J. 1953 Direct chromatographic determination of acetic, propionic and butyric acids in cheese. J. Dairy Sci., *36:* 808.

HERSHBERGER, T. V., O. G. BENTLEY AND A. L. MOXON 1955 A rapid volumetric method for the determination of cellulose in studies with rumen microorganisms *in vitro*. J. Animal Sci., *14:* 725.

HUHTANEN, C. N., M. R. ROGERS AND L. S. GALL 1952 Improved techniques for isolating and purifying rumen organisms. J. Bact., *64:* 17.

HUNGATE, R. E. 1950 The anaerobic mesophilic cellulolytic bacteria. Bact. Revs., *14:* 1.

LOOSLI, J. K., H. H. WILLIAMS, W. E. THOMAS, F. H. FERRIS AND L. A. MAYNARD 1949 Synthesis of amino acids in the rumen. Science, *110:* 144.

MCNEILL, J. J., R. N. DOETSCH AND J. C. SHAW 1954 Some nutritional requirements of bovine rumen bacteria. J. Dairy Sci., *37:* 81.

MOYLE, V., E. BALDWIN AND R. SCARISBRICK 1948 Separation and estimation of standard C_2-C_8 fatty acids by buffered partition columns. Biochem. J., *43:* 308.

RUF, E. W., W. H. HALE AND W. BURROUGHS 1953 Observations upon an unidentified factor in feedstuffs stimulatory to cellulose digestion in the rumen and improved liveweight gains in lambs. J. Animal Sci., *12:* 731.

DIETARY LEVELS OF PANTOTHENIC ACID AND REPRODUCTIVE PERFORMANCE OF FEMALE SWINE [1]

D. E. ULLREY, D. E. BECKER, S. W. TERRILL AND R. A. NOTZOLD

Illinois Agricultural Experiment Station, University of Illinois, Urbana

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INTRODUCTION

Although widely distributed in the tissues of many species, pantothenic acid is frequently found at a low concentration in feedstuffs fed to swine. Under conditions of dry-lot feeding, natural diets for growing pigs must often be supplemented with this vitamin to achieve optimum performance. It is also a frequent practice to add pantothenic acid to the diets of pregnant and lactating swine, although the minimum requirement of this vitamin for normal reproduction has not been established.

Hodgkiss et al. ('50) using a purified diet attempted to produce pantothenate deficiency in pregnant gilts. However, the basal diet proved inadequate and pigs born in the control litters were weak at birth and died within 36 hours. Nelson and Evans ('46) reported that pantothenate deficiency instituted on the 13th day of gestation did not interfere with reproduction in rats. When the deficiency was instituted 16 to 23 days before mating or as late as the day of mating, failure of implantation, resorption, or defective litters always resulted. Inanition was eliminated as a contributing factor by use of the paired-feeding technique.

[1] From a thesis submitted by the senior author to the Graduate College of the University of Illinois in partial fulfillment of requirements for the Ph.D. degree, 1954.

Approved for publication by the director of the Agricultural Experiment Station.

Unpublished observations by our group suggested that there might be more than a casual relationship between pantothenate deficiency and reproductive pathology in female swine. The following investigation had as its objectives to establish an approximate requirement of pantothenic acid for reproduction, and to characterize and elucidate further the deficiency symptoms previously noted.

MATERIALS AND METHODS

Experimental design. Forty gilts of approximately equal age and weight were assigned to the test. They were divided into two replicates of 4 groups each, and the 5 gilts (litter mates or half-sisters) of each group were divided at random among the 5 pens constituting a replicate. One replicate contained Durocs; the second contained Hampshires and Hampshire-Yorkshire crossbreds.

For 28 days they were hand fed the basal diet plus a vitamin supplement, omitting calcium pantothenate (table 1). At the end of this depletion period, the number of gilts was reduced to 32 by removing one member of each group. The bases upon which gilts were discarded were: (1) positive or suspect reaction to the brucellosis test; (2) lameness associated with housing on concrete; and (3) substandard weight gains. The 16 Duroc gilts remaining in replicate 1 were allotted at random from related groups to lots 1 through 4. Similarly, the 7 Hampshires and 9 Hampshire $\times$ Yorkshire gilts in replicate 2 were allotted to lots 5 through 8. Treatments were initiated as follows: lots 1 and 5, no calcium pantothenate; lots 2 and 6, 4.4 mg of calcium pantothenate/kilogram of feed; lots 3 and 7, 11.0 mg of calcium pantothenate/kilogram of feed; lots 4 and 8, 17.6 mg of calcium pantothenate/kilogram of feed.

Feed intake was equalized between lots as far as possible during the prenatal period. Those gilts which farrowed were fed to the limit of their appetites during lactation.

During the first 8 weeks of the test (4 weeks depletion and 4 weeks treatment), the gilts were checked every second day with a boar for the occurrence of oestrous. At the end of the 8th week, breeding was initiated. Two different boars served each gilt during a single oestrous. The gilts were first bred on the initial day of heat and bred the second time 12 to 24 hours later. Individuals which continued to exhibit oestrous were bred in the same manner until oestrous periods ceased or until they were slaughtered.

TABLE 1

Basal diet [1] *and daily vitamin supplement* [2]

Ingredient		Ingredient	
	%		mg
Corn starch	61.4	Thiamine HCl	9.0
Drackett protein 220 [3]	18.4	Riboflavin	18.0
Cerelose	10.0	Nicotinic acid	120.0
Wood flock	3.0	Folic acid	3.0
Crude corn oil	2.0	Pyridoxine HCl	9.0
Vit. A and D oil (3000A, 600D/gm)	0.5	Vit. B_{12} in mannitol (1 mg B_{12}/gm)	90.0
Choline chloride	0.2	Biotin	0.18
DL-methionine	0.5	Para-amino benzoic acid	60.0
Mineral mixture [4]	4.0	Inositol	540.0
Alpha tocopherol acetate (11 mg/kg feed)		2-methyl-1, 4-naphthoquinone	5.4
	100.0	Ascorbic acid	100.0

[1] Microbiological assay of Skeggs and Wright ('44), with intestinal phosphatase and chick pancreatic enzyme digestion for total pantothenic acid, indicated a free pantothenic acid content of 1.1 μg/gm and a bound pantothenic acid content of 0.4 μg/gm.

[2] Provided in a premix of cerelose and fed at the rate of 5 gm of premix/gilt/day during gestation. Amount was doubled during lactation. The appropriate amount of Ca pantothenate was fed daily in proportion to the amount of basal diet fed. In addition, once a week each gilt received 55,000 I.U. of vitamin A, 11,000 I.U. of vitamin D, and 210 mg of alpha-tocopherol acetate (420 mg during lactation).

[3] An isolated soybean protein obtained from The Drackett Products Co., Cincinnati, Ohio. Crude protein content, 81.38%.

[4] $CaHPO_4$, 29.5 kg; NaCl (iodized), 7.3 kg; K_2CO_3, 6.4 kg; $MgCO_3$, 1557.2 gm; $FeSO_4 \cdot H_2O$, 454.0 gm; $MnSO_4 \cdot H_2O$, 136.2 gm; $CoCl_2 \cdot H_2O$, 45.4 gm; $CuSO_4$, 45.4 gm; NaF, 9.1 gm; $ZnCO_3$, 18.2 gm; KI, 4.5 gm.

Pregnant gilts were weighed on the 107th day of gestation, washed, and moved to individual pens, where they remained until their pigs were weaned at 5 weeks of age.

The gilts which had farrowed were rebred as soon after weaning as they would accept the boar. It was assumed that the gilts had conceived if oestrous did not recur, and they were slaughtered 24 to 28 days after breeding.

Among those gilts which had not farrowed, the individuals exhibiting estrus were bred and killed within 5 days. The individuals in which oestrous had ceased were slaughtered at approximately the same time.

Housing. The gilts were housed continuously on concrete with wood shavings as bedding. The pens were cleaned daily and washed with water at least once a week.

Collection of milk samples. A composite sample of milk from all teats of each gilt was obtained during the third or 4th week of lactation. This was done by hand milking the gilts after intramuscular administration of 1 to 2 ml of a posterior pituitary extract.[2] The milk samples were mixed, frozen, and stored at —20°C. for two months until assayed for free and bound pantothenic acid.[3]

Hematology. Blood was collected at the time of slaughter and was prevented from clotting by mixing with 0.8 mg of sodium heparin per milliliter of blood. Erythrocyte and leucocyte counts were made with a hemocytometer. The per cent hematocrit was determined by centrifuging blood-filled Wintrobe tubes at 3,000 r.p.m. for 30 minutes. Hemoglobin was determined by the acid hematin method of Cohen and Smith ('19).

Five milliliters of unheparinized blood were mixed with 5 volumes of cold (0°C.) 10% trichloroacetic acid solution for the purpose of determining blood pyruvate. The method of Friedmann and Haugen ('43) was used.

[2] Manufactured by Allied Laboratories Division of Pitman-Moore, Indianapolis, Indiana.

[3] These assays were performed by the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey.

Post-mortem examination. A careful inspection of the animal carcass was made both before and after opening the body cavities. The adrenals and ovaries were excised and weighed. A complete description of the ovaries was recorded, including number and diameter of corpora lutea and follicles. The reproductive tract was examined for the presence of ova or embryos. Within 30 minutes of death, the hypophysis was removed and refrigerated until it could be frozen (usually less than two hours).

Samples of tissues exhibiting gross abnormality were fixed either in a solution of FAA [4] or Bouin's fluid [5] for microscopic examination. It was standard procedure to fix samples of the following tissues whether gross lesions were visible or not: liver, spleen, ventricular myocardium, kidneys, adrenals, ovaries, uterus (both under and between embryos if the uterus was gravid), embryos, and adductor muscle. The tissues were imbedded in paraffin, sectioned at 8.5 μ, cleared in xylene, and stained with hematoxylin and eosin for microscopic examination.

Hormone assay. The gonadotrophic and thyrotrophic activity of hypophyses removed from gilts fed different levels of calcium pantothenate was compared using the two-day-old male chick assay described by Robinson and Nalbandov ('51). The pituitary powder was suspended in 0.5 ml of water at each injection. Both water-injected and non-injected controls were employed.

RESULTS AND DISCUSSION

Weight gains and feed consumption. The mean daily gain of the 40 gilts during the last 14 days of depletion was 0.45 ± 0.14 kg. Average daily feed consumption per gilt was 1.8 kg.

The accumulated average daily gains during the first 140 days of treatment are presented in table 2. This period was chosen since it included the period when deficiency symptoms

[4] Formalin, 15%; 95% ethanol, 80%; glacial acetic acid, 5%.

[5] Glacial acetic acid, 5%; formalin, 24%; saturated aqueous solution of picric acid, 71%.

were developing in those gilts receiving low levels of pantothenic acid. It may be observed that the gilts in replicate 1 receiving 1.5 mg of pantothenic acid per kilogram of diet were affected earlier and more severely than those in the corresponding lot in replicate 2.

The average daily feed consumption per gilt during the first 140 days of treatment is presented in table 2. Individuals in lot 1 first exhibited anorexia 82 days after initiating the

TABLE 2

Accumulated average daily gain and average feed consumption, during the first 140 days of treatment[1]

LOT NO.	PANTOTHENIC ACID	PERIOD OF TREATMENT IN 4-WEEK INTERVALS					AV. DAILY FEED/GILT
		1	2	3	4	5	
	mg/kg	*kg*	*kg*	*kg*	*kg*	*kg*	*kg*
				Replicate 1			
1	1.5	0.53(4)	0.26(4)	0.18(2)	0.11(1)	0.07(1)	1.49
2	5.9	0.40(4)	0.37(4)	0.43(4)	0.43(4)	0.49(4)	2.08
3	12.5	0.48(4)	0.42(3)	0.49(3)	0.48(3)	0.51(3)	2.07
4	19.1	0.51(4)	0.32(4)	0.48(4)	0.47(4)	0.48(4)	2.09
				Replicate 2			
5	1.5	0.44(4)	0.34(4)	0.31(4)	0.30(4)	0.25(3)	1.94
6	5.9	0.50(4)	0.41(4)	0.48(4)	0.48(4)	0.45(4)	2.08
7	12.5	0.45(4)	0.32(4)	0.38(3)	0.37(3)	0.42(3)	2.07
8	19.1	0.48(4)	0.40(4)	0.49(3)	0.49(3)	0.49(3)	2.06

[1] The values were obtained by dividing the total lot gain or feed by the number of gilt-days. The number of gilts surviving at the end of each interval is enclosed in parentheses.

experiment. Lot 5 gilts lost their appetite after 107 days, regained it 4 days later, and next went off feed on the 151st day. The appetites of the gilts in the other lots remained generally good throughout the test.

Gestation performance. Data pertaining to gestation performance may be found in table 3. No pigs were born to gilts receiving 1.5 mg of pantothenic acid per kilogram of feed although all but one individual exhibited oestrous and were bred. After the initial breeding, gilts in lot 1 did not again exhibit oestrous.

Gilts in lot 5 continued to cycle longer although they were bred at each oestrous. Autopsy data indicated conception had occurred in one individual which had been bred during each of 8 estrual cycles, but degeneration of the blastocysts had followed within a week of the last breeding. In a second

TABLE 3

Gestation and lactation performance

	REPLICATE 1				REPLICATE 2			
LOT NO.	1	2	3	4	5	6	7	8
PANTOTHENIC ACID MG/KG FEED	1.5	5.9	12.5	19.1	1.5	5.9	12.5	19.1
Services for conception	–	2.0	2.0	2.5	–	2.0	2.0	2.0
Litters farrowed	0	3	3	4	0	4	2	3
Pigs per litter	–	8.3	8.7	9.5	–	6.8	8.0	6.0
Birth weight, kg	–	1.4	1.0	1.4	–	1.2	1.3	1.0
Litters weaned	–	1	3	4	–	4	2	2
Pigs weaned/litter farrowed	–	1.7	6.0	9.2	–	3.5	8.0	4.5 [1]
Weaning weight of litters farrowed, kg	–	8.9	45.1	62.8	–	26.0	57.4	39.3 [1]
Free pantothenic acid in milk, μg/ml [2]	–	2.1	3.8	3.7	–	1.1	2.8	3.1
Total pantothenic acid in milk, μg/ml [2]	–	3.4	7.1	7.9	–	3.5	8.4	6.3

[1] One gilt of very nervous temperament killed her pigs shortly after birth and was not included in calculating this figure.

[2] Assayed according to the method of Skeggs and Wright ('44) with intestinal phosphatase and avian liver enzyme digestion for the determination of total pantothenic acid.

case, implantation had occurred and 8 out of 10 embryos appeared normal at 28 days. This gilt had previously exhibited oestrous three times; oestrous had ceased for three months and then reappeared.

Five gilts receiving 5.9 mg or more of pantothenic acid per kilogram of diet did not farrow. An individual in lot 2

aborted 4 partially-resorbed fetuses on the 127th day of gestation. Using humerus length to estimate age, it appeared that development of these fetuses had stopped before the 60th day of prenatal life.

One gilt in lot 3 died of an acute infection after 39 days on test.

A gilt in lot 7 which did not conceive, although bred at approximately 21-day intervals until slaughtered, was found to be ovulating normally. Chronic salpingitis and adhesions between the fimbriae and the ovaries precluded conception.

One gilt in lot 7 and another in lot 8 died after 78 days on test. At autopsy, necrotic gastric ulcers were found, from which extensive hemorrhage had occurred.

There was no significant difference between treatments in the number of pigs farrowed per litter. Birth weights were essentially the same.

Although strength of the pigs at birth was not closely related to treatment, definite signs of physiological abnormality were noted in the offspring of gilts in lot 2. Nearly all individuals exhibited locomotor incoordination. Some pigs could not stand at all and lay with their legs outstretched. Others moved with spraddled rear legs. In general, the skeletal muscles lacked tone, allowing the rear quarters to weave unsteadily from side to side as the pigs walked. Those pigs which showed the greatest muscular coordination walked with a typical "goose-step." Occasional individuals exhibited a persistent tremor of the head and body, resembling paralysis agitans. Diarrhea and frequent loss of blood from the anus was observed within a few hours after birth.

Symptoms of deficiency were not noted at birth in pigs farrowed in lot 6. One gilt had considerable difficulty in farrowing and gave birth to 9 dead pigs, but the two which were alive appeared completely healthy. This difference between lots 2 and 6 in their reaction to the same treatment was noted in varying degrees throughout the test.

Lactation performance. Data pertaining to lactation performance are given in table 3. It may be noted that pigs in

only one of the three litters farrowed in lot 2 survived to 5 weeks of age. Fifty-six per cent of the pigs born alive died before their 14th day of life. No such heavy loss occurred in the other lots.

The average weaning weights of the litters farrowed were chosen as the measurements which reflected most completely the adequacy of the pantothenic acid levels to support total gestation-lactation performance. These measurements included, indirectly, an evaluation of the number of live pigs farrowed, the survival to 5 weeks, and the milk production of the gilts. The data were analyzed by the method of least squares for treatment differences, replicate differences, and treatment-replicate interaction. A highly ($P < 0.01$) significant difference was found among treatments. The performance of pigs from gilts receiving 12.5 or 19.1 mg of pantothenic acid per kilogram of diet was significantly ($P < 0.01$) greater than those receiving 5.9 mg. Differences between the two higher treatment levels, differences between replicates, and treatment-replicate interaction were not significant.

Results of the assay of the gilts' milk for pantothenic acid are reported in table 3. Analysis of the free pantothenic acid data by the method of least squares established a significant ($P < 0.05$) difference between the 5.9 mg and higher levels. There was no significant difference between the two higher levels or between replicates, nor was there significant treatment-replicate interaction. Differences between treatments in the amount of total pantothenic acid were not significant.

Ante-mortem deficiency symptoms. Soft feces, diarrhea, and bleeding from the anus were noted in lots 1 and 5 about 9 weeks after initiation of the experiment. Two weeks later locomotor incoordination appeared in lot 1. It gradually progressed to "goose-stepping" and to eventual inability to rise. One gilt in lot 2 developed a similar condition after two weeks of lactation, and shortly after weaning her pigs was unable to rise. The other gilts in lot 2 developed a neuromuscular sensitivity which was expressed as spastic movements of the rear legs upward toward the thigh.

Lot 5 gilts did not exhibit evidence of impaired locomotion until nearly 4 months after initiation of the experiment. None of the other lots appeared to be affected.

The condition at birth of pigs born to gilts in lot 2 has already been described. The desire to nurse was somewhat reduced. Severity of muscular weakness, incoordination, and diarrhea increased until most of the pigs died. Dermatitis, alopecia, and the dark exudate about the eyes noted by Wiese et al. ('51) in their study with the baby pig were not noted here.

Hematology. Of the blood values studied, only the concentration of pyruvate appeared to have any relation to treatment. The limited data gathered in this study indicated that 4 mg % was the upper limit of the normal range of pyruvate concentration in the blood of these gilts. Five individuals exceeded this value, two in each of lots 1 and 5 and one in lot 2. The highest value recorded was 9.72 mg %. From current knowledge of the biochemical role of pantothenic acid and thiamine in pyruvate oxidation, it might be expected that deficiencies of these two vitamins would be similar in their ability to inhibit pyruvate metabolism. However, an increased concentration of blood pyruvate was not a sensitive indicator of pantothenic acid depletion, occurring late in the deficiency and associated perhaps more closely with Selye's general adaptation syndrome.

Post-mortem observations. Gross pathology frequently noted in gilts from lots 1, 2 and 5 included fatty livers, enlarged adrenal glands, intramuscular hemorrhage, eccentric dilatation of the heart, rectal congestion, atrophic ovaries, and infantile uteri. The condition of the reproductive organs resembled the genital atrophy observed in pantothenic acid-deficient rats by Figge and Allen ('42).

Gross pathology noted in pigs born to gilts in lot 2 included fatty liver and kidneys, flaccid cardiac muscle, intestinal congestion, ulceration and hemorrhage of the colon and rectal mucosa, excessive accumulation of urates in the renal pelvis, ureters, and bladder, and edematous and ischemic skeletal

muscle. In one instance, small hemorrhages were noted in the surface of the adrenal glands.

Observations of the histo-pathology include the following: centro-medial-lobular fatty degeneration of the liver both in gilts and in pigs, accumulation of urates in the renal collecting tubules of the pigs, edematous cardiac muscle with cellular degeneration and indistinct striations, and pyknotic cells in the glomerulosa layer of some adrenals. The areas of intramuscular hemorrhage, found primarily in the adductor muscle group but once in the shoulder muscles of the deficient gilts, were accompanied by extensive cellular degeneration. The adductor and gracilis muscles of pigs born in lot 2 were edematous, the striations were indistinct, and extensive vacuolation of the cytoplasm was evident.

The ovaries of gilts in lots 1 and 5 were characterized by a high percentage of atretic follicles and few corpora lutea. Small ovary weight appeared to be related to loss of functional activity. No corpus luteum was present when the combined weight of the ovaries was less than 7 gm. The proportion of interstitial tissue had increased to the detriment of the area occupied by follicles. In the most severely affected ovaries, from lot 1 gilts, dissolution of the follicular granulosa cells had occurred. Extensive degeneration of the theca folliculi was also evident. The small, turgid uteri removed from these same gilts were found to be unusually vascular. The endometrial glands were small in number and the uterine epithelium was low and compact.

Hormone content of the pituitaries. Because the reproductive systems of the deficient gilts exhibited such drastic changes, it was suspected that there might be abnormal production of gonadotrophins or thyrotrophins by the anterior pituitaries. An increased production of the gonadotrophic hormones might be expected, if the inhibitory influence of the ovarian steroids was greatly reduced or no longer present.

Results of the assay are presented in table 4. No significant difference among treatments, as measured by chick testes or thyroid weight, was found. Unfortunately, pituitaries from

gilts in lot 1 were not available for assay. A trend of high gonadotrophin on low levels of pantothenic acid to lower gonadotrophin on higher levels of pantothenic acid was evident in replicate 2. More extensive investigation of this relationship is required.

TABLE 4

Hormone content of gilt hypophyses

GILTS							ASSAY ANIMALS (CHICKS)	
LOT NO.	NO. OF GILTS	OVARY WEIGHT	FOLLICLE INDEX [1]	CORPORA LUTEA	ANTERIOR PITUITARY WT.		TESTES WEIGHT	THYROID WEIGHT
					WET	DRY		
		gm			*mg*	*mg*	*mg*	*mg*
				Replicate 1				
1	3	4.6	10	5.3	–	–	–	–
2	4	10.9	104	11.0	265	70.8	39.6	8.5
3	3	11.3	161	15.0	303	79.5	34.6	5.8
4	4	9.6	132	11.8	304	76.7	39.6	7.8
				Replicate 2				
5	4	14.3	145	6.0	213	58.3	41.9	7.6
6	4	13.8	152	10.8	326	86.4	36.8	7.2
7	3	11.6	97	12.7	315	85.1	33.5	7.3
8	2	12.6	128	14.0	269	74.4	28.1	8.2
Controls, water injected							15.9	6.1
Controls, non-injected							16.0	5.0

[1] The sum of the grossly visible follicles in both ovaries multiplied by their respective diameters.

SUMMARY

The effect of 4 dietary levels of pantothenic acid on the reproductive performance of female swine was investigated. After 4 weeks of pantothenate depletion, 32 sexually-mature gilts were divided among two replicates with 4 treatments per replicate. The total pantothenic acid intake in each treatment was 1.5, 5.9, 12.5, or 19.1 mg of pantothenic acid per kilogram of diet.

A dietary intake of 1.5 or 5.9 mg was not sufficient to prevent development of pantothenate-deficiency symptoms. The deficiency affected the Durocs in replicate 1 more severely than

the Hampshires and Hampshire-Yorkshire crossbreds in replicate 2.

Gilts fed the 5.9 mg level conceived, and gestation was supported to term. However, abnormal pigs were farrowed.

Total gestation-lactation performance, as measured by the average litter weaning weight of litters farrowed, was equal in lots receiving 12.5 and 19.1 mg of pantothenic acid per kilogram of diet and was superior to that of lots receiving 5.9 mg or less.

The free pantothenic acid content of milk collected during the third and 4th week of lactation was significantly less when the gilts received only 5.9 mg of pantothenic acid as compared with higher levels of intake. Differences in the total pantothenic acid content of the milk were not significant.

Under the conditions of this investigation, it appears that 12.5 mg of pantothenic acid per kilogram of diet are adequate to support normal reproduction of female swine.

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LITERATURE CITED

COHEN, B., AND A. H. SMITH 1919 The colorimetric determination of hemoglobin. J. Biol. Chem., *39:* 489.

FIGGE, F. H. J., AND E. ALLEN 1942 Genital atrophy during pantothenic acid deficiency and responses to gonadotropic and estrogenic hormone. Endocrinology, *30:* S1028.

FRIEDEMANN, T. E., AND G. E. HAUGEN 1943 Pyruvic acid, II. The determination of keto acids in blood and urine. J. Biol. Chem., *147:* 415.

HODGSKISS, H. W., M. E. ENSMINGER, R. W. COLBY AND T. J. CUNHA 1950 Inadequacy of purified diets for reproduction by swine with observations on an added deficiency of pantothenic acid. J. Animal Sci., *9:* 619.

NELSON, M. M., AND H. M. EVANS 1946 Pantothenic acid deficiency and reproduction in the rat. J. Nutrition, *31:* 497.

ROBINSON, G. E., JR., AND A. V. NALBANDOV 1951 Changes in the hormone content of swine pituitaries during the estrual cycle. J. Animal Sci., *10:* 469.

SKEGGS, H. R., AND L. D. WRIGHT 1944 The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. J. Biol. Chem., *156:* 21.

WIESE, A. C., W. P. LEHRER, JR., P. R. MOORE, O. F. PAHNISH AND W. V. HARTWELL 1951 Pantothenic acid deficiency in baby pigs. J. Animal Sci., *10:* 80.

FURTHER OBSERVATIONS ON THE IMPROVEMENT OF POLISHED RICE WITH PROTEIN AND AMINO ACID SUPPLEMENTS[1]

P. D. DESHPANDE, A. E. HARPER, FELIPE QUIROS-PEREZ AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture
University of Wisconsin, Madison

ONE FIGURE

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INTRODUCTION

Pecora and Hundley ('51) have reported that the rate of gain of rats fed a rice diet, supplemented with lysine and threonine, was substantially greater than that of a control group receiving rice alone. In that, and in a similar study (Harper et al., '55), it was pointed out that the limiting amino acids in rice diets, as judged from the chemical composition of rice proteins, are not necessarily limiting from the physiological point of view, and that very complex imbalances may be produced when certain of the amino acids calculated to be limiting are included in the diet. The latter authors, who studied the deposition of liver fat in rats fed rice diets containing choline, concluded that the relatively small supplements of lysine and threonine which brought about an increase in the growth rate failed to prevent the accumulation of fat in the liver. On the other hand, if the

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level of lysine in the diet was increased to twice the original amount (from 0.2 to 0.4%), there was a significant decrease in the deposition of liver fat; however, lysine at this higher level retarded growth. Of a number of mixtures tested, only a combination of all of the essential amino acids was found to prevent the growth retardation.

The following report represents an extension of the above work and includes further information concerning the imbalance of amino acids in rice diets.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain, weighing from 40 to 50 gm, were used. The animals were divided, with respect to weight, into similar groups of 6 and were housed in individual cages with raised screen bottoms. They were fed ad libitum and weighed at weekly intervals during the experimental periods which varied from two to 7 weeks.

At the end of the experimental period, the rats were stunned and decapitated. The livers were removed and stored at —4°C. Fat was determined by ether extraction of the dried and ground liver (Hawk and Elvehjem, '53).

The rations used for the experiments had the following basic composition: Corn oil 5, salts 4 (Hegsted et al., '41) 4, choline 0.15 and vitamin mixture 0.25%; rice and the supplements of different amino acids and purified proteins as indicated in the tables of results and a small quantity of sucrose were added to make up 100%. The vitamin mixture supplied in milligrams per 100 gm of the ration: thiamine HCl 0.5, riboflavin 0.5, niacin 2.5, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, folic acid 0.02, vitamin B_{12} 0.002, and inositol 10.0. Two drops of halibut liver oil fortified with vitamins E and K were given orally each week (Harper et al., '54b).

RESULTS

Since the inclusion of histidine, tryptophan and methionine, which appeared to be the most limiting amino acids in the rice diet after lysine and threonine, had proven ineffective in

preventing the growth retardation caused by lysine (Harper et al., '55), the amino acid calculated to be next limiting was included. The data summarized by Flodin ('53), Pecora and Hundley ('51), Block and Bolling ('51) and Rose ('37), suggested that phenylalanine should be the next most limiting. The results presented in table 1 (Exp. 1) show that the addition of phenylalanine along with histidine, tryptophan and methionine to a diet containing the higher levels of lysine and threonine (0.4 and 0.5% respectively) did not in any way improve growth over that observed with only lysine and threonine. When the remaining essential amino acids arginine, leucine, isoleucine, and valine were added *individually* together with the amino acids provided for group 5, the liver fat values remained low, but there was still no improvement in growth; in fact, a slight depression in growth, particularly noticeable in group 8 which received 0.3% of L-leucine, was observed. Only group 10, which received all of the essential amino acids concomitantly showed a satisfactory growth response.

The problem was then approached in a different way by using diets that contained supplements of all of the essential amino acids except one, and the results presented in table 1 (Exp. 2) show that isoleucine, leucine, histidine and, to a lesser extent, valine were limiting in these rice diets. Methionine and tryptophan held an intermediate position whereas arginine and phenylalanine seemed to be relatively unimportant as supplements to rice diets.

Whether these amino acids exerted their effects individually or in combination was further studied using diets containing the higher and the lower levels of lysine and threonine. The data presented in table 1 (Exp. 3) show that isoleucine, leucine, histidine and valine together made a very good supplement to the rice diet containing lysine and threonine and that the supplementary value of these 4 amino acids was more marked when lysine and threonine were present at the higher levels. The individual members as well as the incomplete combinations did not give any such response. Supple-

TABLE 1

Growth and liver fat deposition in rats fed rice diet supplemented with amino acids

EXP. NO.	GROUP NO.[1]	COMPOSITION OF THE DIET				LIVER FAT	GAIN IN WT.
		Rice	L-lysine HCl	DL-threonine	Other amino acids [2]		
		%	%	%	%	% *dry wt.*	*gm/wk.*
1	1	87	..	...	.....	33.9 ± 1.4	8.5 ± 1.4
	2	87	0.2	0.24	.....	25.0 ± 1.3	22.0 ± 1.2
	3	87	0.2	0.5	.....	24.2 ± 1.8	22.0 ± 0.6
	4	87	0.4	0.5	.....	10.6 ± 0.7	16.0 ± 1.3
	5	87	0.4	0.5	Histidine + tryptophan + methionine + phenylalanine	9.5 ± 0.5	16.5 ± 1.6
	6	87	0.4	0.5	Histidine + tryptophan + methionine + phenylalanine + arginine	10.3 ± 0.4	15.0 ± 0.9
	7	87	0.4	0.5	Histidine + tryptophan + methionine + phenylalanine + valine	9.7 ± 0.5	14.0 ± 1.0
	8	87	0.4	0.5	Histidine + tryptophan + methionine + phenylalanine + leucine	8.1 ± 0.6	6.5 ± 0.1
	9	87	0.4	0.5	Histidine + tryptophan + methionine + phenylalanine + isoleucine	9.6 ± 1.0	12.2 ± 1.2
	10	87	0.4	0.5	Essential amino acids ≅ 3% casein	13.6 ± 1.3	26.6 ± 1.9
2	11	87	0.4	0.5	Essential amino acids ≅ 3% casein	14.9 ± 1.0	26.0 ± 1.8
	12	87	0.4	0.5	Essential amino acids minus methionine	11.5 ± 0.4	23.0 ± 1.0
	13	87	0.4	0.5	Essential amino acids minus tryptophan	12.1 ± 0.5	20.0 ± 2.7
	14	87	0.4	0.5	Essential amino acids minus histidine	10.5 ± 0.3	12.5 ± 0.7
	15	87	0.4	0.5	Essential amino acids minus arginine	14.2 ± 0.6	26.0 ± 1.4
	16	87	0.4	0.5	Essential amino acids minus leucine	10.4 ± 0.6	11.0 ± 0.9

	17	87	0.4	0.5	Essential amino acids minus isoleucine	10.8 ± 0.6	7.7 ± 2.0
	18	87	0.4	0.5	Essential amino acids minus valine	13.9 ± 1.5	16.7 ± 2.1
	19	87	0.4	0.5	Essential amino acids minus phenylalanine	11.8 ± 1.2	25.0 ± 2.0
	20	87	0.4	0.5	.....	12.7 ± 1.1	14.0 ± 1.4
3	21	87	..	..	.....	27.6 ± 1.8	6.6 ± 0.6
	22	87	0.2	0.24	.....	28.0 ± 2.1	19.1 ± 1.6
	23	87	0.2	0.24	Leucine + isoleucine + histidine + valine	24.5 ± 1.0	22.6 ± 1.1
	24	87	0.4	0.5	.....	12.6 ± 0.5	17.5 ± 1.8
	25	87	0.4	0.5	Isoleucine	12.0 ± 0.8	20.0 ± 1.0
	26	87	0.4	0.5	Isoleucine + histidine	11.4 ± 0.8	16.0 ± 1.2
	27	87	0.4	0.5	Isoleucine + histidine + leucine	15.3 ± 1.5	24.8 ± 1.3
	28	87	0.4	0.5	Isoleucine + histidine + leucine + valine	13.8 ± 0.7	27.2 ± 1.6
	29	87	0.4	0.5	Isoleucine + histidine + leucine + valine + methionine + tryptophan	16.6 ± 0.9	27.0 ± 1.3
4	30	84.6	0.2	0.24	.....	19.1 ± 1.0	25.0 ± 3.6
	31	84.6	0.4	0.5	.....	19.0 ± 0.9	12.7 ± 0.3
	32	84.6	0.4	0.5	0.3 L-leucine + 0.44 DL-isoleucine + 0.14 L-histidine + 0.46 DL-valine	25.6 ± 1.2	12.9
	33	84.6	0.4	0.5	0.6 L-leucine + 0.88 DL-isoleucine + 0.28 L-histidine + 0.92 DL-valine	23.6 ± 1.2	12.2
	34	84.6	0.4	0.5	0.6 L-leucine + 0.88 DL-isoleucine + 0.28 L-histidine + 0.92 DL-valine + 0.1 DL-methionine + 0.05 DL-tryptophan + 0.2 DL-phenylalanine	29.8 ± 0.3	12.8 ± 0.7
	35	84.6	0.6	0.75	0.6 L-leucine + 0.88 DL-isoleucine + 0.28 L-histidine + 0.92 DL-valine	21.3 ± 0.8	12.2

[1] Six rats in each group.

[2] The levels of these amino acids in experiments 1, 2 and 3 were: L-arginine HCl, 0.16%; L-histidine HCl, 0.14%; DL-tryptophan, 0.05%; DL-phenylalanine, 0.2%; DL-methionine, 0.1%; L-leucine, 0.3%; DL-isoleucine, 0.44%; DL-valine, 0.46%.

mentation with methionine and tryptophan did not further improve growth.

Although the rate of gain of rats fed the rice diet supplemented with all of the essential amino acids in amounts equivalent to those provided by three per cent of casein was definitely improved over that of the control group, the maximum growth obtained in any of the experiments was about 27 gm per week. Harper et al. ('55) have reported a similar growth response in rats fed a rice diet supplemented with various proteins at a level of three per cent. As these values were lower than those obtained with a good purified diet (about 33 to 35 gm per week for a two-week period), it was of interest to see whether the rice diet could be improved sufficiently to support a similar rate of growth by increasing the levels of certain animal protein supplements.

Figure 1 shows the relative rates of gain of rats fed the rice diet supplemented with fibrin, casein, pork, fish meal, kidney meal or gelatin at levels of three and 6% over a period of two weeks. The results show that 6% of any of the proteins used gave an increase in the rate of growth over that obtained with the corresponding three per cent level. When the level of the supplement was further increased up to 12% (studied only with fibrin which is nutritionally an excellent protein) little further improvement in growth was obtained. The rates of growth obtained with the supplements of 6% of pork, fibrin, casein and kidney meal were equal to that obtained with a good purified diet; fish meal proved somewhat less adequate. The content of liver fat of each of the groups that received 6% or more of any of the protein supplements approached a normal value.

Gelatin, which from a nutritional point of view is an incomplete protein, gave a substantial improvement in growth. However, several attempts to further increase the rate of growth of rats receiving rice and gelatin by adding small amounts of what were considered to be the limiting amino acids were without success. A supplement containing several

essential amino acids was required to give a rate of growth as good as that obtained with the more complete proteins.

In view of these observations, it was important to determine whether by further increasing the quantities of leucine, iso-

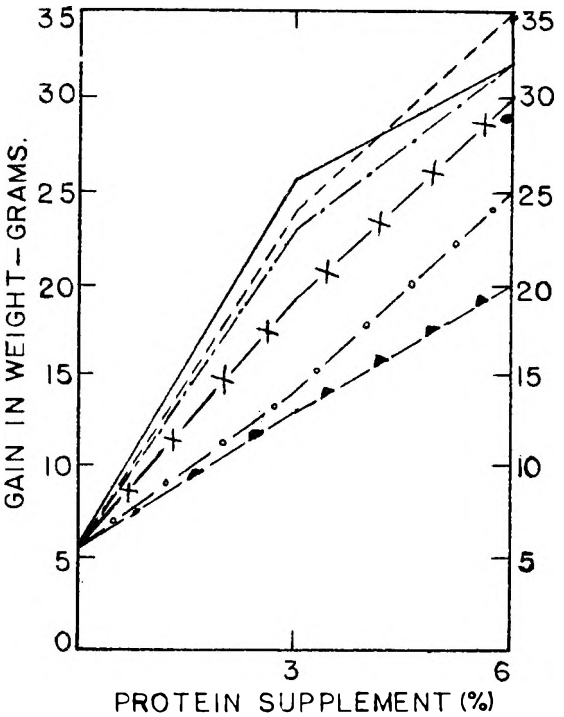

Fig. 1 Rate of growth of rats fed rice diet supplemented with various proteins at two different levels.

leucine, histidine and valine a larger growth response could be obtained. The results given in table 1 (Exp. 4) show that growth was depressed to a small extent when the levels of these amino acids were increased and that this was not remedied by concomitant increases in the levels of lysine and

threonine. A better rate of growth was obtained, however, when methionine, tryptophan and phenylalanine were also added to the diet.

The effect of age on the growth response to certain of these amino acid supplements was studied and the results are given in table 2. The figures for the average gain show the superiority of the more complete mixture. It is also interesting to note that the growth depression observed during the

TABLE 2

Effect of age on the rate of growth of rats[1] *fed the rice diet supplemented with amino acids*

WEEK	GROUP I [2]	GROUP II	GROUP III	GROUP IV
	Gain in wt.	Gain in wt.	Gain in wt.	Gain in wt.
	gm	*gm*	*gm*	*gm*
1	5.8	17.6	10.7	18.1
2	7.8	22.7	21.2	25.5
3	6.7	24.0	25.5	29.9
4	6.5	23.0	26.3	28.5
5	5.8	21.9	23.5	36.5
6	8.0	21.9	23.0	23.0
7	4.4	19.4	22.7	16.6
Total wt. gain	45.0	150.5	152.9	178.1

Gr. I 87% rice diet with no supplement.
Gr. II 87% rice + 0.2% L-lysine + 0.24% DL-threonine.
Gr. III 87% rice + 0.4% L-lysine + 0.5% DL-threonine.
Gr. IV 87% rice + 0.4% L-lysine + 0.5% DL-threonine + 0.3% L-leucine + 0.44% DL-isoleucine + 0.14% L-histidine HCl + 0.46% DL-valine.

[1] The average initial weight was 45.5 gm.
[2] Six rats in each group.

first two weeks with the higher levels of lysine and threonine was not evident after three weeks and that thereafter this group grew slightly more rapidly than the group receiving the lower levels of these amino acids. It may be noted that as the diet was improved maximum growth occurred later; in group 1 this was during the second week; group 2, third week; group 3, 4th week and group 4, 5th week. As with the two-week experiments, the fat content of the liver of rats

receiving either the unsupplemented rice diet or that supplemented with the lower levels of lysine and threonine was high while that of the groups receiving the diets containing the higher levels of lysine and threonine was low.

As the growth of rats fed diets containing leucine but no isoleucine was retarded and as a leucine-isoleucine antagonism had been reported by Harper et al., ('54a), the ability of isoleucine to counteract this growth retardation was investigated using different levels of these two amino acids. From

TABLE 3

Growth depressing effect of leucine in rats fed rice diet supplemented with lysine and threonine [1]

GROUP NO.[2]	L-LEUCINE	DL-ISOLEUCINE	GAIN IN WT.
	%	%	*gm/wk.*
1	...	...	19.2 ± 1.4
2	0.3	...	13.6 ± 1.4
3	0.3	0.44	23.0 ± 1.3
4	0.6	...	6.1 ± 0.9
5	0.6	0.22	16.3 ± 1.4
6	0.6	0.44	19.6 ± 1.3
7	1.0	...	7.7 ± 1.1
8	1.0	0.4	16.2 ± 1.0
9	1.0	0.8	16.0 ± 0.5

[1] The diet contained 87% rice + 0.2% L-lysine + 0.24% DL-threonine.

[2] Six rats in each group.

the results summarized in table 3 it may be seen that when leucine was fed at levels of 0.3, 0.6 and 1.0% in the rice diet, growth was retarded in all instances. Except at the highest level of leucine fed, the retardation was completely overcome by isoleucine. Isoleucine, when fed at higher levels (2% in the diet), also had a similar though less pronounced growth-retarding effect.

DISCUSSION

The results of the liver fat determinations are in agreement with the earlier observations (Harper et al., '55) that the fat content of the liver of rats fed rice diets supplemented with

the higher levels of L-lysine-HCl and DL-threonine (0.4 and 0.5% respectively) are in the normal range, and that this is primarily an effect of the additional lysine. The observation that the deposition of liver fat followed the same trend in the longer experiments as in the shorter ones, i.e. fat accumulated in the livers of rats given either no supplement or the lower levels of lysine and threonine while the livers of those receiving the higher levels were in the normal range, indicates that, in contrast to the situation with rats fed 9% casein diets (Harper et al., '54b), the fatty infiltration is of considerable duration when the lysine content of the rice diet is low. Shils and Stewart ('54) have also reported that amino acid deficiencies such as tryptophan and lysine, in rice diets, cause fatty infiltration of the liver, and Arulanantham ('53, '54), using a longer experimental period, has demonstrated that deficiencies of either amino acids or fatty acids may cause an accumulation of liver fat in rats fed rice diets. These observations may be of significance with regard to the fatty liver associated with kwashiorkor.

Since the retardation in growth caused by the higher level of lysine, which was previously overcome in short experiments only by adding all of the essential amino acids to the diet, was prevented by the addition of a mixture of leucine, isoleucine, histidine and valine, it is evident that additional amounts of these 4 amino acids are sufficient to correct the imbalance caused by the higher level of lysine. The observation that the decreased rate of growth associated with the higher levels of lysine and threonine was not evident after two weeks indicates that this imbalance is of significance only during the early growth period.

The retardation in growth caused by an excess of leucine and the influence of isoleucine alone in counteracting this effect appears similar to the observations made using a 9% casein diet (Harper et al., '54a). Whether this represents a direct antagonism between leucine and isoleucine, or an imbalance created by an excess of leucine is difficult to deter-

mine because of the limiting nature of leucine and isoleucine in the rice diet.

The growth results confirm many of the observations reported previously but necessitate some modification of the earlier conclusions. Thus while Pecora and Hundley ('51) reported that all of the other essential amino acids along with lysine and threonine must be added to the rice diet to obtain a further growth response, the present investigation shows that leucine, isoleucine, histidine and valine bring about the same effect as was obtained with all of the essential amino acids. Although a supplement of 6% of a well-balanced protein, making a total of approximately 11% of protein in the rice diet, was sufficient to support growth equivalent to that obtained with a good purified diet, supplementation of the rice diet containing lysine and threonine with quantities of leucine, isoleucine, histidine and valine equivalent to those provided by 6% of casein (table 1, Exp. 4) caused an imbalance which retarded growth. Since increasing the amounts of lysine and threonine still further did not improve this condition, whereas addition of methionine, tryptophan and phenylalanine did improve growth, it is apparent that very complex situations may be encountered in studies of amino acid supplementation of cereal proteins. Similar observations have been reported by other workers. Henderson et al. ('53), using zein diets, observed imbalance in which valine and lysine were involved and with acid hydrolysed casein an imbalance involving threonine was found. Salmon ('54), who investigated the tryptophan requirement of rats under various conditions, concluded that the imbalance effect is a general phenomenon that may alter the requirement of any essential amino acid. It thus seems possible that the higher levels of amino acids used as supplements by Pecora and Hundley could have caused imbalances in certain of their rice diets which might account for their failure to obtain an improvement in growth with incomplete mixtures of essential amino acids.

With regard to the nutritive value of cereal proteins it is significant that the analytical data show the order of

deficiency of the amino acids in rice to be lysine, histidine, methionine, tryptophan, threonine, phenylalanine, valine, leucine, isoleucine and arginine; whereas the results of growth studies indicate the following order: Lysine, threonine, leucine, isoleucine, histidine, valine, tryptophan, methionine, phenylalanine and arginine. This emphasizes the need for increased knowledge of the availability of amino acids from intact proteins as a prerequisite to any attempt to determine from chemical data the limiting amino acids in diets composed largely of cereals.

It is interesting to note that when rats were fed diets containing 11% of protein derived entirely from pork, growth was less than when the same level of protein was provided as a combination of rice and pork. This stresses the importance in practical nutrition of using mixtures of proteins. The kidney meal and the fish meal used in this study are of particular interest as supplements to plant proteins in view of their availability at low cost and the possibility of their production on a commercial scale in places where cereals are the main ingredients of low protein diets.[2] The problem, however, may not be as simple as it appears because most vegetarian diets lack certain minerals and vitamins as well as protein, and a study of the effect of these dietary essentials is dseirable before any long range plan for the improvement of such diets is undertaken.

SUMMARY

The effects of various supplements of protein and amino acids on growth and on the deposition of liver fat in rats receiving a rice diet have been investigated and the imbalance created when the rice diet was supplemented with sufficient lysine to prevent the accumulation of liver fat has been studied in greater detail.

A rice diet supplemented with 6% of various animal proteins supported an excellent rate of growth and main-

[2] Mr. Ezra Levin, Viobin Corporation. Personal communication.

tained liver fat at normal levels. A supplement of several essential amino acids was required to give similar results.

In short experiments (2 weeks) the retardation in growth caused by including 0.4% of L-lysine·HCl in the rice diet was prevented by increasing the levels of leucine, isoleucine, valine and histidine. In longer experiments the growth-retarding effect of additional lysine disappeared without the addition of other amino acids to the diet.

Supplementation of the rice diet with various combinations of amino acids from which one or more of leucine, isoleucine, valine and histidine had been omitted resulted in a retardation of growth but in all instances in which the diet contained at least 0.4% of additional L-lysine · HCl the fat content of the liver approached a normal value. Even after 7 weeks an accumulation of liver fat occurred unless the rice diet was supplemented with this level of lysine.

The sequence in which the amino acids of polished rice became limiting for growth was found to differ markedly from that calculated from amino acid analyses. This stresses the need for investigation of the availability to the animal of the amino acids of intact plant proteins.

It is suggested that the most practical way of improving the nutritive value of rice diets is by supplementation with foods containing nutritionally well-balanced proteins.

LITERATURE CITED

ARULANANTHAM, R. 1953 Supplementary effect of protein and amino acids with the poor rice diet of South India., Ph. D. Thesis, Northwestern University, Chicago, Illinois.

———— 1954 Supplementary effect of protein and amino acids with 'poor' rice diet of South India. Fed. Proc., *13:* 1.

BLOCK, R. J., AND B. BOLLING 1951 The amino acid composition of proteins and foods. Charles C. Thomas, Publisher, Springfield, Ill. 2nd Ed.

FLODIN, N. W. 1953 Amino acids and proteins. Their place in human nutrition problems. J. Agr. Food Chem., *1:* 222.

HARPER, A. E., D. A. BENTON, M. E. WINJE AND C. A. ELVEHJEM 1954a Leucine-isoleucine antagonism in the rat. Arch. Biochem. Biophys., *51:* 523.

HARPER, A. E., D. A. BENTON, M. E. WINJE, W. J. MONSON AND C. A. ELVEHJEM 1954b Effect of threonine on fat deposition in the livers of mature rats. J. Biol. Chem., *209:* 165.

HARPER, A. E., M. E. WINJE, D. A. BENTON AND C. A. ELVEHJEM 1955 Effect of amino acid supplements on growth and fat deposition in the livers of rats fed polished rice. J. Nutrition, *56:* 187.

HAWK, E. A., AND C. A. ELVEHJEM 1953 The effects of vitamin B_{12} and B_{12r} on growth, kidney hemorrhage and liver fat in rats fed purified diets. Ibid., *49:* 495.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HENDERSON, L. M., O. J. KOEPPE AND H. H. ZIMMERMAN 1953 Niacin-tryptophan deficiency resulting from amino acid imbalance in non-casein diets. Ibid., *201:* 697.

PECORA, L. J., AND J. M. HUNDLEY 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. J. Nutrition, *44:* 101.

ROSE, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298–300.

SALMON, W. D. 1954 The tryptophan requirement of the rat as affected by niacin and level of dietary nitrogen. Arch. Biochem. Biophys., *51:* 30.

SHILS, M. E., AND W. B. STEWART 1954 Preventive influence of certain amino acids on experimental fatty liver of portal type. Proc. Soc. Exp. Biol. Med., *87:* 629.

EFFECT OF HIGH DIETARY MANGANESE ON HEMOGLOBIN FORMATION[1, 2]

R. H. HARTMAN, GENNARD MATRONE AND G. H. WISE
Animal Nutrition Section of Department of Animal Industry, North Carolina Agricultural Experiment Station, Raleigh

FOUR FIGURES

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Effects of different dietary levels of manganese are of interest because of the evidence of antagonisms existing between this mineral and other elements and because of the reports of extreme and variable concentrations of manganese in forages (Beeson and Matrone, '50; Beeson, '54; Blakemore et al., '37; Russell, '44; Stuart et al., '50). Previous work (Hawkins et al., '55) indicated that feeding high levels of manganese to the calf affected the concentration of serum magnesium and interfered with calcium and phosphorus metabolism. Subsequent studies, in which lambs were used, revealed an apparent interference of manganese with iron utilization. Data from these studies form the basis for the present report.

EXPERIMENTAL

The effects of manganese on hemoglobin formation were investigated in two experiments. The first was conducted with lambs restricted to a fortified milk diet and the second, with lambs fed a roughage diet.

[1] Published with the approval of the Director of Research, N. C. Agricultural Experiment Station, as paper No. 650 of the Journal Series. The data were taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the Master of Science degree.

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

Experiment 1

Procedure. Eight ewe and 8 wether lambs, ranging in age from 7 to 19 days, were the test subjects. The lambs were assigned to individual wooden pens having elevated floors of aluminum-painted wire screen. During a preliminary period of one month, these lambs were fed fresh cow's milk, from nippled bottles, at a rate of one pound of milk per 5 pounds of body weight daily. Additional vitamins, A and D, were administered in capsules (5000 I.U. of vitamin A and 500 I.U. of vitamin D per capsule [3]) on alternate days.

TABLE 1

Levels of manganese added to the basal diet at different periods in the experiment

PERIODS		MANGANESE SUPPLEMENTATION [1]			
No.	Weeks	Mn_0	Mn_1	Mn_2	Mn_3
		ppm	*ppm*	*ppm*	*ppm*
1	0 – 12	0	5	15	45
2	12 – 20	0	5	30	90
3	20 – 24	0	–	450	900
4	24 – 35	0	–	2500	5000

[1] Supplements of manganese ($MnSO_4 \cdot H_2O$) were added to the whole milk diets on the basis of the total solids content.

The principal component of the experimental diets was whole milk, which was fortified with 50 ppm of iron,[4] 5 ppm of copper,[4] and 0.1 ppm of cobalt [4] on a dry matter basis. As shown in table 1, the dietary treatments consisted of 4 levels of manganese, designated as Mn_0, Mn_1, Mn_2, and Mn_3, during periods 1 and 2, and three levels, Mn_0, Mn_2, and Mn_3, during periods 3 and 4. The supplements of manganese were increased gradually in successive periods. Two ewe and two wether lambs were randomly assigned to each experimental diet. Because of the death of three test lambs (two on Mn_0 and one on Mn_1) during periods 1 and 2, the Mn_1 treatment

[3] Code No. 200 capsules supplied through the courtesy of the Gelatin Products Division of R. P. Scherer Corporation, Detroit, Mich.

[4] $FeSO_4.H_2O$, $CuSO_4.5H_2O$ and $Co(C_2H_3O_2)_2.4H_2O$ were the compounds used.

was discontinued after period 2. The lambs on this treatment were reassigned to the remaining diets. The amount of the diet offered was adjusted to body weight at weekly intervals until the maximum intake was reached. From this time until the end of the experiment the lambs were fed as much as they would consume at each feeding.

Responses to the treatment were evaluated from data on the hemoglobin concentrations of the whole blood and the iron and copper concentrations in the blood serum. Samples of blood were collected from the jugular vein of the lambs before the experimental regimen was initiated and at regular two-week intervals thereafter. Hemoglobin concentrations were determined by the method described by Shenk et al. ('34). Iron and copper were measured by a method described by Matrone et al., ('47). At the end of the experiment, 6 wether lambs, two from each treatment, were slaughtered. Samples of the liver, kidney, spleen, and heart were analyzed for iron and copper.

RESULTS

Hemoglobin. As shown in figure 1, the mean biweekly concentrations of hemoglobin of the test lambs receiving the Mn_3 level of manganese were lower than those of the lambs fed the other levels. An analysis of variance of these data indicated that the difference between the highest level, Mn_3, and the other levels was significant ($P \leq 0.01$) in all periods. A further analysis of the data indicated that there was no significant difference among treatments Mn_0, Mn_1 and Mn_2 in any of the periods.

Iron. The serum iron concentrations for the lambs on treatment Mn_3 decreased during period 1 and had the lowest mean concentration during period 2, as shown in figure 2. An analysis of the linear regression coefficients, measuring the change in serum iron with time, revealed a significant ($P \leq 0.05$) linear difference among diets for period 1. This difference is interpreted to mean that the level of serum iron of the animals on treatments Mn_0, Mn_1, and Mn_2 increased

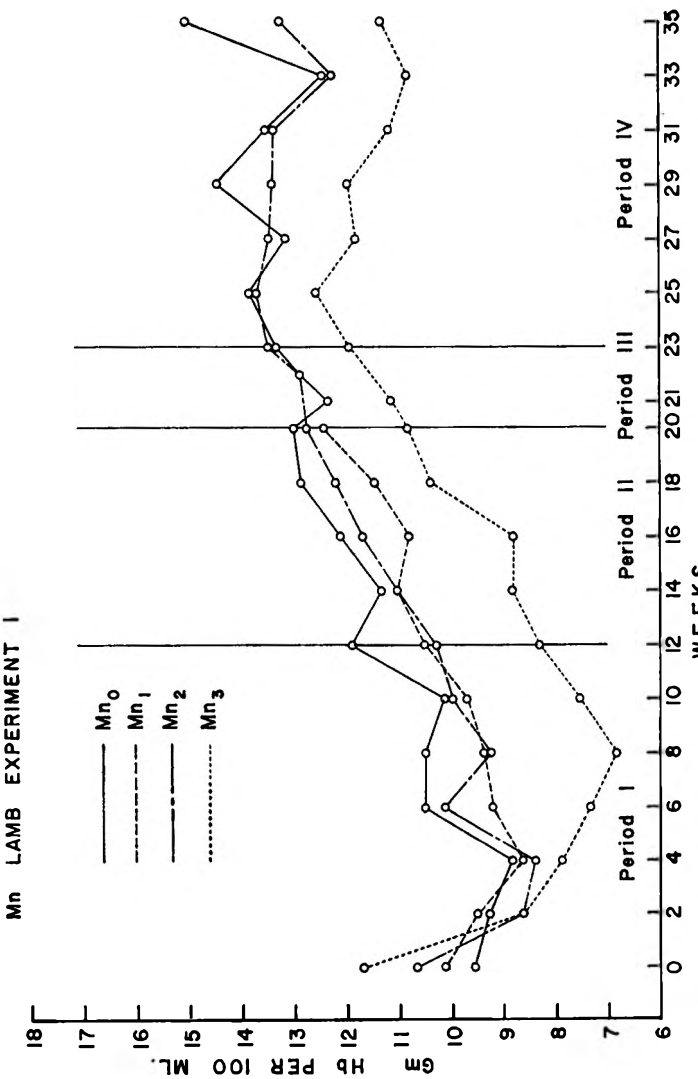

Fig. 1 Mean values of hemoglobin of the lambs fed various levels of manganese during the 4 periods.

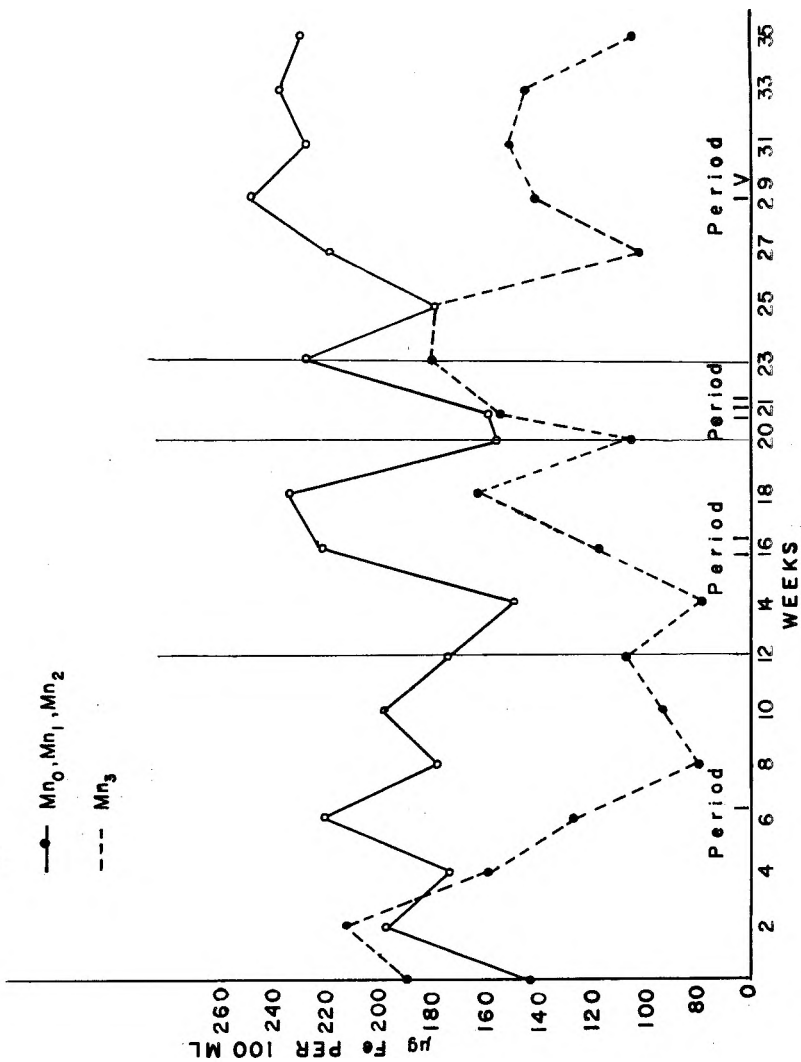

Fig. 2 Mean values of serum iron of the lambs fed various levels of manganese during the 4 periods. Since the responses to the Mn_0, Mn_1 and Mn_2 were not significantly different, the serum iron values were combined into one curve.

with time, whereas the serum iron of animals on Mn_3 decreased. Thus, the highest intake of manganese was associated with the lowest serum iron value.

Copper. The experimental treatments had no significant effect on the serum copper concentrations of the lambs in any of the periods. The mean concentration of serum copper for all experimental lambs increased with each successive period; the mean values during periods 1, 2, 3 and 4 were 69, 112, 123 and 142 μg per 100 ml of serum, respectively.

TABLE 2

Concentrations of iron and copper in the liver, kidney, spleen and heart of the lambs (experiment 1)

DIETARY TREATMENT [1]	CONCENTRATION OF IRON AND COPPER IN ORGANS							
	Liver		Kidney		Spleen		Heart	
	Fe	Cu	Fe	Cu	Fe	Cu	Fe	Cu
	μg per gm dry weight							
Mn_0	178	622	173	29	803	11	156	29
Mn_0	152	734	187	39	879	8	122	14
Mn_2	110	1185	110	27	198	8	125	18
Mn_2	94	1251	125	19	202	3	109	18
Mn_3	94	2751	23	22	171	9	65	7
Mn_3	99	2521	174	28	236	18	123	13

[1] Each treatment represents the values obtained from one lamb.

Composition of organs. The results of the analysis of the organs are shown in table 2. From these data it is apparent that the high dietary levels of manganese were related to decreased concentrations of iron in the organs. In the liver the decrease in the concentration of iron was associated with an increase in the concentration of copper.

Experiment 2

Procedure. Eight lambs from 6 to 9 days of age were nursed on unsupplemented cow's milk for approximately two months prior to feeding of the experimental diets. During the second month of this preliminary period, the lambs were bled periodically until the concentration of hemoglobin de-

creased to 4 to 6 gm per 100 ml of whole blood, at which stage they were placed on experiment.

The basal diet of the experiment consisted of 60% excellent quality chopped soybean hay, 32% glucose,[5] 4% casein, and 4% cottonseed oil.[6] The three manganese treatments under study were: (1) basal, (2) basal + 1000 ppm of Mn, and (3) basal + 2000 ppm Mn (hereafter referred to as Mn'_0, Mn'_2 and Mn'_3). The lambs were randomly assigned to the diets. Three lambs received the Mn'_0, three the Mn'_3 and two the Mn'_2 diet.

As the animals increased their consumption of the experimental diets, the whole milk was reduced gradually to 500 ml per day and the roughage diets were increased to the level of one pound per day per lamb. One thousand parts per million of manganese, based on the dry matter content, were added to the milk supplement of these lambs receiving the Mn'_2 and Mn'_3 diets. Because of this procedure the Mn'_3 diet contained somewhat less (1986 ppm) than 2000 ppm of manganese. This small discrepancy was ignored in the results presented.

Each lamb remained on the experimental treatments for 11 weeks. Observations on the concentrations of blood hemoglobin, serum iron, and serum copper were made immediately before feeding the test diets, throughout the feeding period and for a period of three weeks after the manganese additions were discontinued.

Results

Hemoglobin. As shown in figure 3, the weekly mean concentrations of hemoglobin in blood of lambs fed the basal diet increased more rapidly than in animals receiving either the 1000 ppm of manganese or the 2000 ppm. These data indicate that feeding high levels of manganese retarded the regeneration of hemoglobin. It is apparent, however, that

[5] "Cerelose," Corn Products Refining Company, New York, N. Y.

[6] Wesson Oil and Snowdrift Sales Company, New Orleans, La.

the difference between the treatments of 1000 ppm and of 2000 ppm manganese was negligible.

Iron. The biweekly mean values of serum iron from the second through the 14th week are shown in figure 4. The lambs receiving 1000 ppm and those receiving 2000 ppm of manganese had lower concentrations of serum iron than those fed the basal diet. There was no significant difference, however,

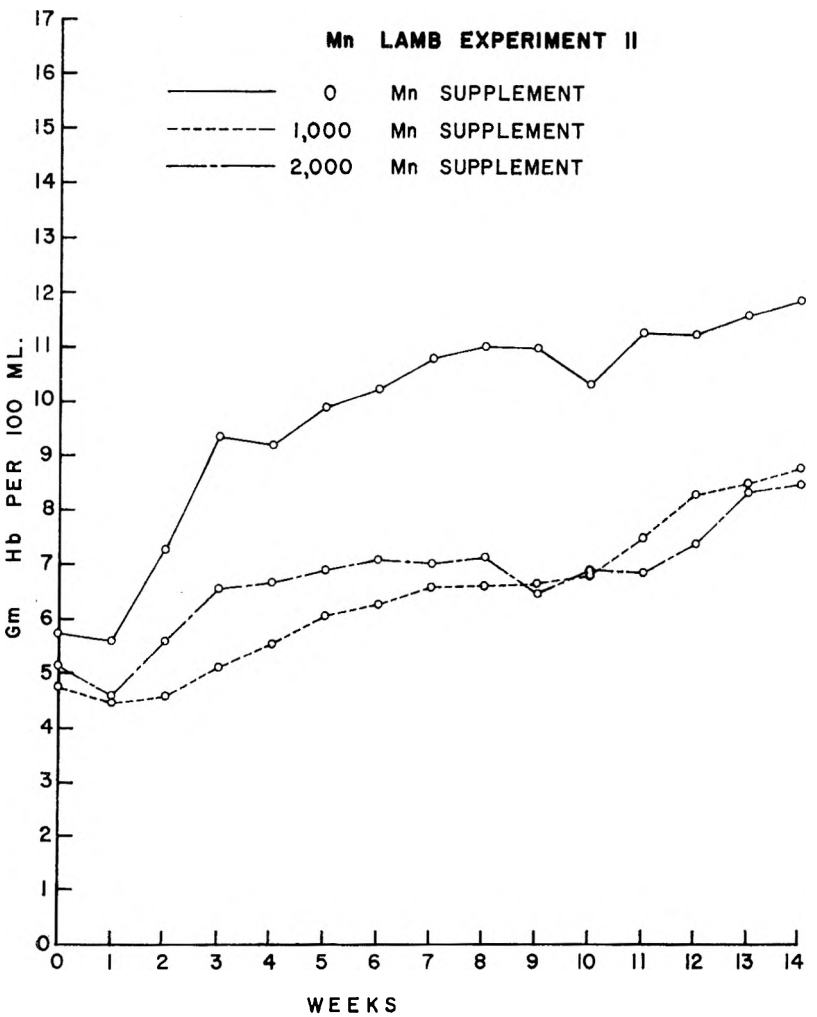

Fig. 3 Mean values of hemoglobin of the lambs fed three levels of manganese.

between the concentrations of serum iron from the 1000 and the 2000 ppm manganese treatments. The removal of supplemental manganese from the diets (11th week) was followed by a rise in the level of serum iron of the lambs previously fed manganese, but this response occurred earlier in the group fed 1000 ppm manganese than in the group fed 2000 ppm.

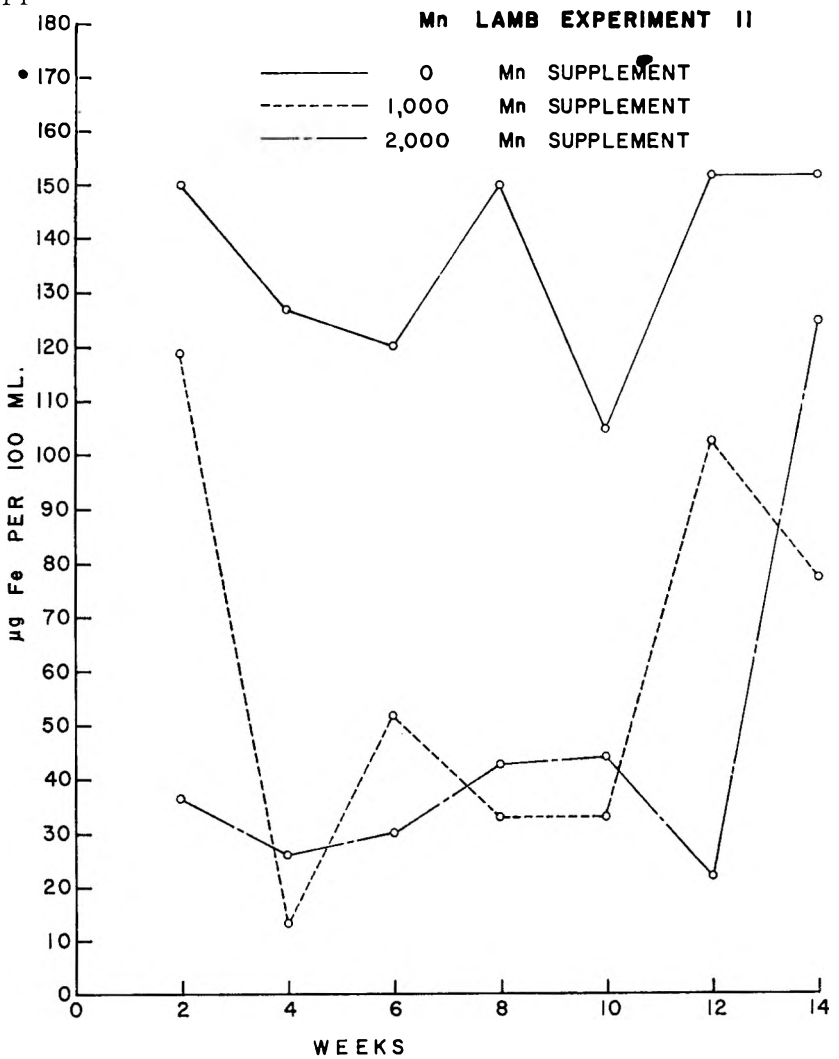

Fig. 4 Mean values of serum iron of the lambs fed three levels of manganese.

Copper. The concentrations of serum copper showed no effects ascribable to the manganese treatments. The mean serum copper concentration of all the lambs decreased from 84 μg per 100 ml of serum at the beginning of the experiment to 55 μg per 100 ml of serum on the 12th week.

DISCUSSION

When lambs depleted of iron were fed this element either as an inorganic salt in the milk diet or as a natural constituent of a roughage diet, the presence of excessive manganese depressed or retarded hemoglobin formation but had no perceptible effect on productive functions, including growth. The adverse hematopoietic responses may be explained in several ways: the excessive manganese may interfere with (1) the absorption of iron, (2) the formation of hemoglobin, and (3) the combination of (1) and (2).

Since the concentrations of iron in the sera, livers, and spleens of lambs fed appreciable quantities of manganese were low, the probabilities are that manganese interfered with iron absorption rather than with hemoglobin formation *per se.* A further indication that iron absorption was reduced in lambs fed manganese was the decreased concentration of iron in the liver accompanied by an increase in copper stores, a relationship synptomatic of iron deficiency (Hahn and Fairman, '36). It is suggested that excessive manganese either converts iron to a form physiologically unavailable or in some manner antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption. Further studies on the mode of action of excessive manganese in the utilization of iron are being conducted.

SUMMARY

In two experiments young growing lambs were depleted of iron either by an iron deficient diet or by a combination of the diet and phlebotomy. In the first experiment, a whole milk diet fortified with iron, copper, cobalt and vitamins A

and D was supplemented with various levels of manganese. A level as low as 45 ppm of manganese fed to young lambs brought about a decrease in the concentration of hemoglobin and of serum iron. Higher levels of manganese, up to 5000 ppm, were associated with decreased concentrations of iron in the liver, spleen and kidney.

In the second experiment anemic lambs were fed a roughage diet supplemented with three levels of manganese, 0, 1000, and 2000 ppm. Hemoglobin regeneration was markedly retarded and serum iron depressed in lambs fed diets containing either 1000 ppm or 2000 ppm of manganese.

The data indicate that manganese interferes with iron absorption rather than with hematopoiesis. It is postulated that excessive manganese antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption.

LITERATURE CITED

BEESON, K. C. 1954 Unpublished data.

BEESON, K. C., AND GENNARD MATRONE 1950 The nutrient element content of native forage in relation to land forms and soil types in the North Carolina Coastal Plain. Copper metabolism, a symposium on animal, plant and soil relationships. Johns Hopkins Press, Baltimore, Md., pp. 370–398.

BLAKEMORE, F., J. A. NICKLESON AND J. STEWART 1937 Some effects of high manganese content in the diet of animals, with special reference to lactation tetany. Vet. Record, *49:* 415–422.

HAHN, P. F., AND E. FAIRMAN 1936 The copper content of some human and animal tissues. J. Biol. Chem., *113:* 161–165.

HAWKINS, G. E., G. H. WISE, G. MATRONE, R. K. WAUGH AND W. L. LOTT 1955 Manganese in the nutrition of young dairy cattle fed different levels of calcium and phosphorus. J. Dairy Sci., *38:* 536–547.

MATRONE, GENNARD, W. J. PETERSON, H. M. BAXLEY AND C. D. GRINNELLS 1947 Copper and iron in the blood serum of dairy cows. Ibid., *30:* 121–126.

RUSSELL, F. C. 1944 Minerals in pasture deficiencies and excesses in relation to animal health. Imperial Bureau of Animal Nutrition, Tech. Comm., *15:* 69–85.

SHENK, J. H., J. L. HALL AND H. H. KING 1934 Spectrophotometric characteristics of hemoglobin. I. Beef blood and muscle hemoglobin. J. Biol. Chem., *105:* 741–751.

STUART, M., F. G. HARBAUGH AND J. DENNIS 1950 A quantitative investigation of some mineral components of wheat plants. Am. J. Vet. Res., *11:* 400–401.

THE EFFECT OF CHRONIC INANITION AND FREQUENCY OF FOOD INGESTION ON THE DENTAL CARIES EXPERIENCE IN THE RAT

JOSEPH C. MUHLER
Department of Chemistry, Indiana University, Bloomington

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During the investigations of the effect of hypophysectomy on dental caries a pronounced failure to gain weight normally resulted in the young hypophysectomized rats, as would be expected (Shafer and Muhler, '55). Since the hypophysectomized rats ate considerably less food, and since dental caries production in the rat is intimately related to the ingestion of a coarsely ground corn or rice diet, it became of interest to determine what effect caloric restriction of an adequate diet known to induce caries would have on the incidence of dental caries. This problem has not been directly studied previously, but is considered to be of importance in order to establish more clearly the role of food intake and dental caries in the rat. If food intake is of importance it may well explain some of the variability in dental caries incidence always encountered in such studies.

Stephan and Harris ('55) have recently commented on the relationship of the growth of rats to their caries susceptibility and report that in experiments with animals of the Sprague-Dawley strain the greatest amount of smooth surface caries was generally found in individuals which gained the least weight. However, fissure caries showed no such relationship. Rats of the "Yale" strain showed no correlation of weight gain with susceptibility to dental caries of any type. Furthermore, since frequency of food ingestion

in the human is considered to be related to the dental caries experience, it was also of interest to study these variables.

The purpose of this investigation, then, was to study the effect of chronic inanition as well as the effect of frequency of food ingestion on the dental caries experience in rats receiving a diet known to induce a moderate degree of caries (Muhler, '55).

EXPERIMENTAL

A total of 160 weanling rats of the Sprague-Dawley strain were divided into 5 series for use in these experiments. Each series was further subdivided according to sex. Series I was the control group and received food ad libitum. The animals of series II received 60% of the food eaten by the control group while those in series III received 40% of the food eaten by the control group. This was accomplished by housing the animals in individual cages and dividing them into sets of three each in such a manner as to be assured that each restricted food mate of the control group received exactly 60 or 40% of the food eaten each day by the control member fed ad libitum. The animals of series IV received food ad libitum three times a day for a one-half-hour period beginning at 8 A.M., 12 noon and 6 P.M., while those in series V received food ad libitum but for only one and one-half hours once a day beginning at 8 A.M. All of the animals received the same stock corn cariogenic diet (Muhler, '55) and fluorine-free re-distilled water. The animals were kept in an air-conditioned room. The duration of the experiment was 140 days, after which time the animals were sacrificed by ether and the heads examined for dental caries by methods previously described (Muhler, Nebergall and Day, '53).

RESULTS AND DISCUSSION

The combined results from these experiments are found in table 1. The male control animals had a mean number of 9.4 cavities and the females 7.4. In the rats of both series II and series III a pronounced reduction in the incidence of

dental caries was found. These data show that, under these conditions, the amount of food eaten is correlated with the development of experimental dental caries. Since these data clearly indicate that the caloric intake of the coarse corn diet must be related to the incidence of dental caries in the rat, it may well be that female rats of the Sprague-Dawley strain show a lower caries incidence because of the ingestion

TABLE 1

The effect of food restriction on the incidence of experimental dental caries in the rat

	SERIES	NO. OF RATS	SEX	NO. OF LESIONS	FINAL WEIGHT	TOTAL FOOD CONSUMED
					gm	*gm./rat/day*
I	Ad libitum, control	12	Male	9.4 ± 2.1	357	14.5
		18	Female	7.4 ± 1.6	232	10.8
II	60% restriction	12	Male	2.6 ± 0.12	248	8.7
		18	Female	1.2 ± 0.06	179	6.9
III	40% restriction	12	Male	1.0 ± 0.09	178	5.9
		18	Female	0.4 ± 0.01	130	4.7
IV	Ad libitum,[1] 3 times daily	12	Male	2.7 ± 0.10	300	...
		14	Female	1.2 ± 0.05	190	...
V	Ad libitum,[1] once daily	12	Male	1.9 ± 0.08	239	...
		16	Female	0.4 ± 0.02	158	...

[1] The animals in series IV were allowed food for three one-half-hour periods each day; those in series V for one period of one and one-half hours.

of less food. These data suggest that more careful consideration be given to the total amount of food eaten by rats which are under investigation for dental caries.

There appears to be a tendency for the animals receiving 40% of the amount of food eaten by those in the control group to have less dental caries than those receiving 60%, but a statistical analysis of these data indicate them to be significant only at the 0.5 level of confidence.

The frequency of food consumption also seems to affect the experimental production of dental caries, for when the food was available three times a day there was a tendency for more caries than when the food was available only once a day but for the same period of time. This latter observation is consistent with the caries experience that might have been expected since there appeared to be a tendency for the animals receiving food three times a day to eat more than those eating but once a day. This is indicated also by the fact that the animals in series IV weighed considerably more than those in series V. However, these differences are not significant even at the 0.05 level and thus indicate only a tendency for more caries in the animals receiving food three times a day. It appears that these differences can probably be explained on the basis of differences in quantity of food eaten rather than frequency of feeding. However, this point needs further investigation.

In a previous study in which the incidence of dental caries was determined in hypophysectomized animals (Shafer and Muhler, '55) there was a significant increase in the operated animals in comparison to similar unoperated animals. In the former group there was a pronounced reduction in food consumption and also in body weight. It was estimated that in these animals the food reduction was approximately 60%. If caloric restriction was responsible for the increase in dental caries a similar increase should have occurred in the animals of series II in this study. Thus it appears that the increase in dental caries noted in the hypophysectomized animals was a result of the hypophysectomy and not food restriction alone.

These findings are interesting also in any consideration of the mechanism of caries initiation. If dental caries in the rat is a result of cusp fracture and subsequent dissolution of the enamel and dentin resulting from the coarse corn diet, then one would expect to find a reduction in dental caries in the animals receiving less food, but probably not of the order of magnitude as reported here. Furthermore, it was consistently found that as high a number of fractured cusps

occurred in the animals of series II and III as in the controls, but with no caries progression. Instead, there was an unusually high number of arrested caries. In short, there appeared to be the same amount of fractured cusps, also caries initiation in other areas of the tooth besides the fractured areas, but little caries progression in these animals. Additional work is needed to clarify the mechanism by which dental caries is initiated and extended in the rat.

CONCLUSIONS

The total amount of dental caries occurring in the rat was in large measure dependent upon the amount of food eaten. When the cariogenic diet was restricted to 60% of the food eaten by the animals in an ad libitum control group there was approximately 80% less dental caries, and when the diet was restricted to 40% there was approximately 90% less dental caries.

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LITERATURE CITED

MUHLER, J. C. 1955 The cariogenicity of various corn diets in the rat. J. Dent. Res., *34:* 364.

MUHLER, J. C., W. H. NEBERGALL AND H. G. DAY 1953 Preparations of Stannous flouride compared with sodium fluoride for the prevention of dental caries in the rat. J. Am. Dent. Assn., *46:* 290.

SHAFER, W. G., AND J. C. MUHLER 1955 Experimental dental caries. VI. The effect of hypophysectomy on dental caries and the salivary glands of the rat. J. Dent. Res., *34:* 531.

STEPHAN, R. M., AND M. R. HARRIS 1955 Location of experimental caries on different tooth surfaces in the Norway rat, in *Advances in Experimental Caries Research,* 47–65, American Association for Advancement of Science Publication.

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