THE RELATION OF THE 'GRASS JUICE FACTOR' TO GUINEA PIG NUTRITION [1]

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FIVE FIGURES

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Earlier work in this laboratory (Elvehjem, Hart, Jackson and Weckel, '34) demonstrated a seasonal variation in the nutritive value of mineralized milks. Rats fed a mineralized milk, produced by cows on good pasture, grew at a rate of 4 to 4.5 gm. per day during a 6-week experimental period. When milk, produced by cows receiving a winter barn ration, was used as the basal diet, growth of only 2 to 2.5 gm. per day was obtained. Later it was demonstrated (Kohler, Elvehjem and Hart, '36) that the addition of fresh lawn clippings or the pressed juice from grass to the mineralized winter milk produced a rate of growth comparable to that obtained on summer milk.

In a more recent publication (Kohler, Elvehjem and Hart, '37) results were presented to show that various supplements rich in the known vitamins produced little or no growth response in rats on a basal winter milk diet. This led to the conclusion that the growth stimulating factor of grass was distinct from all the known vitamins. At this time it was pointed out that considerable variation was encountered in different groups of rats when placed on the basal ration, presumably due to differences in the storage of certain factors in the young rats when started on the experiment. Since

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

guinea pigs are herbivorous, we felt that this species might be a more suitable animal for the assay of a factor which is found most abundantly in fresh green plant tissue.

The present paper deals with the growth of guinea pigs on mineralized winter milk and the growth-promoting action of supplements similar to those used in our earlier work with rats.

EXPERIMENTAL

Two preliminary experiments were made in order to study the reaction of guinea pigs to diets made up largely of whole milk. The milk used was obtained fresh each morning from the same cow (Holstein) at the dairy barn. The whole milk was fed twice daily in sufficient quantities to allow ad libitum consumption. In addition to the milk each guinea pig received a daily mineral supplement consisting of 1 mg. of Fe as ferric pyrophosphate, 0.1 mg. of Cu as copper sulfate, and 0.1 mg. Mn as manganese sulfate. The salts were incorporated into a small amount of dextrin and fed in a small gelatin capsule. The animals were weighed daily.

In the first trial four guinea pigs weighing about 300 gm. each were used. Two pigs were placed on the basal mineralized milk alone, one on the basal plus 2 cc. of orange juice daily and one on the basal plus 5 gm. of fresh oat grass which had been grown in the greenhouse.

For the second experiment six guinea pigs were divided into three groups of two pigs each. Two pigs received the basal mineralized milk diet and the four remaining pigs were given the same diet except that the milk was aerated for 1 hour at 60 to 65°C. Two of the pigs receiving the aerated milk were given 1 mg. of ascorbic acid per animal daily. The aerated milk was used to determine if a variation in the vitamin C intake would alter the results. Growth curves for the ten pigs are given in figure 1.

The sequence of events observed when the pigs were placed on the milk diets was very similar in each animal. During the first week or two the animals lost considerable weight. After

this preliminary period, they apparently became accustomed to the liquid diet and consumed fairly large amounts of milk. For the next few weeks they remained at constant weight or in some cases gained slightly until their original losses were regained. Between the fourth and seventh weeks the animals began to lose weight rapidly, some losing as much as 70 to 80 gm. in 1 week. At this stage respiratory trouble was noted in most cases. Immediately preceding death, clonic contractions of the legs were observed.

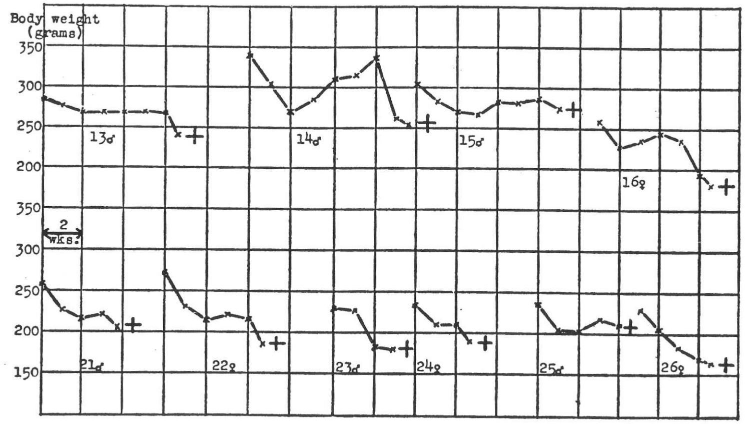

Fig. 1 Weight records of guinea pigs receiving mineralized winter milk (ad lib.). Nos. 13, 14, 25 and 26 received whole raw milk. No. 15 received raw milk + 5 gm. greenhouse oat grass daily. No. 16 received raw milk + 2 cc. orange juice daily. Nos. 21 and 22 received aerated milk. Nos. 23 and 24 received aerated milk + 1 mg. pure vitamin C daily.

Autopsies were made on all the animals and no signs of scurvy or rickets could be detected. In many cases the lungs showed advanced states of inflammation and congestion and, in some animals, necrotic areas in the lungs were evident. Since all animals showed a temporary favorable response on the milk after the first week or two, it appears that the animals actually suffered from a dietary deficiency which brought on the extreme debility and susceptibility to infection.

Since the addition of orange juice or oat grass did not alter the changes observed, it is obvious that neither vitamin C nor vitamin P (Armentano et al. '36) were limiting factors. The oat grass was tested on rats simultaneously and found to be inactive as a source of the 'grass juice factor.' Thus the failure of this oat grass to stimulate growth in guinea pigs did not eliminate the importance of this factor in the nutrition of guinea pigs.

The animals receiving the aerated milk died a little sooner than the other pigs. However, no signs of scurvy were evident even in these animals. The addition of generous amounts of ascorbic acid gave no better results than those observed in the pigs on the untreated milk. The uniformity of the results obtained in the preliminary trials indicates that winter milk is deficient in one or more essential factors necessary for the normal development of guinea pigs.

Various supplements were fed to guinea pigs receiving a basal diet of winter milk plus iron, copper and manganese in an attempt to correct the deficiency observed. In all of the following experiments the salt solutions together with the supplement were placed in a clean dish with a little milk each morning. Later in the day, when the animal had completely consumed the contents of the dish, enough milk was added to insure ad libitum feeding. Since milk contains 2 to 2.5 mg. of vitamin C per 100 cc., and the guinea pigs consumed 80 to 200 cc. of milk per day, there should be no danger of an inadequate supply of this vitamin. Unpublished work in our laboratory has shown that 0.3 mg. of pure ascorbic acid per day is sufficient to prevent the onset of scurvy and to support a good rate of growth. However, as a precautionary measure all animals were given ½ cc. of orange juice daily by pipette.

In figure 2 are presented growth curves of guinea pigs which received a series of supplements chosen to supply ample quantities of various known vitamins. The supplements were as follows: 5 gm. whole wheat meal, 5 gm. white cornmeal, 2 gm. brewers' yeast, 1 gm. vacuum dried whole

liver (pork),[2] 0.5 gm. liver extract powder,[2] and 0.5 gm. 92% alcohol soluble liver extract.[2] The wheat and cornmeal were found to be ineffective at the original levels, but when 7.5 gm. per day were fed, fair rates of growth were obtained. The vacuum dried whole liver gave some growth when the level was increased to 1.5 gm. per day, but neither of the liver extracts was effective. Apparently, the active principle of the liver is not extracted, or is destroyed by the commercial

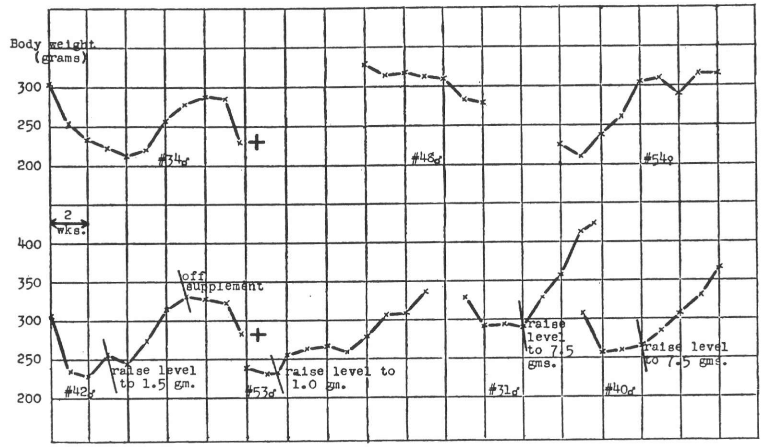

Fig. 2 Weight records of guinea pigs receiving mineralized winter milk (ad lib.) plus supplements containing liberal quantites of known vitamins. The daily supplements were as follows: No. 34, control; no. 48, 0.5 gm. 92% alcohol-soluble liver extract; no. 54, 2.0 gm. brewers' yeast; no. 42, 1.0 gm. vacuum dried whole liver; no. 53, 0.5 gm. liver extract powder; no. 31, 5.0 gm. whole wheat meal; no. 40, 5.0 gm. white cornmeal. All received 0.5 cc. orange juice daily.

processes used in the preparation of liver extracts. The inferior response given by the yeast together with the remarkable growth produced by grasses, shown in figure 3, adds credence to the hypothesis that the 'grass juice factor' described for rats is the same as the limiting factor concerned here.

[2] We are indebted to Dr. David Klein, Wilson Laboratories, Chicago, for the samples of liver and liver extract used.

The effects of feeding dried grasses as supplements to guinea pigs on the mineralized winter milk are shown in figure 3. The three cereal grasses (nos. 462, 458, 455)[3] were produced under comparable conditions. They were grown at the same time on plots of the same field so that they were exposed to identical weather conditions. They were cut at the same stage of growth, 1 month after emergence, and were dried at 82°C. for 4 hours.

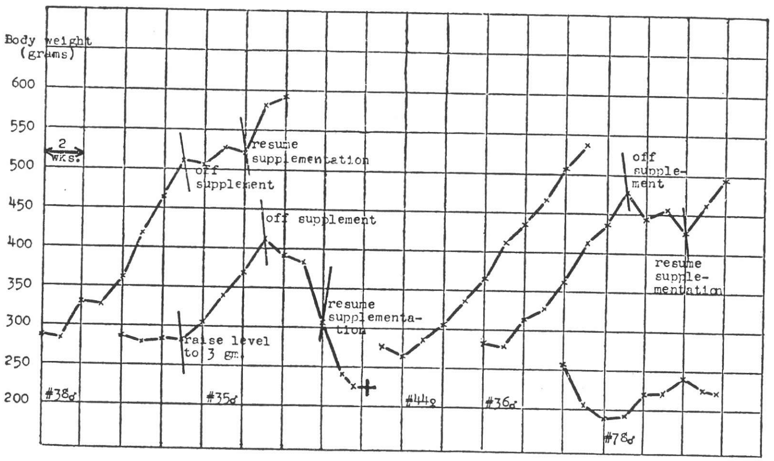

Fig. 3 Weight records of guinea pigs receiving mineralized winter milk (ad lib.) plus dehydrated grasses. The daily supplements were as follows: no. 38, 2.0 gm. barley grass no. 462; no. 35, 2.0 gm. oat grass no. 455; no. 44, 2.0 gm. barley grass no. 462; no. 36, 2.0 gm. wheat grass no. 458; no. 78, control. All received 0.5 cc. orange juice daily.

The barley grass, which was the most effective, produced a growth rate of 5.3 gm. per day from the second to the seventh week of the experiment. The wheat grass was only slightly less potent than the barley grass. However, in the case of the oat grass, it was necessary to raise the level to 3 gm. per day to produce good growth.

[3] These grass samples were generously supplied by Mr. C. F. Schnabel of the American Butter Company.

After 7 weeks on experiment, pigs nos. 35, 36 and 38 were taken off the supplement and fed the mineralized milk alone. Growth stopped almost immediately. After 3 weeks on the basal ration alone, during which period these animals actually lost weight, supplementation was resumed. Once again remarkable growth resulted in the animals receiving the barley and wheat grasses (pigs nos. 36 and 38). The animal receiving the oat grass (pig no. 35) was in such poor condition

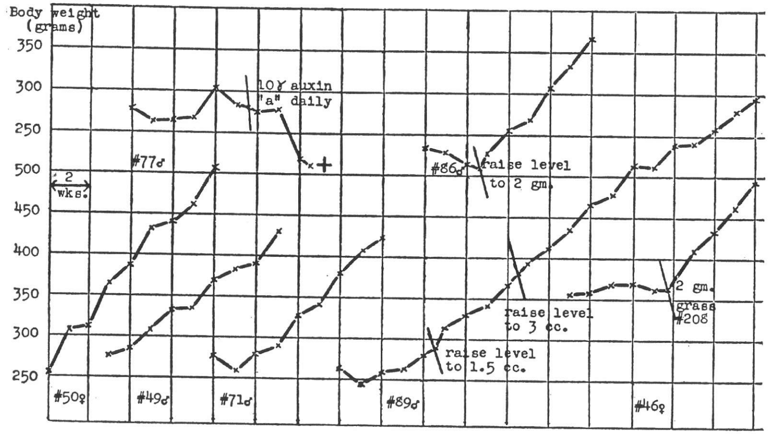

Fig. 4 Weight records of guinea pigs receiving mineralized winter milk (ad lib.) plus various preparations of grass or auxin. The daily supplements were as follows: no. 50, 20 gm. fresh lawn clippings; no. 49, 20 cc. juice pressed from lawn clippings; no. 71, 1.0 gm. frozen oat grass pulp no. 207 (batch 1); no. 89, 0.75 cc. juice of oat grass pulp no. 207; no. 46, control for 4½ weeks, 2.0 gm. dehydrated oat grass no. 208 (Arnold drier); no. 77, control for 5½ weeks, then 10 gamma auxin 'a'; no. 86, 1.0 gm. frozen oat grass pulp no. 207 (batch 2). All received 0.5 cc. orange juice daily.

at this time that it refused to eat the supplement when it was offered. This animal died about 2 weeks later. These results show definitely that the grasses contain a nutritional factor which is essential for maintenance as well as growth of guinea pigs.

The growth curves in figure 4 show that guinea pigs do not need roughage in their diet, at least when milk is used as the basal ration. One guinea pig received 20 gm. of fresh lawn clippings (mostly Kentucky blue grass) per day as the supplement, while another received 20 cc. of the centrifuged press juice from fresh lawn clippings. Twenty cubic centimeters of this juice contained about 0.2 gm. solids. Both the clippings and the juice were quite effective, although neither was as good as barley grass no. 462, which was fed at a comparable level. Pulped oat grass no. 207 [4] which had been frozen immediately after cutting and pulping was tested and found to be more potent than the freshly cut lawn clippings. Two batches of this pulp were obtained. Of the first batch, 1 gm. was sufficient to promote good growth. The second batch was less potent, 2 gm. per day being required. Since this pulp contained only about 25% solids, the effective levels were 0.25 to 0.50 gm. per day on the dry basis. The centrifuged press juice from this pulp was also effective; 1.5 to 3 cc. per day, containing 8% of solids or 0.12 to 0.24 gm. dry matter, gave definite growth stimulation. A portion of the grass used to prepare pulp no. 207 was dried in an Arnold drier [5] and designated as grass no. 208.[6] This dehydrated oat grass was fed to a guinea pig on the winter milk basal ration, and it was found that about 2.0 gm. daily were necessary to produce good growth. Hence in the dehydration process some destruction occurred.

Since green plant tissues which are growing most rapidly are the most potent sources of the factor in question, it was thought that it might be worthwhile to test the plant hormone, auxin. Hence, auxin 'a' [7] was fed at a level of 10 gamma per day to a guinea pig on the winter milk basal diet. The growth

[4] See footnote 3, page 450.

[5] In the Arnold drier (also called Heil drier and made by the Heil Company), the initial temperature was 1400°F. and the final temperature 250°F. The total time of drying the grasses was 3 minutes. During this period, evaporation proceeds at such a rate that the actual temperature of the grass is considerably less than that of the surrounding gas. Natural gas was used as fuel. The combustion gases are drawn through the drying drum, and give a partial CO_2 atmosphere.

[6] See footnote 3, page 450.

[7] Kindly furnished us by Dr. F. Kögl, Utrecht, Holland.

curve is also given in figure 4. Although no response was obtained, the possibility that auxin is the factor is not excluded since the level fed may have been too low. Our supply was not great enough to feed other pigs at higher levels.

Some idea as to the stability and solubility of the active principle of the grasses was obtained in later experiments, the results of which are given in figure 5. The effect of temperature during storage of grass is shown by the growth of the animals receiving grasses no. 75 and no. 76.[8] These samples were portions of the same batch of dehydrated rye grass, which were stored under different conditions for a period of 6 months. Grass no. 75 was kept in a cooler at a freezing temperature, while grass no. 76 was kept at room temperature which at one time rose as high as 95°F. It is evident that considerable loss occurred during storage at room temperature, while the sample stored in the cold retained its activity.

Lot no. 73,[8] a mixture of wheat and rye grasses, was divided into two parts. One-half of the mixture was fed to a guinea pig as a supplement to mineralized winter milk, while the other half was autoclaved for 1 hour at a pressure of 15 pounds per square inch, dried in the drying room (65°C. for 24 hours), and tested on another guinea pig. The weight records indicate that much of the activity was destroyed by this treatment.

Three samples of dried alfalfa were tested, two of which had been produced under ordinary farm conditions as contrasted to the third, sample no. 380,[8] which had been cut at the stage of most rapid growth and dried in an Arnold drier.[9] The latter sample was quite potent, while the field-dried samples were very poor in growth promoting activity. This difference cannot be ascribed entirely to the method of drying, since the samples were not cut at comparable stages of growth, and further the inactive samples had been stored at ordinary barn temperatures, while sample no. 380 was kept in the

[8] See footnote 3, page 450.

[9] See footnote 5, page 452.

cooler until the feeding tests were made. Also the samples were grown on different soils in different sections of the country.

Other growth curves given in figure 5 show that the factor is not extracted from dehydrated barley grass (no. 462) by

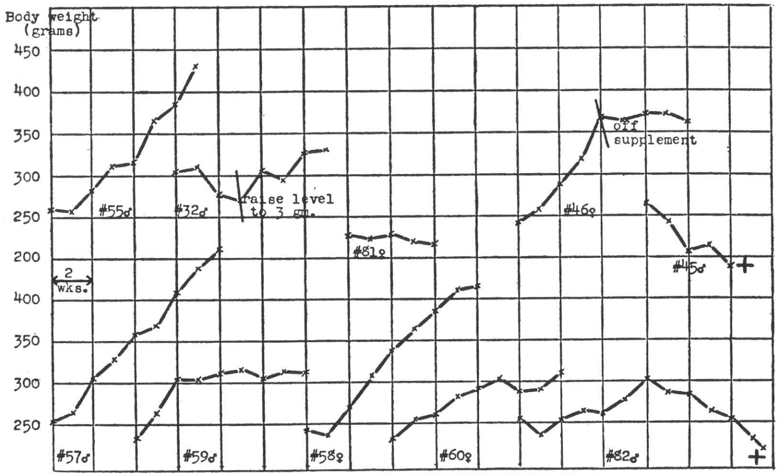

Fig. 5 Weight records of guinea pigs showing the stability of the grass juice factor to heat and its solubility in petroleum ether. The daily supplements were as follows: no. 55, 2.0 gm. dehydrated alfalfa no. 380 (Arnold drier); no. 32, 2.0 gm. alfalfa hay, sample 1; no. 81, 2.0 gm. alfalfa hay, sample 2; no. 46, residue from petroleum ether extraction of grass no. 462; no. 45, petroleum ether extract of grass no. 462; no. 57, 2.0 gm. dehydrated rye grass no. 75 (stored in cooler for 6 months); no. 59, 2.0 gm. dehydrated rye grass no. 76 (stored at room temperature for 6 months); no. 58, 2.0 gm. wheat and rye grass no. 73; no. 60, 2.0 gm. autoclaved grass no. 73; no. 82, control. All received 0.5 cc. orange juice daily.

petroleum ether (Soxhlet extraction for 24 hours). The fractions were fed at levels corresponding to 2 gm. per day of the original grass. The petroleum ether extract, which constituted about 5% of the original grass, was inactive, while the residue retained most of the potency.

DISCUSSION

Although at present there is no indisputable proof that the rat and guinea pig factors are the same, the following considerations indicate that this is the case.

The same basal ration, winter milk supplemented with minerals, has been used to produce the deficiency in both species.

Sources of the potent factor (or factors) are parallel in activity for the two species. Grass samples which were effective for one species have proved to be potent for the other, and, conversely, grass samples which have been inactive for one have also proved to be inactive for the other species. Further, yeast showed inferior activity for both species.

The limited data available at present on loss of activity of grasses upon heat treatment, drying, and storage are parallel for the two species.

The pressed-out juice of potent grasses contains the active factor for both rats and guinea pigs.

Up to the present we have not been able to obtain good growth in guinea pigs receiving mineralized whole milk produced by cows on pasture (Summer, '37). This can probably be attributed to the fact that the pastures were in a very poor condition due to a sustained dry spell. Further, the guinea pig's requirement for the factor is apparently much greater than that of the rat, so that a 'summer milk' which would support good growth in the rat might not be good enough to support growth in the guinea pig. Further work will be done with 'summer milk' when better pastures are available.

In this connection it is interesting that Riddell et al. ('36), in studies designed to show the effects of winter and summer rations of cows on the vitamin C content of the milk, report that, although no appreciable difference was noticeable in incidence of scurvy, 40 cc. of pasture milk fed as a supplement to the basal scorbutic diet produced much better growth in guinea pigs than did a similar supplement of milk produced by cows on a dry ration. Cows receiving silage produced a milk of intermediate growth promoting qualities. It is probable that these differences were associated with the growth factor with which we have been working.

In view of the indications pointed out above we have assumed, as a working hypothesis, that the rat and guinea pig factors are the same, and further studies are being carried out on stability, chemical properties, and concentration using the latter species for assay.

As was pointed out in our previous paper, the combination of mineralized winter milk, orange juice and yeast supplies in adequate quantities all of the essential minerals, protein and energy, as well as the following known vitamins, C, P, A, B_1, B_6, nicotinic acid amide (anti-blacktongue factor), factor W, flavin, choline and the chick antipellagra factor. Further, unpublished data from this laboratory have shown that several grasses which are rich in the 'grass juice factor,' are very poor sources of the chick pellagra factor, vitamin B_4, the chick gizzard factor, and the highly unsaturated essential fatty acids. That vitamin D was not involved was shown in earlier work by feeding cod liver oil to rats on mineralized winter milk. It is on the basis of the above facts that we postulate the existence in grasses, and less abundantly in other food materials, of a new essential factor in nutrition.

An interesting fact brought out by this work is that guinea pigs can be raised on an all liquid diet in spite of the fact that their digestive tract is equipped to handle large amounts of roughage. Thus, the animals receiving mineralized milk, orange juice and grass juice grew at a good rate and no abnormalities were observed. When the grass juice was omitted the animals died. In contrast to guinea pigs, rats on a mineralized winter milk diet do not die, but grow continuously, although at a slow rate.

Several earlier workers (Bartenstein, '05; Moro, '07) attempted to raise guinea pigs and rabbits on all milk diets, and were uniformly unsuccessful. Their work, however, was done before it was appreciated that milk is low in iron, copper and manganese, so that deficiencies of these elements may have complicated the picture.

Several workers have attempted to raise calves on a milk diet supplemented with iron and copper. Bennet ('32) and Herman ('36) reported that, even with the addition of Fe, Cu, and Mn, calves will not live more than 8 to 13 months on an exclusive milk diet. Herman stated that although the calves grew better than normal on the mineralized milk diet for the first 6 to 8 months, they began to show signs of debility and lost weight after this period. All the animals died by the thirteenth month except one which lived to the seventeenth month. It would be interesting to repeat his experiment adding supplements of a potent grass juice to his ration. By such a method it might be possible to raise a calf on a liquid diet.

Duncan, Huffman and Robinson ('35) attribute the failure of complete nutrition in calves, limited to a milk diet, to a low blood magnesium.

Virtanen ('36) and Virtanen and Lane, ('36 a, '36 b) have published a series of papers showing that various green plant constituents reach maximum concentrations when plant growth is most rapid. Thus, protein, tryptophane, aspartic acid, carotene, and vitamin C are present in the largest quantities just before the plant reaches the flowering stage.

Hunt, Record and Bethke ('36) have shown similar variations in the vitamin B_1 and flavin content of pasture grasses and hays which were correlated with the rate of growth of the plants.

We would like to emphasize the fact that the potency of grasses in the growth stimulating factor with which we have been working also seems to vary in a similar manner with the stage of growth, the mature plants being much less effective than rapidly growing ones. Ordinarily, farmers allow their hay crops to reach a mature stage of growth before harvesting and at this stage the growth-stimulating activity is relatively low. This fact together with destruction of the active principle during drying and storage accounts for the seasonal variation in milk which has been discussed in an earlier paper. This seasonal variation led to the discovery of the grass juice factor which later work has shown to be distinct from other known nutritional essentials.

SUMMARY

1. Winter milk supplemented with iron, copper, and manganese is an inadequate diet for young guinea pigs. In contrast to rats, which grow slowly on mineralized winter milk, guinea pigs die on such a diet.

2. Orange juice, brewers' yeast, and liver extract produce little or no beneficial effect when fed as supplements to this diet.

3. Various grasses contain a factor (or factors) which is essential for maintenance and growth of guinea pigs. Small supplements of such grasses enable guinea pigs on a mineralized winter milk diet to grow normally.

4. The active principle of grasses is soluble in the plant juices since centrifuged grass press juice is active.

5. The activity of grasses disappears upon storage at room temperature. It is fairly stable at lower temperatures. It is destroyed to a large extent by autoclaving.

6. From experience with the guinea pig it is probable that this species can be used to good advantage in further studies on the 'grass juice factor.'

We are indebted to Mr. S. B. Randle for help in carrying out some of the more recent work reported in this paper.

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