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## VITAMIN B<sub>2</sub> DEFICIENCIES AS AFFECTED BY DIETARY CARBOHYDRATE

AGNES FAY MORGAN, BESSIE B. COOK AND HELEN G. DAVISON  
*Laboratory of Household Science, University of California, Berkeley*

FIVE FIGURES

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In the course of certain attempts to produce and to prevent cataracts in rats by dietary means there were observed in this laboratory striking differences in the response of rats to basal diets similar in all respects except as to the carbohydrate constituent. Lactose was used in one group of experiments because of the now well-established cataractogenic effect of that sugar (Mitchell and Dodge, '34; Yudkin and Arnold, '35) and vitamin B<sub>2</sub> deficiencies were produced both with this and with the more usually employed cornstarch diet, pursuant to Day, Langston and O'Brien's ('31) finding that cataracts may occur in rats having such deficiencies. It was found at once that while cataracts are produced more or less regularly on lactose-containing diets, the other more usual B<sub>2</sub> deficiency symptoms could not be obtained. This led to the use of sucrose as well as of lactose and cornstarch with varying single and multiple B<sub>2</sub> deficiencies.

The whole problem of the nature, function and even the number of factors included under the designation vitamin B<sub>2</sub>, as the British writers have employed the term, is at present too confused for any attempt at review here. Suffice it to say that we have been satisfied by the evidence brought forward by Lepkovsky, Jukes and Krause ('36), Elvehjem and Koehn ('35), Birch, György and Harris ('35), Gorter ('36), and others that at least three distinct factors are involved,

1) riboflavin, 2) an adsorbable heat stable probably basic substance called B<sub>6</sub> by György ('34), and factor 1 by Lepkovsky, Jukes and Krause ('36), 3) one or more unadsorbable factors called filtrate factor by Lepkovsky, Jukes and Krause ('36), B<sub>2</sub> by Elvehjem and Koehn ('35), antiblacktongue or P-P factor by Birch, György and Harris ('35). We have chosen to call these flavin, B<sub>6</sub> and filtrate factors respectively.

The plan involved the use of three similar basal diets with sucrose, lactose or cornstarch as carbohydrate constituent, adequate amounts of the fat-soluble vitamins fed as cod liver oil, crystalline vitamin B<sub>1</sub> (Merck) (10 micrograms per rat per day) as source of vitamin B<sub>1</sub> and one, two or all three of the B<sub>2</sub> factors. There were thus three groups of rats each on the following four regimes: without any of the B<sub>2</sub> factors; with only one, that is flavin or B<sub>6</sub> or filtrate factor; with each of the three possible combinations of two, that is flavin and B<sub>6</sub>, flavin and filtrate factor, filtrate factor and B<sub>6</sub>; and finally with all three factors. Twenty-seven groups were used, each made up of four to ten animals. Several of the comparisons were repeated three to five times. The growth curves used in this report, however, were taken from concurrent groups carefully chosen from comparable litters since it has become clear that all feeding experiments of this kind must be judged by the parallel performance of littermate controls.

The diet used had the following composition: casein, purified by repeated extractions with hot 95% alcohol and with cold 60% alcohol, 22; Crisco, 9; Osborne and Mendel salt mixture, 4; carbohydrate, 65. In some of the lactose diets a lower proportion of casein was used, 18 or 15%, but no differences in the relative severity of the vitamin deficiencies resulted. The lactose was exposed to strong daylight in thin layers for 7 or 8 days before it was incorporated in the diet and exhibited no fluorescence under 'black light.'

There was little coprophagy among the rats fed these diets. Wide-mesh floor screens on individual cages were used and careful observation made to detect any feces consumption. A small number of suspected animals was removed from the groups.

In most cases the rats, taken at 21 to 28 days of age, were depleted by use of the B<sub>2</sub>-free diet for 3 or 4 weeks at which time the weights were found to be stationary or declining except in the case of the lactose groups.

A single level of dosage was chosen for each of the three supplements and only one source of each supplement. Pure crystalline lactoflavin furnished by Dr. S. Lepkovsky and by Vitab Products, Inc. of San Francisco was used at the level of 20 micrograms daily. A series of assays of this material established its high potency, comparable with that reported by Copping ('36), by Ansbacher, Supplee and Bender ('36), and of course, by Lepkovsky and Jukes ('36).

The B<sub>6</sub> preparation was made from autolyzed wheat germ according to the suggestion of Birch and György ('36) and was fed in daily amount equivalent to 0.5 gm. of wheat germ. This extract was tested by Dr. T. H. Jukes on chicks and found to be free from the filtrate factor. Rat assay has confirmed this. The filtrate factor was a rice bran concentrate furnished by the kindness of Vitab Products, Inc. This product was practically free from flavin but contained appreciable amounts of B<sub>6</sub>. In all the tests involving the filtrate factor therefore the presence of a small amount of B<sub>6</sub> must be postulated. The standard dose used was 0.5 cc. of the concentrate per rat per day.

The feeding experiments were carried on for varying periods from 12 to 36 weeks, but usually for 14 weeks. Growth, blood and urine sugar, blood and urine calcium, eye lens changes, skin and hair changes were recorded.

#### THE EFFECT OF LACTOSE

The outstanding findings in the groups on the lactose diet were the complete absence of dermatitis, frequent appearance of cataracts, and subnormal but persistent growth even on the entirely B<sub>2</sub>-deficient diet (Morgan and Cook, '36). Addition of either flavin or B<sub>6</sub> or both had little or no effect in improving the growth of these animals. When the filtrate factor was given however either alone or with the other two

factors, a large increase in growth rate resulted and a definite decrease in severity and speed of appearance of the cataracts. With filtrate factor alone added (it being understood that  $B_1$  is present in all cases) the growth achieved as seen in figure 1 is the same as that with 0.5 gm. brewers' yeast (Northwestern) daily. When the flavin and  $B_6$  were also given, the growth is

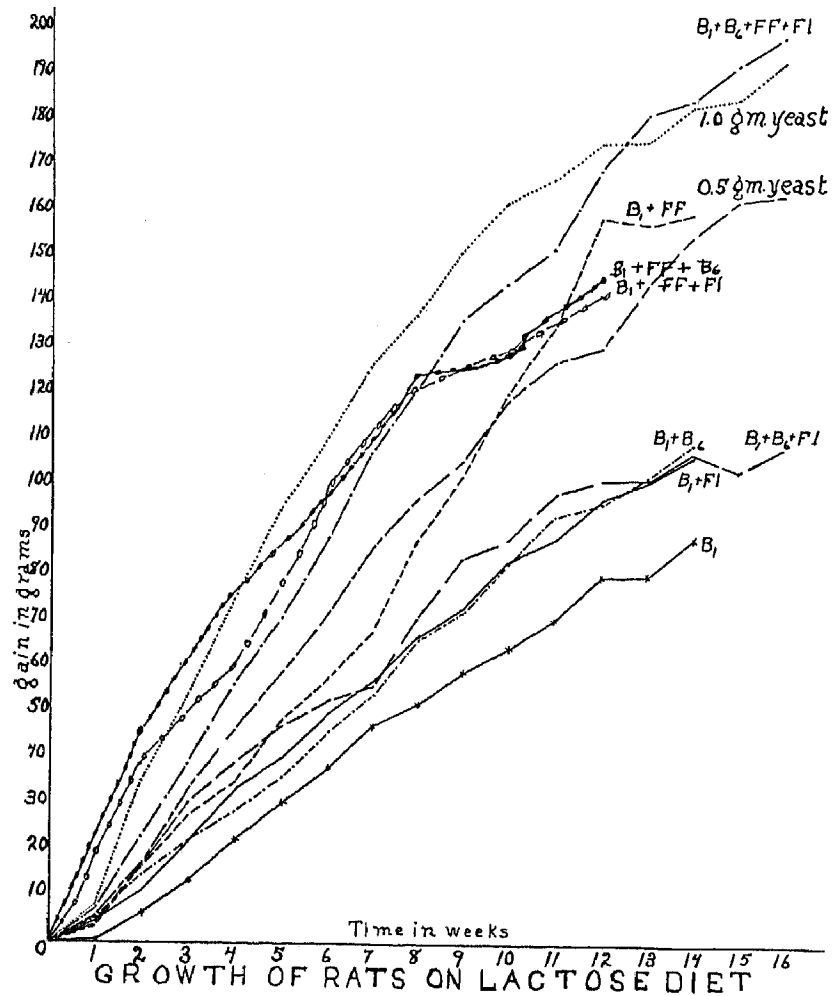


Fig. 1 Growth of young rats on lactose diet with one two, three or all four of the B vitamins:  $B_1$ , flavin (FI), filtrate factors (FF), vitamin  $B_6$  ( $B_6$ ).

seen to be equal to that promoted by 1.0 gm. daily of the yeast. Apparently 0.5 gm. yeast supplies insufficient amounts of one of the B<sub>2</sub> accessories since 1.0 gm. of yeast is needed to equal the performance of the group given the four factors separately.

All of the lactose-fed rats had much enlarged ceca filled with yellowish white semi-solid contents, apparently mostly unabsorbed lactose. Early in the feeding period some showed diarrhea and a few died during these attacks. Usually after a week or two the diarrhea ceased and the animals appeared to be normal in all respects except for the cataracts and a sub-normal rate of growth.

In an earlier experiment groups of rats were fed a similar diet but with 50% lactose and 20% cornstarch instead of 70% lactose and with tiki-tiki as source of all the B vitamins. These had less mature and more delayed cataracts but considerably improved growth when compared with those on the 70% lactose diet. When the proportion of lactose was further reduced to 30%, the cataracts were fewer and less severe but growth was much decreased. These relations are shown in figure 2. Apparently the tiki-tiki was supplemented successfully by the vitamin-forming activity of the lactose at the 50% level, insufficiently at the 30% level and at the 70% level the toxic effect of the lactose became more pronounced than its favorable effect. With cornstarch alone the tiki-tiki allowed very little growth, no cataracts developed but severe dermatitis or hair loss usually occurred. It is probable that the tiki-tiki was lacking chiefly in flavin.

Since B<sub>6</sub> or flavin or both along with the 70% lactose diet brought about little improvement, it would appear that either the lactose contained considerable amounts of these two factors or that they were formed possibly by bacterial activity in the intestine from which adequate quantities were absorbed. Since the addition of the filtrate factor brought about obvious improvement, it must be assumed that the lactose carries none of this and that none is elaborated in the intestine.

In order to determine whether the intestinal contents contain the B<sub>6</sub> and flavin the contents of the ceca of sixty-five lactose-fed rats were washed out with 60% alcohol, filtered, the filtrate concentrated and fed to six rats depleted on the cornstarch diet. These animals resumed growth at a rate

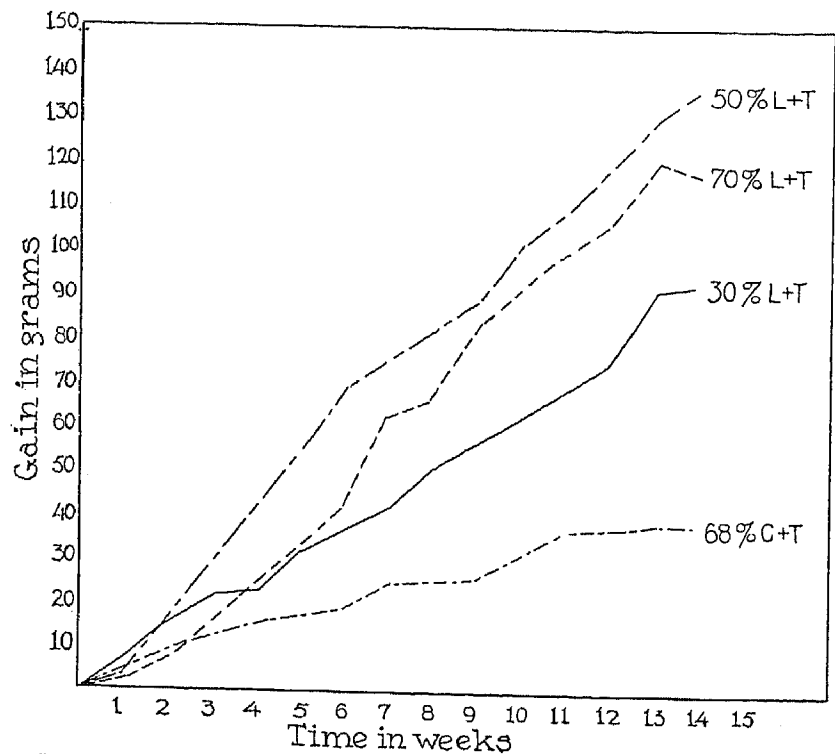


Fig. 2 Growth of young rats on diets containing 30, 50 and 70% lactose (and 40, 20 or 0% cornstarch) and 68% cornstarch with tiki-tiki (rice bran concentrate) as sole source of the B vitamins.

closely paralleling that of littermates on the lactose diet without supplements. The equivalent of the contents of one caecum per rat promoted growth for 3 or 4 days. When the caecum material was no longer given, the growth of these rats continued for 1 week then leveled off as in the negative cornstarch group.<sup>1</sup>

<sup>1</sup> These observations were made by Dorothy V. Rundle.

THE EFFECT OF CORNSTARCH

The rats on the cornstarch diet grew very little without supplements, sometimes developed dermatitis but had no cataracts. See growth curves in figure 3. Their growth was improved by flavin but the dermatitis became more severe and

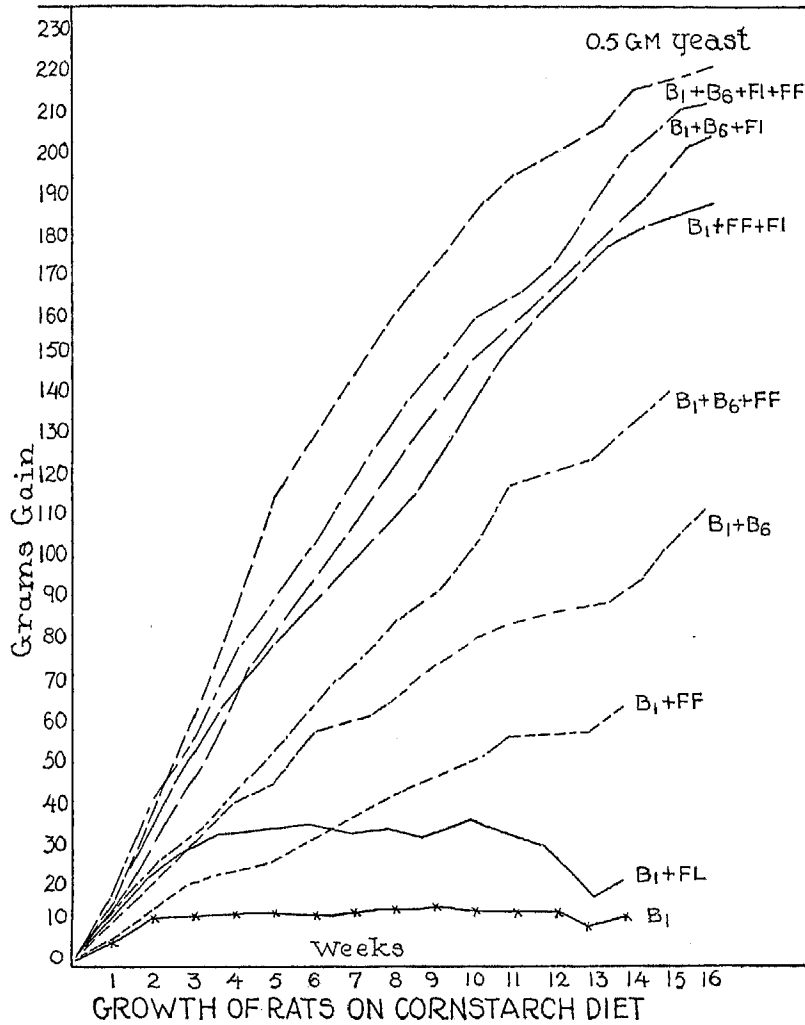


Fig. 3 Growth of young rats on cornstarch diet with one, two, three or all four of the B vitamins: B<sub>1</sub>, flavin (Fl), filtrate factors (FF), vitamin B<sub>6</sub> (B<sub>6</sub>).

was of the usual 'specific' type with swollen red paws from which the skin was sometimes sloughed off, scabby denuded nose and forelegs and swollen encrusted ears. When  $B_6$  was also given, the dermatitis did not appear and close to normal growth resulted. Addition of the filtrate factor merely increased the growth rate somewhat but alone produced little improvement.

The filtrate factor preparation which was used was assayed<sup>2</sup> for  $B_6$  and flavin by being fed at two levels with flavin to one group of rats and with  $B_6$  to another. No improvement was found in the latter by doubling or quadrupling the quantity of filtrate factor, which was taken to mean that the latter contained little if any flavin. Definite improvement in growth, sufficient to make these groups equal in performance to those receiving the standard dose of  $B_6$  supplement, resulted however when the filtrate factor was doubled and quadrupled for the group given flavin but no  $B_6$ . Apparently the filtrate factor in the quantity fed, 0.5 cc. per day, contained one-half the amount of  $B_6$  in the daily dose of wheat germ preparation used as source of  $B_6$ , that is one-half of the equivalent of 0.5 gm. wheat germ. It is not surprising therefore that when filtrate factor and flavin were given, fairly good growth resulted on all diets.

We were puzzled by the good growth resulting from the addition of flavin and  $B_6$  to the cornstarch diet and by the relative ineffectiveness of additions of filtrate factor either alone or with the other supplements, as shown by the growth curves in figure 3. The supposition must be made either that the cornstarch carries appreciable amounts of filtrate factor or that it promotes intestinal formation of this factor. Since the  $B_6$  preparation appeared to be free from filtrate factor by chick assay and by its effect with the sucrose and lactose diets, no other source of the filtrate factor than the starch can be found.

Hogan and Richardson ('34) have reported cure of florid dermatitis in rats fed a sucrose diet by concentrated hot

<sup>2</sup> These assays were made by Dorothy V. Rundle.

alcohol extracts of cornstarch, the condition cured being possibly due at least in part to filtrate factor deficiency. Certainly the factor which might be expected to adhere to cornstarch is not the filtrate factor which has begun to be identified through clinical cures of pellagra and blacktongue with the P-P of Goldberger. Several recent reports moreover, emphasize the relative poverty of cereals in general and maize in particular in all these factors except B<sub>6</sub>.

Many of the studies with rats in which pellagra-like conditions were produced have been carried on with basal diets containing large amounts of cornstarch, rice starch or cereal mixtures. The work of Birch, György and Harris ('35) which led to their conclusion that rats do not need or can synthesize the third factor, that is additional to flavin and B<sub>6</sub>, was done with a diet containing 68% cornstarch. If the filtrate factor is not actually carried by the starch usually employed, it may be that the bacterial flora favored by the presence of the starch in the rat's intestine synthesize it.

The obvious inconsistency of the presence of the filtrate factor in cornstarch along with the role of corn (maize) as a prominent part of pellagra-producing diets raises again the question of the specificity of the filtrate factor as the pellagra-preventing substance or at least of the singleness of character of this accessory or accessories.

#### THE EFFECT OF SUCROSE

The rats fed the sucrose diets supplemented with B<sub>1</sub> only developed dermatitis regularly if they survived long enough, grew not at all and usually died in a few weeks. Some growth resulted when any one of the three factors was given, the filtrate factor probably because of its B<sub>6</sub> contamination being most effective (fig. 4). When any two of the factors were given together, improvement over that seen on any one alone resulted, and when all three were given, normal animals were produced nearly as large as those given 0.5 gm. yeast daily. The improved growth obtained by the addition of each factor is in almost mathematical relation to the growths obtained

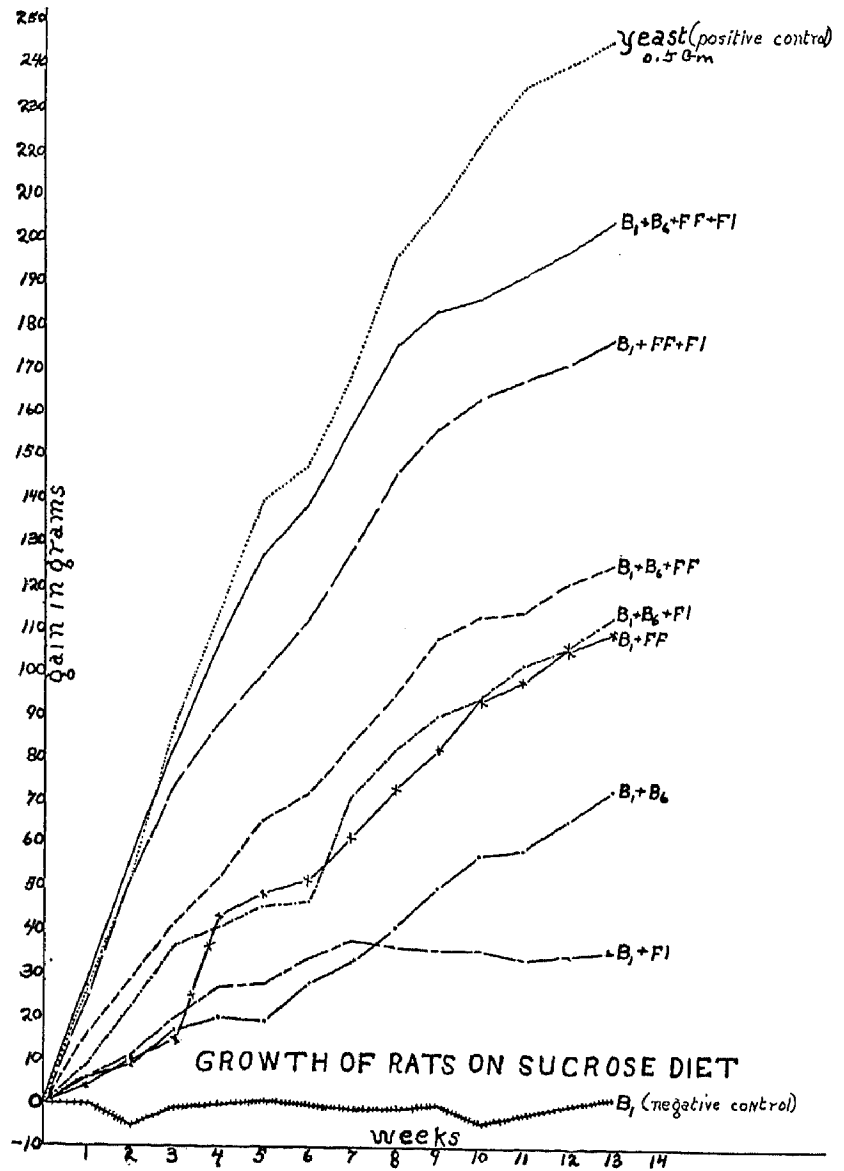


Fig. 4 Growth of young rats on sucrose diet with one, two, three or all four of the B vitamins: B<sub>1</sub>, flavin (FI), filtrate factors (FF), vitamin B<sub>6</sub> (B<sub>6</sub>).

when one or two are progressively missing. Thus in 14 weeks flavin alone promoted an average gain of 35 gm., B<sub>6</sub> alone, a gain of 72 gm., filtrate factor alone, a gain of 108 gm. In the latter case it must be remembered that about one-half the standard dose of B<sub>6</sub> was present in the filtrate factor supplement. When flavin and B<sub>6</sub> were given together, the gain was 112 gm., almost an exact addition of the separate gains. When flavin and filtrate factor were given together, the gain was 177 gm., close to the normal obtained with flavin, B<sub>6</sub> and filtrate factor, 204 gm. When B<sub>6</sub> and filtrate factor were given together, the gain was 125 gm., 73 of this ascribable to the B<sub>6</sub>. Apparently the sucrose diet is completely free from all of these factors and also produces none of them in the intestine. It would seem therefore to be the carbohydrate of choice for basal diets for rats in the study of the vitamin B<sub>2</sub> complex. This conclusion was reached also by Halliday and Evans ('37) who found the depletion period shorter and deficiency symptoms more severe with sucrose instead of cornstarch diets.

The studies of Guerrant, Dutcher and Tomey ('35) with diets containing various carbohydrates and deficient in all the B vitamins have indicated the synthetic activities of the flora promoted by dextrin and to a slighter extent, lactose. The overwhelming effect of vitamin B<sub>1</sub> deficiency apparently obscured the effect upon the B<sub>2</sub> factors in this case. The recent work of Bender, Ansbacher, Flanigan and Supplee ('36) has differentiated the effect of dextrin however since B<sub>1</sub> and lactoflavin were supplied. No dermatitis occurred on the dextrin ration but with a similar sucrose diet dermatitis was quite severe. The addition of rice polish concentrate cured the dermatitis in the latter animals and produced practically normal growth. But a flavin-free milk concentrate had no such effect. Whether the addition of the milk concentrate improved the growth of the dextrin-fed rats is not evident and there is no statement as to the use of the rice polish concentrate with the dextrin ration. It is impossible to judge therefore whether the dextrin provides both B<sub>6</sub> and filtrate factor in adequate amounts. Obviously it does not provide

flavin as does the lactose. There is apparently some difference between the intestinal activities or the original contaminations of these two carbohydrates. It seems clear however from the absence of dermatitis in both lactose and dextrin-fed rats that B<sub>6</sub> is produced in both cases.

#### BLOOD AND URINE ANALYSES

As determined by the method of Shaffer and Somogyi ('33) the blood sugar of the rats on the sucrose diet was within the usual normal range, 97 to 121 mg. per 100 cc., in all cases both with and without deficiencies. The same was true of those on the cornstarch diet in which the range was 108 to 125 mg. per 100 cc. Those on the lactose diet however had blood reducing sugar values of 158 to 241 mg. per 100 cc. Such high blood sugar values are in agreement with the findings of Day ('36) and of Mitchell, Merriam and Cook ('37). No attempt was made to differentiate glucose from galactose in the blood of the lactose-fed rats. A few animals on lactose plus vitamin B<sub>1</sub> were given small frequent doses of insulin zinc for 3 or 4 weeks in order to determine whether the cataracts produced by such a diet might be thus averted. No change in severity or early appearance of the cataracts resulted but the reducing sugar in the blood of these animals was found to be lowered to the same extent as in cornstarch-fed animals. This observation is contrary to the report of Mitchell, Merriam and Cook ('37), who found blood galactose, assuming that most of the hyperglycemia was due to galactose, unaffected by insulin.

Urine sugars as determined in samples of undiluted rat urine collected with special precautions to avoid contamination were found to be lowest on the starch diet, 44 to 117 mg. per 100 cc. of urine, intermediate on the sucrose diet, 159 to 480 mg. per 100 cc. of urine and highest on the lactose diet, 254 to 956 mg. per 100 cc. of urine. With sucrose the larger urine sugar output was seen on the deficient diets.

Urine calcium was determined also on the undiluted urine and was found to be largest on lactose diet, 59 to 171 mg.

per 100 cc. of urine. On the starch and sucrose diets the range of excretion was the same, 3 to 27 mg. per 100 cc. of urine. This effect of the lactose is perhaps only the result of the increased calcium absorption first reported by Bergeim ('26) as resulting from the presence of lactose in the intestine.

#### THE GRAYING OF RAT HAIR

An interesting symptom which developed in many of the filtrate factor-deficient animals was the graying of hair in regular patterns. This was most evident in the black rats but could be seen in gray and hooded animals as well. In very few cases was the graying evident in those which received only vitamin B<sub>1</sub>. The rats which grew well and were relatively free from dermatitis or alopecia in nearly all cases became gray. These were chiefly the rats on sucrose diet which received no filtrate factor. Those on cornstarch in many cases grayed also but only after a prolonged period. The phenomenon was first observed in animals given the starch diet and small amounts of wheat germ, and all of these were cured of the depigmentation by the filtrate factor preparation from rice bran. In all cases when B<sub>6</sub> was present in the diet as well as flavin the graying was most severe.

Three black rats of the same litter fed the sucrose diet are shown in figure 5. Rat no. 10 was given all three factors throughout the experiment which continued for 24 weeks. This animal was normal in all respects at all times and continued to have a glossy black coat. Rat no. 11 was given the B<sub>6</sub> preparation only for the first 12 weeks during which time its fur became streaked with gray. The filtrate factor preparation was then added in standard amount to the diet of no. 11. After 8 weeks the fur of this animal had again become glossy and black. Rat no. 12 was given B<sub>6</sub> and flavin only for these 20 weeks with increasing graying of the fur, bloody ears and nose and emaciation. During the last 8 weeks 0.17 mg. of copper as copper sulfate was given rat no. 12 daily. The photograph shown in figure 5 was taken after the experiment had progressed for 20 weeks. Obviously the two rats

given the filtrate factor have normal fur while the rat lacking the filtrate factor is in poor condition with completely depigmented fur. The copper had no curative effect on the depigmentation. Gorter ('35) has recently claimed that the depigmentation is due to lack of copper and that it may be prevented or cured by the administration of minute amounts of copper

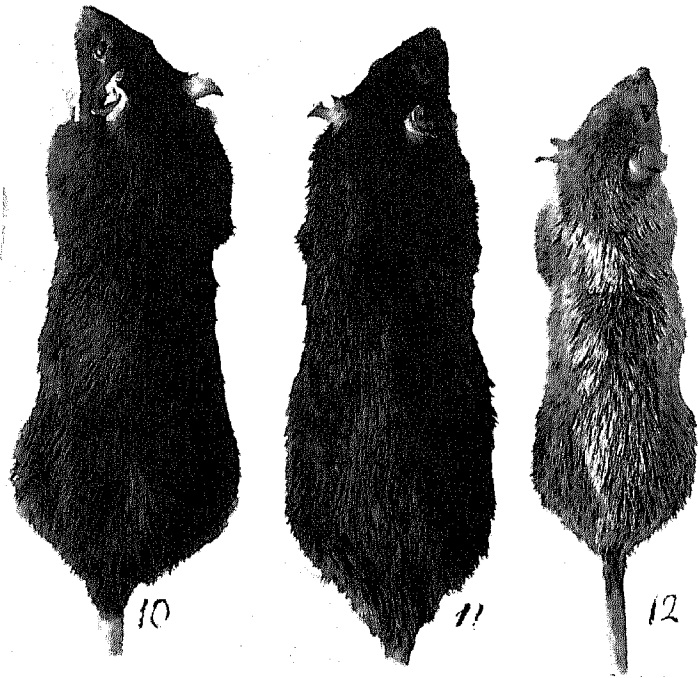


Fig. 5 Littermate black rats on the sucrose diet. No. 10 was given all of the  $B_2$  vitamins throughout the experiment which continued for 24 weeks. No. 11 was given  $B_2$  only for 12 weeks, then filtrate factor. No. 12 was given  $B_2$  and flavin (no filtrate factor) but was given additional Cu latter part of experiment.

salts. The specificity of copper may be questioned in these cases since all rats in this laboratory obtain their water from bottles equipped with pure copper drinking tips. After the photograph shown in figure 5 was taken, rat no. 12 was given the filtrate factor in double the standard daily dose for 4 weeks at which time its fur had become black again, it had increased in size and had become normal in all respects.

Another group of some forty young black rats have been placed on the sucrose diet with vitamins B<sub>1</sub> and B<sub>6</sub> and flavin but without filtrate factor. Nearly all of these animals have shown marked graying of the fur within 8 to 12 weeks and rapid resumption of the normal color when filtrate factor preparations made from rice bran and from liver were fed. Control animals given the filtrate factor from the beginning have retained normal fur color. We conclude that the filtrate factor or factors prevents and cures graying of rat hair.

#### SUMMARY

When lactose in sufficient amount is incorporated in the basal diet, of the three necessary factors now recognized in the vitamin B<sub>2</sub> complex, both flavin and vitamin B<sub>6</sub> need not be given separately and are apparently elaborated in the intestine of the animals. The filtrate factor however must be given in order to obtain normal growth. In no case did dermatitis develop in lactose-fed rats even when the proportion of lactose was lowered to 30%, but a large proportion of the rats developed cataracts. The severity of the cataract occurrence was mitigated when all the vitamin B factors were supplied, and this prophylactic effect seemed to inhere in the filtrate factor particularly.

When cornstarch is used as the carbohydrate of the basal diet, dermatitis and relative failure of growth result unless B<sub>6</sub> and flavin are supplied. The filtrate factor appears to be present in limited amount in the starch or to be produced in the intestine when cornstarch is used. No cataracts occurred on any of the starch or sucrose diets.

When sucrose is used as the carbohydrate of the basal diet, earlier and more severe dermatitis and growth failure occur unless all three of the factors are supplied. Better growth response was obtained with sucrose than with starch when one or more of the factors was supplied and better response with starch than with lactose.

Graying of the hair of black rats was seen on most of the diets which lacked the filtrate factor and this was prevented or

cured by administration of the rice bran concentrate which was used as source of the filtrate factor.

Blood and urine reducing sugar of the rats on lactose were much increased over that of the starch-fed group. Urine calcium was also much increased in this group. The sucrose diets produced normal blood sugar values but when one or more of the B<sub>2</sub> factors was lacking, high urine sugars. Some clue to the mode of action of these factors may be found in this behavior.

#### CONCLUSIONS

Lactose favors the production in the intestine of rats, probably by microorganisms, of both flavin and vitamin B<sub>6</sub> but not the filtrate factor.

Cornstarch either carries with it or favors the production of the filtrate factor only.

Sucrose neither carries nor produces any of the vitamin B<sub>2</sub> factors and is therefore the carbohydrate of choice for the study of these factors.

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