ESSENTIAL FATTY ACIDS, VITAMIN B_6, AND OTHER FACTORS IN THE CURE OF RAT ACRODYNIA*

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Dermal abnormalities in the rat have been recognized as having a dual origin; *viz.*, from an "essential" fatty acid deficiency (Burr and Burr, 1929), or a vitamin B_6 deficiency (György, 1935). That some relationship exists between these two deficiencies was suggested by the finding of Birch and György (1936) that certain fats cured the dermatitis as produced by vitamin B_6 deficiency. The point was emphasized when Quackenbush, Platz, and Steenbock (1939) showed that linoleic acid, curative in "essential fatty acid deficiency," was also curative for acrodynia. Birch (1938) examined the relation of vitamin B_6 and the fatty acids of corn oil in the cure of acrodynia and concluded that at least two factors were operative, vitamin B_6 and a second factor present in corn fatty acids. This was based on the observation that a vitamin B_6 preparation failed to cure rat acrodynia unless the fatty acids from corn oil were fed as well. However, Quackenbush, Platz, and Steenbock (1939) had shown that the fatty acids from corn oil cured acrodynia when fed alone and hence apparently no additional vitamin B_6 was needed. This suggested that either (1) fatty acid preparations from corn oil contained vitamin B_6 in addition to the second factor postulated by Birch or (2) corn oil fatty acids were capable of supplanting vitamin B_6 plus any other factor necessary in the cure of acrodynia. It was the purpose of this investigation to examine these possibilities and to study further

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the relationship between acrodynia and the Burr and Burr syndrome.

EXPERIMENTAL

Acrodynia was produced on a fat-free, vitamin B_6-deficient diet as follows: Weanling rats, 35 to 40 gm., as prepared by the technique of Quackenbush, Platz, and Steenbock (1939) were placed on Diet V. This diet consisted of a mixture of cerelose (commercial glucose) 78, casein (alcohol-extracted) 18, Wesson's salts (1932) 4, supplemented daily with 10 micrograms of thiamine and 20 micrograms of riboflavin dissolved in 1 drop of 0.02 N acetic acid, and 10 micrograms of carotene and 5 micrograms of calciferol dissolved in 1 drop of liquid hydrogenated coconut oil. On this diet the rats developed an acute acrodynia in 4 to 5 weeks. Materials were tested for antiacrodynic potency by feeding them as supplements to Diet V while the animal was continued on the diet. If a cure resulted in 3 weeks of such supplementation and was maintained for an additional 3 weeks, the material was regarded as potent. The reliability of this curative test for antiacrodynic potency has been demonstrated with more than 500 rats. A material once shown to be potent was consistently confirmed in its potency when subjected to retest. Spontaneous cures were never observed.

For the study of the antiacrodynic potency of fats two oils were selected as examples; *viz.*, corn oil (Mazola) and cottonseed oil (Wesson oil). These oils were curative at levels of approximately 5 and 10 mg. per day. If this curative action was dependent upon a content of vitamin B_6, then the removal of the vitamin, if it existed in the fat, should have resulted in a loss of activity of the fat. Although at this point in our investigation the basic, water-soluble properties of vitamin B_6 did not strengthen the view that fats contained the vitamin, Kuhn and Wendt (1938) demonstrated that vitamin B_6 contained two alcoholic and one phenolic group which made ester combinations at least a possibility. Although Keresztesy and Stevens (1938) showed that vitamin B_6 was unaffected by alkali and hence could presumably survive saponification, it was difficult to understand how the vitamin could appear in fatty acid preparations such as were fed by Birch. These doubts were confirmed by an extended series of

experiments which will not be detailed here. Suffice it to say that when corn or cottonseed oil was used as a source of active fat, continuous ether extraction of fatty acid soaps, continuous extraction of fat with dilute acid, refluxing with dilute acid, electrodialysis of the saponification mixture of an active fat, and the use of adsorbing agents in ethereal solutions of fat, all failed to diminish the antiacrodynic potency of the fatty acids. That vitamin B_6 was not part of the antiacrodynic potency of fats seemed certain after the following experiments were performed.

Kuhn and Wendt (1938) had shown that a methyl ether of vitamin B_6 was formed by treatment with diazomethane. According to Möller (1938) the methyl ether of vitamin B_6 is biologically inactive. This suggested the possibility of preparing methyl esters of antiacrodynic fatty acids which would be free of active vitamin B_6 by treating the crude fatty acids with diazomethane in ether solution. If, for some obscure reason, vitamin B_6 had been brought into the solution of the fatty acids, treatment with diazomethane would have resulted in the formation of the methyl ether. Then, if the potency of the fatty acids was indeed dependent upon a content of vitamin B_6, there would be a resultant loss in antiacrodynic activity. However, esters of corn and cottonseed oil fatty acids (fed to four and two rats respectively), as well as the ester of linoleic acid (fed to two rats), prepared by the action of diazomethane on the free acids were all curative within 3 weeks when 20 mg. of the preparation were administered daily.

That the antiacrodynic activity of fatty acids was not due to vitamin B_6 *per se* was further confirmed by the demonstration that the antiacrodynic activity of fatty acids from corn oil was retained after three successive precipitations with barium in alkaline solution. 25 gm. of corn oil were saponified with alcoholic KOH and the soaps poured into a saturated solution of $Ba(OH)_2$. The barium soaps were filtered off, and the acids regenerated with HCl and extracted with ether. The ether was removed under reduced pressure and the potassium and barium soaps formed again as before. This was repeated again and the fatty acids were finally esterified with ethyl alcohol by refluxing with sulfuric acid. The esters were active in curing acrodynia in two rats given 20 mg. daily. Cure was effected within 3 weeks. Similar

precipitation of the acids of cottonseed oil and of linoleic acid resulted in active ester preparations which when administered to three and two rats respectively at the rate of 20 mg. daily cured acrodynia within 3 weeks. As vitamin B_6 itself does not form an insoluble barium salt, the conclusion seemed inescapable that vitamin B_6 was in no way involved in the cure of acrodynia when it was effected by certain fats.

The curative properties of the fatty acids of corn and cottonseed oil were thus again referred to the fatty acid fraction of which linoleic acid had been demonstrated as a potent member. Since linoleic acid, as well as arachidonic (Turpeinen, 1938) and linolenic acids, is prepared from active oils, the possibility has been existent that their activity was dependent upon the inclusion of the true active material. Synthesis of these materials would eliminate these objections but as yet no practicable syntheses have been devised. It was thought desirable, therefore, to examine an active oil, corn oil, for constituents which might form a part of the fatty acid fraction as ordinarily prepared and have antiacrodynic activity, and yet not be an unsaturated fatty acid of the linoleic, linolenic, or arachidonic acid series. Two such types of compounds were investigated; *viz.*, phenols and certain lactones. The phenolic compounds in corn oil (Mazola) were obtained as follows: 1 kilo of corn oil was saponified, the unsaponifiable compounds were removed by ether extraction, and the potassium soaps poured into an excess of $Ba(OH)_2$ solution. The Ba soaps were filtered off and the Ba removed from the filtrate by H_2SO_4. The acidified filtrate was then extracted with ether and the combined ether extracts washed, dried with Na_2SO_4, and the ether removed under reduced pressure. Approximately 400 mg. of residue remained, insoluble in water but soluble in 5 per cent Na_2CO_3. Addition of CO_2 to saturation permitted the ether extraction of materials which gave an intense blue color with Folin's phenol reagent. When the material was fed, it was found devoid of antiacrodynic activity (Table I).

Another group of compounds which might be found in fatty acid fractions from oils is composed of certain lactones which are reformed on acidification of the saponification mixture, such as the coumarins. True fatty acids are separable from such compounds by extraction from lipid solution with aqueous K_2CO_3.

Possible lactones present in corn, cottonseed, and wheat germ oil were prepared for feeding in the following manner. The fat was saponified with alcoholic KOH, diluted, and the non-saponifiable fraction removed by petroleum ether extraction. The solution was then acidified and the fatty acids removed with petroleum ether, washed, and the fatty acids extracted from the petroleum ether solution with successive portions of 5 per cent K_2CO_3. The K_2CO_3 was then washed out with water and the petroleum ether solution was dried with Na_2SO_4. The solvent was removed under reduced pressure. The residue was taken up in liquid hydrogenated coconut oil for feeding. Test (Liebermann-Burchard) showed that the residues contained some sterols which

TABLE I

Antiacrodynic Potency of Crude Phenols and Lactones Present in Antiacrodynic Oils

Preparation	Amount fed daily	Original oil equivalency	Effect on acrodynia (3 wks.) (4 rats)
		mg.	
1. Crude phenols of corn oil	0.4 mg.	1000	Negative
2. " lactones " " "	140 γ	40	"
K_2CO_3-extracted acids from (2)	20 mg.	20	Curative
3. Crude lactones of cottonseed oil	90 γ	40	Negative
K_2CO_3-extracted acids from (3)	20 mg.	20	Curative
4. Crude lactones of wheat germ oil	240 γ	40	Negative
K_2CO_3-extracted acids from (4)	20 mg.	20	Curative

were incompletely removed with the non-saponifiable fraction. Yields of residue per gm. of fat were corn oil 3.5, cottonseed oil 2.3, and wheat germ oil 6.0 mg. When these residues were fed, they proved to be totally inactive, while the fatty acids which had been removed by the K_2CO_3 extraction proved active when fed as the ethyl esters (Table I).

The preceding experiments thus redemonstrated that the antiacrodynic activity of certain vegetable oils was resident in the fatty acid fraction as a fatty acid. These fatty acids had usually been fed as the ethyl esters as prepared by refluxing the acids with anhydrous ethyl alcohol and sulfuric acid. Formation of the methyl esters from the free acids by diazomethane had left

the antiacrodynic activity unimpaired. It was thought that some clue as to the chemical nature of the active fatty acid might be obtained, other than that it was probably unsaturated, by preparing the ethyl or methyl esters of the active fatty acids in other ways. Therefore, methyl esters of fatty acids from corn and cottonseed oils were prepared by formation of the silver soaps and refluxing with methyl iodide. The resulting esters were active (Table II). The ethyl esters of the same acids were also active when prepared from the sodium salt by refluxing with diethyl sulfate (Table II).

Quackenbush, Platz, and Steenbock (1939) showed that elaidinization of linoleic acid resulted in a loss of antiacrodynic potency. It appeared desirable to determine whether another type of molecular alteration, hydrogenation, would destroy the activity of

TABLE II

Esters of Antiacrodynic Fatty Acids As Prepared by Different Esterifying Agents Curative within 3 Weeks

Preparation (20 mg. fed daily)	No. of rats	Iodine value (Hanus)
Methyl esters of corn oil acids by methyl iodide.....	2	117.6
" " " cottonseed oil " " "	2	96.0
Ethyl " " corn oil by diethyl sulfate.....	5	115.0
" " " cottonseed oil by diethyl sulfate.....	5	98.0

linoleic acid. Consequently a previously assayed preparation of ethyl linolate (iodine value = 159) was hydrogenated over platinum. Hydrogen absorption was complete in $\frac{1}{2}$ hour. The ethyl stearate thus formed (iodine value = 0) was tested for antiacrodynic potency on five rats at 4 times the original curative level and failed to cure.

Water-Soluble Factors in Cure of Acrodynia—It seemed fairly well established by the above that the antiacrodynic potency of certain fats was resident in the unsaturated fatty acids of which linoleic acid was representative. To determine under what conditions vitamin B_6 could effect a cure of rat acrodynia was the object of the succeeding experiments.

Birch (1938) failed to cure rat acrodynia on a fat-free diet when

he fed a vitamin B_6 preparation obtained by alcoholic extraction of yeast. However, in experiments similar to those of Birch, we have been able to cure rat acrodynia with another source of water-soluble B vitamins. When rats exhibiting acrodynia as produced in this laboratory on Diet V were fed 100 mg. of a rice bran concentrate (vitab[1]) per day, the acrodynia was cured in

TABLE III

Antiacrodynic Potency of Water-Soluble Factors of Rice Bran Concentrate (Vitab)

	Preparation	Vitab equivalent fed daily	No. of rats	Effect on acrodynia (3 wks.)
		mg.		
1.	Rice bran concentrate	100	12	Curative
		50	2	Negative
1,*a*.	" " " (ether-extracted)	100–150	4	Curative
2.	Fullers' earth filtrate from (1)	150	11	Negative
		200	5	"
3.	" " eluate from (1)	600	9	Temporary improvement; relapsed
4.	Combined filtrate and eluate		4	Curative
		γ		
5.	Crystalline vitamin B_6	10	12	Temporary improvement; relapsed
		15	2	" "
		25	1	" "
		50	1	" "
		mg.		
6.	Filtrate factor (2) fed with 10 γ vitamin B_6 to rats having relapsed on 10 γ vitamin B_6	150	4	Curative

3 weeks (Table III). The vitab was completely water-soluble and when diluted formed a clear solution. A diluted sample of vitab was extracted by three shakings with ether (pH = 4.85)

[1] Vitab, Type II, is a commercial B vitamin concentrate prepared from rice bran. It was supplied by Vitab Products, Inc., Emeryville, California.

and concentrated *in vacuo* to its original volume without any loss in potency (Table III).

Since a similarity between "essential fatty acid" deficiency and acrodynia had already been indicated by the curative effects of fats and linoleic acid in both deficiencies, it appeared desirable to determine whether this similarity could be extended to include curative effects of the two deficiencies by the same water-soluble concentrate, ether-extracted vitab. Since in the production of the Burr and Burr syndrome the B vitamins have been fed in a limited amount of yeast (0.7 gm. daily) in comparison with the acrodynia-producing Diet V which supplies merely thiamine and riboflavin, it was possible to study the effect of yeast vitamins on the production of dermal symptoms by progressively increasing the amounts of dried yeast. Accordingly sixteen weanling rats, prepared as usual, were divided into four groups of four. Diet V was modified to include 1, 2, 4, and 8 per cent yeast at the expense of the glucose and was fed to Groups I, II, III, and IV respectively. Carotene, calciferol, thiamine, and riboflavin were fed in addition as usual. Each group was continued on its respective diet until growth stopped and the animals remained stationary or lost weight for a continuous period of 3 weeks. Weight records were kept and the appearance of symptoms noted. When the growth curves had remained at a plateau for 3 weeks, two animals of each group were fed 22 mg. of vacuum-distilled ethyl linolate prepared via the tetrabromides from corn oil by the method of Rollet (1909). The remaining two rats in each group were then fed 100 mg. daily of ether-extracted vitab. The supplementation was continued for a period of 6 weeks during which increase in weight was noted as well as the disappearance of dermal symptoms.

The growth of the animals on the various yeast levels is indicated in Fig. 1. The cessation of growth of the rats receiving 8 per cent yeast at approximately 175 gm. of body weight is similar to that observed by Turpeinen (1938) in a recent study of the essential fatty acids. Like Turpeinen we observed only mild dermal symptoms in the animals receiving these large amounts of yeast. Scaliness of the paws and tail was the chief symptom. At successively lower levels of yeast, however, this mild scaliness was observed to pass into more severe types until at 1 per cent

yeast the dermatitis was so severe, involving the paws, mouth, eyes, and ears, as to be indistinguishable from the acute acrodynia

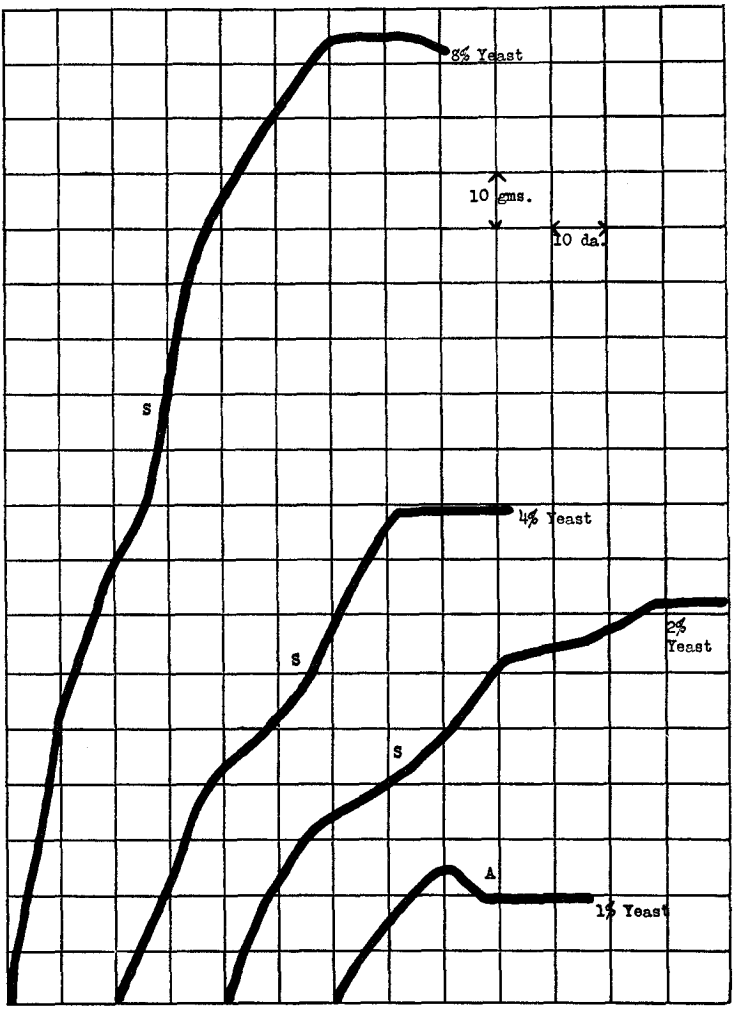

FIG. 1. Growth of rats on low fat diet (Diet V) containing varied amounts of dried brewers' yeast. S = scaliness; A = acrodynia.

produced on Diet V. These results parallel those obtained by Birch (1938) who fed graded amounts of an alcoholic extract of

yeast. The action of yeast vitamins in the production of the Burr and Burr syndrome seems to be one of modification of the acute acrodynia into the typical scaliness.

The connection between acrodynia and the Burr and Burr syndrome was further emphasized by the parallel curative effects of both ethyl linolate and ether-extracted vitab. In the 6 weeks of feeding of these materials all dermatitis symptoms disappeared within the first 3 weeks, and in some cases even earlier (Fig. 2).

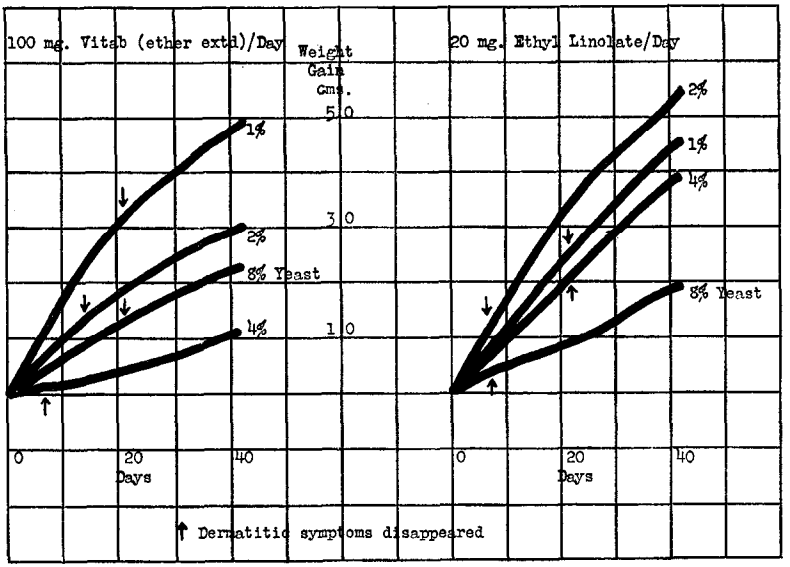

FIG. 2. Growth response to ethyl linolate and ether-extracted vitab of rats that reached a plateau on a low fat diet (Diet V) containing varied amounts of dried brewers' yeast. Each curve represents two rats.

Ethyl linolate and vitab were equally effective in restoring growth, the increments achieved on the 8 per cent yeast level being comparable to the growth obtained by Turpeinen and used by him as a criterion of "essential" fatty acid activity. Increments of growth on the lower levels of yeast were even greater.

With the demonstration that the Burr and Burr syndrome and acrodynia could both be cured by the same agents, it became possible to confine the study of the antidermatitis activity of vitab

to curative studies in acrodynia. Results could thus be obtained in a shorter time and the study much facilitated. It soon became apparent that the antidermatitis potency of vitab was in part dependent upon its vitamin B_6 content. Thus when vitab was diluted with water and shaken with five successive portions of English fullers' earth (pH 4.8), the filtrate had lost its ability to cure acrodynia (Table III). The eluate of the fullers' earth, as prepared by $Ba(OH)_2$ elution, removal of Ba with H_2SO_4, and concentration, was inactive as well. However, when the filtrate and eluate were fed together, antiacrodynic potency was observed once more (Table III). The failure of the fullers' earth eluate (vitamin B_6) to cure acrodynia on a fat-free diet thus confirmed Birch's finding. However, the curative action of vitamin B_6 became possible by the addition of a "filtrate factor." Thus the second factor of Birch is found in a water-soluble form. In fact this second factor which is necessary for the cure of acrodynia has not been shown to exist in fats at all, since originally the presence of this factor in fats was based by Birch on the ability to cure when vitamin B_6 alone had failed. This curative property of fat we have shown to be independent of vitamin B_6 and capable of curing completely in its absence. Thus while Birch's contention that a factor in addition to vitamin B_6 is needed for the cure of acrodynia is confirmed, this second factor is shown to be part of the so called filtrate factor. The importance of filtrate factor in the cure of acrodynia was indicated by György (1938) when he determined the effect of the crystalline vitamin B_6 in the cure of acrodynia. He reported that, "Even the skin effect was not regularly attained unless a further supplement corresponding to the so-called 'filtrate factor' was added."

That vitamin B_6 was indeed incapable of curing acrodynia in the absence of filtrate factor was confirmed when the crystalline vitamin[2] was fed. Although Dimick and Schreffler (1939) had shown that in the presence of a filtrate factor (likewise prepared from a rice bran concentrate) 10 micrograms of vitamin B_6 fulfilled the daily requirement of the rat for this vitamin, when 10 to 50 micrograms of the crystalline vitamin were fed to rats ex-

[2] Crystalline vitamin B_6 as isolated from rice bran concentrate was kindly furnished by Dr. S. Lepkovsky.

hibiting acrodynia on Diet V a temporary cure resulted seldom lasting longer than 2 weeks. It was followed by a relapse into a florid dermatitis as acute as that originally observed and the animals declined and died without ever showing subsequent improvement during the remaining period of vitamin B_6 feeding (Table III).

A few studies have been made of the chemical nature of the factor in the fullers' earth filtrate which is necessary in conjunction with vitamin B_6 for a cure of acrodynia. Continuous extraction of diluted vitab (pH 4.8) with ether in a Kutscher-Steudel apparatus for 48 hours did not diminish the antiacrodynic potency, nor did continuous extraction with ether diminish its potency after it had first been made alkaline with NaOH (pH 8.5). $Ba(OH)_2$ was added to vitab to alkalinity (phenolphthalein) and allowed to stand overnight. Precipitated material was removed by filtration and the barium removed from the filtrate by H_2SO_4. Concentration to the original volume resulted in a preparation which had the complete antiacrodynic potency of the original vitab. The second factor is thus not precipitable by barium.

Since the essential fatty acids are capable of curing rat acrodynia unaided, it might be assumed that the true antiacrodynic substance must be closely related to these acids, possibly as a metabolic product. Since the cyclic, N-containing vitamin B_6 seems far removed from such a type of compound, it would appear that possibly it is the "accessory factor" which is more directly concerned with the cure of acrodynia. Vitamin B_6 would thus be concerned with the conversion of the inactive "accessory factor" into the active, true antiacrodynic substance.

We desire to express our appreciation of the assistance of Mr. Fred A. Kummerow in making some of the preparations.

SUMMARY

Acrodynia, as it is known in the rat, can be cured by two different means.

1. It can be cured by the so called "essential fatty acids." This action is independent of vitamin B_6, since "essential fatty acid" preparations have been shown not to contain any vitamin B_6 and to retain their activity after treatment with diazomethane.

2. It can be cured by rice bran concentrate. This action is independent of fatty acids, but is dependent upon vitamin B_6 plus a second "accessory factor." This second factor has been shown to be included in the filtrate from the fullers' earth treatment of rice bran concentrate.

Vitamin B_6, both in crude preparation and in crystalline form, had only a temporary effect on acrodynia in the absence of the "accessory factor."

Like acrodynia, the Burr and Burr syndrome was cured by both "essential fatty acids" and rice bran concentrate. The Burr and Burr syndrome was produced by the addition of dried brewers' yeast to our acrodynia-producing diet. Gradations between the severe symptoms of acrodynia and the mild symptoms of the Burr and Burr syndrome were obtained by feeding increasing amounts of yeast.

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