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The effect of pectin supplements in avitaminosis A in rats.

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THE EFFECT OF PECTIN SUPPLEMENTS
IN AVITAMINOSIS A IN RATS

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THE EFFECT OF PECTIN SUPPLEMENTS
IN
AVITAMINOSIS A IN RATS

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the degree of
Master of Science

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INTRODUCTION

It is generally recognized that vitamin A is essential in preventing xerophthalmia and keratinization of the mucous epithelium of the body. This substance is also thought to play an important role in the synthesis of mucin by the body because a dehydration of the mucous membrane occurs as a result of avitaminosis A. Manville (1936) has suggested that galacturonic acid plays a role in mucin formation. In view of the fact that the pectin molecule is composed of eight molecules of galacturonic acid, it is thought that ingested pectin when fed in a vitamin A free diet might tend to prevent the characteristic keratinization of the mucous epithelium. It is also thought that the keratinization which occurs in avitaminosis A is due to a lack of mucin. Thus, the object of this investigation is to determine whether or not pectin is effective in preventing, or delaying the development of the typical pathological symptoms which occur in avitaminosis A.

REVIEW OF LITERATURE

Vitamin A is found in plants as the pigment, carotene, which is converted by the animal organism to vitamin A. This is a process that usually takes place in the liver through the action of the enzyme carotenase.

Evidence that vitamin A is not entirely absorbed is

shown by its presence in the blood in colloidal combination with the albumin. It is also found in such organs as the spleen, liver, bone marrow, subcutaneous fat, corpus leuethum, retina of the eye, and in the kidneys according to Eddy and Dalldorf (1937).

Four forms of provitamin A have been discovered, namely, alpha carotene, beta carotene, and gamma carotene, and kryptoxanthine. The empirical formulas for the carotenes is the same, $C_{40}H_{56}$, and for kryptoxanthin $C_{40}H_{55}OH$. However, the beta carotene is apparently the best source of Vitamin A as has been proved by Kuhn and also by Korrer, and quoted by Eddy and Dalldorf (1937). The structural formula of beta carotene, as shown by Kuhn and Brockmann (1934) is as follows:

The suggested formular for vitamin A is: Eddy and Dalldorf (1937)

Most authors agree that the physiological activity of both vitamin A and pure beta carotene can be easily destroyed at high temperatures when oxygen or some oxidizing agent is present. According to the human need for vitamin A in the various age groups can be summarized as follows:*

Age	Maximum calorie need per pound	Maximum weight	Maximum total calorie needed	Vitamin A Need in U.S.P. units
1	45	22	990	2,770
2	43	27	1,160	3,250
3	40	32	1,270	3,560
4	40	35	1,400	3,920
5	37	41	1,515	4,420
6	35	46	1,610	4,500
7	34	51	1,730	4,840
8	35	57	2,000	5,550
9	35	63	2,200	6,160
10	32	69	2,200	6,160
11	32	76	2,430	6,800
12	32	86	2,750	7,700
13	30	100	3,000	8,400
14	25	109	2,720	7,560
15	25	116	2,900	8,120
16	25	119	2,970	8,310
17 and over	18	120 and up	2,160 and up	3,000 and up

* Taken from The Avitaminoses, Eddy and Dalldorf (1937)

The Functions of Vitamin A

Vitamin A is understood to be indispensable to normal growth, vision, and the sustenance of certain normal condi-

tions of specialized tissues. The part that vitamin A plays in the maintenance of vision is twofold. In one instance avitaminosis A produces changes in the cornea which apparently lead to loss of vision by impairing the refractive apparatus, or by promoting a conflagrative ophthalmitis. Insufficient amount of the vitamin is thus related with the function of the eyes. Furthermore, Eddy and Dalldorf (1937), state that it is also necessary to the normal action of the retinal organ, of which it is an indispensable chemical constituent.

Clinical Manifestations of Vitamin A Deficiency

Before delving into the specialized clinical manifestations of vitamin A deficiency it will be best perhaps to give the external pathology of avitaminosis A in rats. The straight and well balanced posture is changed to a humped one, the coat becomes rough and sparse and, in most cases, the animal becomes very thin. Perhaps the most recognizable symptom, as stated by Wolbach and Howe (1925), is the red encrusted eyelids that are almost always prevalent.

One of the first symptoms of avitaminosis A is the abnormal cornification of the vaginal passage which affords a very sensitive and dependable test in the detection of this deficiency disease, according to a report by Mason and Wolfe (1935).

The method of making vaginal smears follows the pro-

cedure outlined by Mason and Ellison (1935a). Vaginal scrapings are taken from the upper vagina and stained with either Ehrlich's or Delafield's hematoxalin for one-half hour. They are then washed with water and subjected to ammonia vapor for bluing. The smear is then washed, faintly counterstained with eosin and washed and dried.

Early cornification shows a protuberance, vacuolation, and cornification of the nucleated epithelial cells. This may be similar to the late metoestrus period. In the normal cycle which varies, but is usually between four to six days, sequent daily smears will usually exhibit stages of the prooestrus, oestrus, metoestrus, followed in one to three days by the dioestrus stage. When deficiency appears in the prooestrus stage, the nucleated epithelial cells take on a faint swollen aspect, and in addition the nuclei may be swollen and pycnotic.

From this stage on the cornification becomes more involved. In the dioestrus stage there is an increase in the number of keratinized epithelial cells (cells undergoing cornification) and leucocytes are present. The material taken is somewhat cheesy in consistency. From here the cycle progresses to advanced cornification following through the prooestrus stage to the oestrus state, which shows complete keratinization, few leucocytes, and partially cornified cells. There next develops an extreme cornified condition characterized by a considerable number of large keratinized cells, very few leucocytes,

and an almost complete absence of nuclei. This stage persists indefinitely and precedes the onset of xerophthalmia as stated by K. E. Mason and E. T. Ellison (1935a)

Steenbock and Bauman (1932) state that upon continuous cornification there is a distinct impairment of the vaginal epithelium, the cells becoming more necrotic with an aggregation of debris and colloidal substances. Leucocytes may reappear but the cornified cells last until death if the deficiency is not corrected. Macy, Outhouse, Long, and Grahm (1927) point to the relation of the opening of the vaginal orifice and the impairment of the vaginal passage. They think that the time of opening of the passage is influenced by the amount of vitamin B in the food. However, in all cases the passage opened before the onset of xerophthalmia.

Evans (1928) shows the relationship of the impaired vaginal tissue resulting from a deficient A diet to the sexual physiology of the female. He believed that incomplete gestation was probably due to the fact that either the follicles were too infirm to be fertilized or that the spermatazoa were incapable of reaching them. Inadequate supply of vitamin A in the diet impairs the reproductive system so that impregnation often fails. He found that 80 per cent of the copulations also failed.

Mason and Ellison (1935) used the lavage method of Guttmacher (1926) of making a vaginal smear, which method included pipeting into the vagina 1 cc. of 1-10,000 solution of neutral red made up in a normal saline solu-

tion. The resulting cellular suspension in the vaginal passage is withdrawn by the pipette and placed on a cover slip and then inverted on neutral red slides as used in the study of blood. These were kept at a temperature of 37.5 degrees Centigrade while the counts were made. They found that in an early stage of cornification the leucocytes were many and active as shown by their ability to take up neutral red. Later, however, the leucocytes decreased in number and appeared to be in a dying state. This was followed by intense cornification. They also showed the presence of bacilli in Brownian movement which upon cultivation proved to be the colon bacillus, and although they appear in the normal vagina of the rat, they were never in such great numbers as in the A deficient rats. There was also a variability in the oestrus cycle wherein there was a little increase in its length. This occurred in the dioestrus interval.

Contrary to the above finding, Aberle (1933) used the Guttmacher technic and he found a lengthening of the oestrus cycle and a shortening of the dioestrus, with a drying of the mucosa.

Ocular Lesions

Perhaps the first symptoms to appear in the eye is the onset of night blindness, hemeralopia, which is quite in evidence when the animal is subjected to bright light, the light irritating the defective apparatus. This is

followed by a muscular twitching of the eyelids. The conjunctive becomes dull and folded, a deterioration takes place in the mucous secreting cells, and a probable impairment of the tear ducts occurs. Lesions appear in the follicular regions as pigmented zones; there is also keratinization of the papule. These symptoms are described by Eddy and Dalldorf (1937).

Wolbach and Howe (1925) showed that keratinized cells appear in the mucous glands which later atrophy and disappear, and there is an abundance of polymorphonuclear leucocytes and mononuclear cells.

Skin

In avitaminosis A the skin becomes very dry due to the degeneration of the sweat glands. This condition is an early manifestation of avitaminosis A, for later a cornification of the hair follicles occurs with masses of papula cornified epithelium.

Other Tissues

According to Sweet and Kang (1935), hemosiderosis of the liver occurs in avitaminosis A. Although Wolbach and Howe (1925) do not think that the liver is involved in avitaminosis A very much they do present evidence of the occurrence of mitochondria pictures.

Sweet and Kang (1935) further show that there is a cornification of the mucous epithelium lining the oesophagus.

Wolbach and Howe (1925) describe a moderate keratinization in the nasal septum but no involvement of the small intestine, with the possible exception of the occurrence of atrophy. Haladay (1923) observed that there is a keratinization of the nasal epithelium preceded by the infiltration of polymorphonuclear cells and also that the epithelium became quite ragged. Van Leersum (1928) and Evans (1932) speak of the deterioration and cornification of the epithelium of the semineferous tubules; and Van Leersum (1928) thinks that keratinization leads to the deposition of calcium calculi, which later modify to larger concretions.

Harris (1937) believes that the cells during avitaminosis A are subject to a metaplastic reaction. Their appearance as well as their character is changed from a moist and active state to a dry flaky cornified state. Thus, he concluded that the primary role of vitamin A is to keep the cells from deteriorating in the manner described. The cause of infestation is due to the degeneration of the mucous membranes which loses moisture, with the result that their ducts become clogged with keratinized cells thus offering a suitable habitat for microorganisms.

Pectin in Relation to Avitaminosis A

Meyers and Baker (1934) have shown that the pectin molecule contains galacturonic acid, arabinose, galactose, methoxyl and acetyl groups.

However, at the present time pectin is believed to be composed of eight molecules of galacturonic acid (Manville, 1936).

Manville (1936) and Manville, Bradway and McMinis (1936) state that galacturonic acid plays a role similar to that of glycuronic acid as a detoxication mechanism in the body. Accordingly, glycuronic acid never exists alone in the body but in combination with mucin, heparin, and chondroitin. In vitamin A deficiency the cells do not produce mucin which acts as a protective mechanism to the mucous membranes of the body by preventing any scrapping or abrasions by harsh particles. In addition, in avitaminosis A there is no production of lysozyme which acts in a bacteri-static and bactericidal capacity. Thus with the loss of mucin and lysozyme, the mucous membranes are very susceptible to infection. In view of the fact that there is a lack of mucin produced there is also a lack of glycuronic acid which is the prosthetic portion of the mucin molecule (Manville, Bradway and McMinis, 1936) which plays so important a part in the detoxication mechanism of the body. This theory was proposed by Manville, Bradway and McMinis, (1936). Chemically, mucin has as its component parts a protein and a carbohydrate fraction. Each mucous cell has as its function the production of mucin. In order to accomplish this synthesis, the main building substances consisting of certain proteins and carbohydrates must be furnished. In avitaminosis A this building process does not function. This is not due to a deficiency of the protein fraction but to the

insufficiency of the carbohydrate, glycuronic acid (Manville, 1937). This investigator (1937) states that there are two sources of glycuronic acid available to the body; namely, endogenous sources synthesized from glycogenic amino acids and exogenous sources present in food material. Due to the fact that galacturonic acid acts similarly to glycuronic acid in detoxication mechanism, it can be substituted for it in the opinion of Manville, Bradway and McMinis (1936).

Pectin shows the characteristics of a hydrophyllic colloid with abundant absorptive qualities, in addition upon its breakdown gives rise to the galacturonic acid molecules. Pectin also can be utilized in the body in detoxicating menthol according to Manville, Bradway and McMinis (1936). Manville, Bradway and McMinis (1937) conclude as follows:

- (1) Pectin possesses an absorptive capacity for bacteria and toxins.
- (2) Because of its colloidal nature it is capable of taking up large quantities of fluid, this provides bulk which helps to sweep out of the intestine harmful materials and to provide a normal stimulus for peristaltic activity.
- (3) By its buffer action it helps to maintain a constant reaction in the intestine.
- (4) It acts as a protective colloid to an inflamed and perhaps ulcerated intestinal musosae.

The chief chemical value of pectin lies in the ability of galacturonic acid to form conjugation products with toxic materials.

Thus, from a review of the literature it would seem that pectin when included in the diet might be effective in

preventing some of the pathological conditions present in avitaminosis A. It is believed that the peculiar property of pectin in this regard would be due to the fact that it is composed of eight molecules of galacturonic acid.

EXPERIMENTAL PROCEDURE

Four groups of white rats were used in the experiment, the rats within each group being litter mates.

In group 1, six rats were placed on a vitamin A free diet, six rats on a vitamin A free diet with the addition of six per cent of pectin, (i.e. 6 per cent of the total diet) and two rats on a control diet containing adequate vitamin A.

In group 2, five rats were placed on a vitamin A free diet with the addition of three per cent pectin (total weight), five more rats on the vitamin A free diet, and two rats on the control diet.

In group 3, four rats were placed on the vitamin A free plus 12 per cent pectin diet; and two rats were placed on the control diet. The 12 per cent pectin diet was employed in order to observe the preventative action of pectin in regard to clinical manifestations of vitamin A. The diets employed were modification of the Sherman and Munsell (1925) deficiency vitamin A-free diet. In the experimental diets some of the starch was replaced by the amount of pectin added to the diet. The composition of the diets is presented in Table 1.

Table I. Composition of the Diets

Material	Diet 1	Diet 2 Control	Diet 3	Diet 4
	Vitamin A		Vitamin A Free	Vitamin A Free
	free		with 3 percent	with 6 percent
	Percent	Percent	pectin	pectin
	Percent	Percent	Percent	Percent
Vitamin A free casein	18	18	18	18
Eradiated yeast	8	8	8	8
Salt Mixture	4	4	4	4
Crisco	5	5	5	5
Starch	65	63	62	59
Pectin			3	6
Cod liver oil		2		

The diets were made in 2,000 gram lots.

The animals were observed carefully during the experiment, care being taken to keep the water and food fresh and amply supplied. The weights and macroscopic changes were recorded every other day, and upon later manifestations of avitaminosis A observations were made daily.

The first part of the investigation consisted in making vaginal smears to detect the earliest possible indication of avitaminous A. The method followed was that described by Mason and Ellison (1935). Scrapings were made with a swab in a rotary motion, care being taken not to cause any injury or irritation to the mucous membrane. With faulty technic a necrotic condition will be produced and there will not be a normal replacing of tissue as occurs in the oestrus cycle. The scrapings were smeared on a slide, and stained in Delafield's hematoxalin for one-half hour. They were then washed and treated with ammonia vapor which produced a deep blue. Again they were washed and this time counterstained in eosin, washed, and observed with the microscope.

At the onset of xerophthalmia in litter mates, the particular litter was sacrificed for the purpose of making histological sections of the tissues; namely, eyelid, nares, vagina, liver, oesophagus, small intestine, and the kidney. The tissues were fixed in Bouin's solution and the method of dehydration in addition to alcohol included the use of dioxan. They were later impregnated with several changes of soft paraffin (45-48°C.) and then immersed in several changes of hard paraffin (50-55°C.) finally being permanently imbedded

in the hard paraffin. The sectioned tissues were stained with Delafield's hematoxylin and counterstained with eosin. The preparations were then observed with the microscope.

DISCUSSION OF RESULTS

The first changes to be observed as a result of avitaminosis A, were found in the abnormal cornification of the vaginal passage. In all cases the orifice of the vaginal passage opened before the occurrence of xerophthalmia. From one to four oestrus cycles in the female rats were determined where necessary, before prolonged cornification took place. The cornification of the oestrus stage of the oestrus cycle is similar to the cornification due to avitaminosis A, hence continued smears must be made so as not to confuse the two. Upon avitaminosis A keratinization of the mucous membrane persists until the particular condition is alleviated. In all cases, the occurrence of cornification in the vagina appeared before the development of xerophthalmia. In some females as soon as the orifice was open and smears made, the presence of continued cornification was observed. However, in others normal oestrus cycles could be determined before the onset of persistent cornification. Evidence of early cornification was observed in that some mucous epithelial cells showed keratinization, while others showed a marked swelling, and vacuolation. As the cornification advanced, more cells became keratinized, the nuclei of other cells became rather pycnotic, and the

cell outlines began to take on a shady appearance. There was also a slight increase in the amount of leucocytes present. In the next stage of cornification there was a decided increase in the number of completely keratinized epithelial cells, the mucous did not seem to appear as readily as before, the epithelial cells were both large and small, and the amount of leucocytes increased. Upon subsequent smears the cornification was somewhat complete, about ninety per cent of epithelial cells were completely cornified and the remaining cells partially cornified. There were few leucocytes present. Further smears showed extreme cornification which remained until the animals were sacrificed. At this stage the epithelial cells were large and flaky, the nuclei had disappeared, and the cell outlines were rough and ragged, and no leucocytes were present. An example of this type of cornification can be seen in Plate 1, Figure 1.

Symptoms of xerophthalmia appeared after the persistence of keratinized cells. The variations in the female were from five to fourteen days. In 80 per cent of the cases, the males showed the symptoms of xerophthalmia first.

The effect of pectin as a delaying and preventing agent in the occurrence of xerophthalmia can now be discussed. Eighty-five per cent of the animals on the straight vitamin A deficient diet developed symptoms of xerophthalmia before the animals fed with the pectin supplement. However, the variation was from one to three days. The one day difference

Figure 1. Weight changes and occurrence of xerophthalmia on vitamin A-free rats with and without pectin supplement as compared to the positive control.

Figure 2. Weight changes and occurrence of xerophthalmia on vitamin A-free rats with and without pectin supplement as compared to the positive control.

Figure 3. Weight changes and occurrence of xerophthalmia on vitamin A-free rats with and without pectin supplement as compared to the positive control.

Figure 4. Growth curve of rats in experiment on curative effect of pectin on vitamin-A deficiency.

was in the three per cent level mostly, whereas the three day difference occurred at the six per cent level. Changes are recorded in Figures 1 to 4.

There were no animals deprived of vitamin A that did not develop the characteristic ocular lesions of avitaminosis

A. In the 12 per cent level of the pectin supplement, the rats were not cured of xerophthalmia. The growth changes were variable as can be observed in Figures 1 through 4.

Further macroscopic changes included an anemic and emaciated appearance with a rough and thin coat, an apparent loss of coordination in movement, hard and forced breathing, and a hunched position of posture.

Microscopic Observation of Specialized Tissues

In general there were no notable changes in the small intestine, liver, oesophagus, or kidney as differing from the normal tissues. However, there were changes in the vagina, eyelid and the nares that were quite marked.

The Vagina, Plate 2, Figures 1 to 4.

In avitaminosis A the mucous membrane of the vagina becomes cornified to a very great extent. This can be readily observed in Figure 2. The keratinized mucous epithelium is sloughing off into the vaginal passage and there is a marked aggregation of leucocytes in the tunica propria. There is also a slight atrophy.

In Figure 3 the condition is somewhat alleviated with

the cornification marked to a lesser degree and with fewer leucocytes present. This represents rats on the three per cent pectin supplement.

The results of the six per cent of pectin in the diet reveal a condition similar to the normal. There is no sloughing of keratinized cells and the mucous membrane appears to be quite normal. This can be observed in Figure 4.

The Eyelid, Plate 3, Figures 1 to 4

In the normal tissue, the mucous membrane is well defined, the cells are structurally well outlined, and the mucous glands present show no signs of impairment (Figure 1). In Figure 2 there are several changes present. The mucous membrane is not clearly defined, there is an abundance of leucocytes present, and the lacrimal gland according to Mori (1922-1923) has undergone an apparent resting stage. The change in the gland is one of the first changes in the ocular organ, thus, without the production of tears there is no bathing fluid for the mucous membrane and this soon results in an irritation which opens the way for bacterial infection. The outer portion of the mucous membrane also appears horny.

In Figure 3, the mucous membrane appears to be normal, with the possible exception of a greater amount of cell layers. There are no abundant leucocytes present. This represents the three per cent pectin level.

In Figure 4, the six per cent level, the tissue appears quite normal. The glands present are well defined, the mucous membrane is sharp and there is a lack of leucocytes.

The Nares, Plate 4, Figures 1 to 4

In the normal tissue there is a slight sloughing off of mucous epithelium which is characteristic of a normal function, i.e. the building of new tissue to replenish the old.

Figure 2 shows typical cornified epithelium. The cell layers have multiplied and many of the cell walls are irregular as compared to the normal. This represents a vitamin A free diet.

Figure 3 represents the three per cent pectin level and the mucous membrane appears normal in the amount of cell layers. There is, however, the presence of a layer of cornified epithelium.

In Figure 4, designating the six per cent pectin level, the same condition prevails as at the previous level.

In the remaining tissues there are no apparent departures from the normal. They are arranged in the following manner:

Figures 1 to 4, i.e normal, vitamin A free, vitamin A free with the 3 per cent pectin supplement, and vitamin A free with the addition of the 6 per cent pectin supplement.

Plate 5 - Kidney

Plate 6 - Liver

Plate 7 - Small intestine

Plate 8 - Oesophagus

SUMMARY

1. From the experimental work it is quite evident that early avitaminosis A in rats can be readily detected by the vaginal smear method.
2. The use of pectin as a supplement in a vitamin A free diet acted as a delaying agent in the occurrence of xerophthalmia. It did not prevent the onset of xerophthalmia nor did it act in a curative capacity.
3. In the liver, small intestine, kidney, and oesophagus there were no marked changes as compared to the normal tissue.
4. In the vagina, nares, and eyelids, there were marked changes in the mucosae and the submucous coat. The clinical manifestations due to avitaminosis A appeared in the vitamin A deficient rats. In the levels that contained the pectin supplements, these conditions in the tissues were not as prevalent.

CONCLUSIONS

Pectin apparently acts as a beneficial supplement to a diet deficient in vitamin A.

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Plate 1.

Vaginal Smear Showing Cornified Cells

Plate 2.

Figure 1. Vagina, Normal

Figure 2. Vagina, Vitamin A Free Diet

Plate 2.

Figure 3. Vagina, 3 Percent Pectin Supplement

Figure 4. Vagina, 6 Percent Pectin Supplement

Plate 3.

Figure 1. Eyelid, Normal

Figure 2. Eyelid, Vitamin A Free Diet

Plate 3.

Figure 3. Eyelid, 3 Percent Pectin Supplement

Figure 4. Eyelid, 6 Percent Pectin Supplement

Plate 4.

Figure 1. Nares, Normal

Figure 2. Nares, Vitamin A Free Diet

Plate 4.

Figure 3. Nares, 3 Percent Pectin Supplement

Figure 4. Nares, 6 Percent Pectin Supplement

Plate 5.

Figure 1. Kidney, Normal

Figure 2. Kidney, Vitamin A Free Diet

Plate 5.

Figure 3. Kidney, 3 Percent Pectin Supplement

Figure 4. Kidney, 6 Percent Pectin Supplement

Plate 8.

Figure 1. Liver, Normal

Figure 2. Liver, Vitamin A Free Diet

Plate 6.

Figure 3. Liver, 5 Percent Pectin Supplement

Figure 4. Liver, 6 Percent Pectin Supplement

Plate 7.

Figure 1. Small Intestine, Normal

Figure 2. Small Intestine, Vitamin A Free Diet

Plate 7.

Figure 3. Small Intestine, 3 Percent Pectin Supplement

Figure 4. Small Intestine, 6 Percent Pectin Supplement

Figure 1. Oesophagus, Normal

Figure 2. Oesophagus, Vitamin A Free Diet

Plate 8.

Figure 3. Oesophagus, 3 Percent Pectin Supplement

Figure 4. Oesophagus, 6 Percent Pectin Supplement

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