THE ISOLATION AND SYNTHESIS OF A FOOD FACTOR OCCURRING IN CREAM AND NECESSARY FOR THE NUTRITION OF THE GUINEA PIG

by

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A THESIS

submitted to the

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1941

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ACKNOWLEDGMENT

The author is indebted to Dr. Rosalind Wulzen for the opportunity of conducting this work and for personally carrying out the assay work.

Sincerest appreciation is expressed to Dr. J. S. Butts for the helpful criticisms and suggestions afforded by him throughout the course of this work.

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THE ISOLATION AND SYNTHESIS OF A FOOD FACTOR OCCURRING IN CREAM AND NECESSARY FOR THE NUTRITION OF THE GUINEA PIG

I. INTRODUCTION

1. Historical. In 1936 Wulzen and Barhs (1)* reported the appearance of a deficiency disease in guinea pigs given supposedly adequate diets. Teh conditions of the disease were similar to those produced in the experimental animals used in the work referred to in this thesis, and will be described in detail later. These workers reported the effects of numerous additions and supplements to the diets employed. They found the factor, which they named pl, to be present in fresh kale, fresh alfalfa, and fresh carrots, and to a less extent in dried alfalfa, cooked alfalfa, dried carrots, yellow corn meal, and milk.

Further investigation of the deficient factor led to the development of a diet which consisted of a basal milk ration arranged as follows: to each hundred cc. of milk was added 10 gm. skim milk powder, and traces of iron and copper. All animals were bedded on straw and were provided with iodized salt. Orange juice (1 cc. per 100 gm. body wt.) and 150 I. U. of carotene were given daily to each animal.

Although the above diet was complete in all of the known nutritional requirements of the guinea pig, it was noticed that the animals developed the characteristic

*Numbers in parenthesis refer to bibliography.

syndrome. Accordingly, this observation was studied using various basal milk rations prepared with different types of milk (ie. skim, raw, whole, and pasteurized) and these were found to produce effects differing radically from one another according to the variety of milk used. Guinea pigs fed whole raw milk ration grew excellently and appeared to be in better condition than the ordinary stock animals. Those fed pasteurized whole milk grew less and developed decided and persistent stiffness of the limbs. The animals receiving pasteurized skim milk ration or raw skim milk ration grew the least and showed consistent stiffness.

2. Description of the Condition. The syndrome produced by the experimental diets was evidently due to a nutritional deficiency. The condition manifests itself as follows: stiffness of the wrists, permanent stiffness of the elbows in a flexed position, loss of ability of the animal to regain an erect standing position when placed on its back, and finally loss of power of locomotion. Death ensued shortly if dropper feeding was not resorted to. In some animals the above conditions would develop in about a month, in other animals as long as a year was required to produce the same condition. In almost all cases the inability to walk finally resulted.

Autopsy revealed extreme calcification. The muscles being finely streaked with calcium deposits running par-

allel to the fibers. Large deposits of tricalcium phosphate were also found attached to the body wall under the skin. These were apt to occur in the joint regions, between the ribs and axilla. Lump deposits which formed along the back posterior to the point of the scapula could be felt through the skin of the living animal. Calcium was found deposited apparently at random in the walls of the digestive tract: the pancreas and spleen might be peppered with granular deposits. The aorta was often thickly incrusted with small lumps projecting in to the lumen.

It is not known, at the present time, whether the calcification is a direct result of the deficiency, or whether it is a result of, or incidental to the syndrome. Nevertheless, it is very definitely present, and may be closely linked with the other symptoms.

3. Previous Experimental Work. The first experimental work of a chemical nature relating to the nutritional deficiency was carried on by A. K. Stout in the Biochemical Laboratory at O. S. C. during the year 1939-40. She found that commercial pasteurized cream was without activity, but raw cream was found to be active in the four cases tried. Butter made from pasteurized cream was not active, but butter from raw cream was active. Additional results and the conclusions reached by her were as follows: (1) the active factor was contained in the butter when raw

cream was churned, (2) the factor was destroyed by heating in the presence of oxygen, (3) heating in an atmosphere of nitrogen did not destroy the activity, (4) the principle was not destroyed when butter was saponified in an atmosphere of nitrogen, (5) acidification of the saponified mixture did not destroy the active factor, and (6) the factor was volatile with steam, and could be concentrated by steam distillation of the free fatty acids obtained from the saponification of the butter. Further work led to her conclusion that the factor was a low-molecular weight unsaturated fatty acid.

4. Method of Assay. The method used to determine the presence of the active principle in different substances was as follows: The fore leg of the guinea pig on the opposite side from the experimenter was extended posteriorly, close to the body wall of the animal, by pressing the thumb on the olecranon process and at the same time supporting the proximal and distal portions of the leg with the fingers. The leg would be as straight as possible. The disengaged hand of the operator was then used to gently superextend the foot by pressing upward on its medial aspect. The foot of a normal animal would bend easily until it formed a right angle with the leg, but if the animal had become quite stiff it was not possible to bend the foot at all. Normal animals were indifferent to the manipulation but nutritionally deficient animals were

very sensitive to the treatment, and manifested pain at once when the foot was forced beyond the point of easy bending. The extent to which the stiffness of the joint disappeared, or failed to disappear, as determined by the method given above, was taken as a measure of the activity of the substance that was being fed. Active substances would produce a decidely significant difference in the angle at which the foot could be bent with respect to the leg, in as short a time as 48 hours. The reliability of the method of assay was dependent on the experience and skill of the operator. All assays were conducted personally by Dr. Wulzen, who developed the method, and has had a great deal of experience with it.

5. Effect of Vitamin E on the Guinea Pig Stiffness. The condition brought about in the guinea pigs fed the deficient diet was similar to the condition described by Goettsch and Pappenheimer (2). This syndrome was produced in guinea pigs and rabbits fed a diet of rolled oats, wheat bran, casein, lard, codliver oil, and inorganic salts. These workers stated that the diet was complete in all known requirements except vitamin E, however, addition of vitamin E did not prevent the development of the disease.

In contrast to these findings, S. Morgulis and Spencer (3) showed that muscular dystrophy could be prevented and cured in rabbits by addition of (1) fresh green

alfalfa, lettuce and Vitamin E, (2) dry alfalfa and wheat germ oil, or (3) whole wheat germ, to an otherwise complete diet.

In order to eliminate the possibility of Vitamin E deficiency as the cause of the condition existing in our experimental animals, studies were carried out using several preparations of Vitamin E, including synthetic alphatocopherol. The conclusions reached were that Vitamin E was somewhat effective in preventing or curing the condition, but did not compare in positive activity with raw cream or active concentrates from cream. (Table I shows some of the known constituents of butter.)

6. Grass Juice Factor. Kohler et.al. (8),(9), and (10) have reported the presence of a water soluble growth promoting substance in young grass necessary for nutrition of the guinea pig. In light of the fact that the basal diet employed by these workers contained fresh unpasteurized milk, it is apparent that they are dealing with a different factor than the one being considered in this paper.

II. EXPERIMENTAL

1. Procedure. The experimental work carried out in this thesis consisted in a confirmation of and continuation of the previous work of A. K. Stout. An attempt was made to gain a more complete knowledge of the syndrome, and

TABLE I.

Compound	No. Carbon Atoms	Percent	Reference
Dioxystearic	18	1.0	(4)
Oleic	18	32.5	(4)
Stearic	18	1.83	(4)
Palmitic	16	38.61	(4)
Myristic	14	9.89	(4)
Lauric	12	2.57	(4)
Capric	10	0.32	(4)
Caprylic	8	0.49	(4)
Caproic	6	2.09	(4)
Butyric	4	5.45	(4)
Diacetyl	4	1-10 mg/kilo	(5)
Acetylmethylcarbinol	4	1-50 mg/kilo	(6)
Vitamin A			(7)
Vitamin B_1			(7)
Vitamin D			(7)

(Several complex fatty acids of molecular weight over 100 and present in quantities less than 1 percent.)

possibly develop a quantitative method of assay based on the excretion of creatine and creatinine in the urine. It has been shown (11) previously, that in cases of muscular dystrophy in guinea pigs that the creatine excretion rises at the expense of the creatinine. A definite creatineuria was found to be present in our guinea pigs which developed typical deficiency symptoms. Inasmuch as the quantitative determination of creatinine and creatine could not be made with sufficient accuracy to permit use as a basis of measure of activity, it was discarded. Table II contains typical data and while strictly quantitative significance cannot be attached to these results, nevertheless, they do show that a creatineuria does exist.

With a few minor exceptions the procedure for the concentration of the active factor was the same as that used by A. K. Stout and is as follows: three gallons of raw cream were churned and the butter (3890 gms.) was saponified by refluxing with 650 gms. of KOH and 3 liters of ethanol for $3\frac{1}{2}$ hours. The saponification was carried on in an atmosphere of nitrogen. The mixture was allowed to cool to room temperature and the excess KOH neutralized slowly, to avoid heating, with dilute sulfuric acid. The resulting suspension of free fatty acids was allowed to stand overnight at zero degrees. The solid fatty acids were filtered off with suction, washed with cold water, and filtered again. The filtrate and washings showed no

TABLE II.

Anl. No.	Sex	Creatinine mgs.	Creatinine & creatine mgs.	Creatine mgs.	Creatine % *	Urine vol. cc.
633	f	12.4	13.9	1.5	11	57
628	f	17.8	29.3	11.5	39.3	73
656	f	20.5	40.3	19.8	49.0	82
630	f	13.5	17.5	4.0	22.8	84
634	f	13.1	17.1	4.0	23.3	66
640	f	22.2	23.3	1.1	4.6	87

Creatine and creatinine excretion of guinea pigs suffering from nutritional deficiency. Analyses were made on 24 hour urine samples.

*Per cent of total creatine and creatinine.

activity. The fatty acid mixture was transferred to a 12 liter balloon flask and steam distilled in an atmosphere of nitrogen for 8½ hours. During the course of the distillation it was noted that a vapor possessing a highly pungent odor was escaping from the receiver. A trap immersed in a solid carbon dioxide ether slush maintained at about -80° was attached to the steam distillate receiver by means of a side-arm. The volume of the condensate in the trap at the end of the distillation was usually less than 2 cc. and consisted mainly of water. However, in every case the material proved to be very active. Due to the small volume condensed in the trap, and the fact that most of it was used in assay feeding, no qualitative test could be made to determine its characteristics.

These results led to the conclusion that the substance being dealt with was exceedingly volatile. Therefore an attempt was made to remove the factor from cream by co-distillation with nitrogen and subsequent condensation in a condenser maintained at -80°. Due to excessive foaming this method was found unsatisfactory. Attempts were also made using butter from raw cream. The butter was kept in a melted condition by placing the distilling flask in a water bath kept at 30-35 degrees. Nitrogen was bubbled vigorously through the melted butter for five hours, the material collected in the trap during this period was tested and found to be inactive. Therefore, this method

of attempted isolation was abandoned.

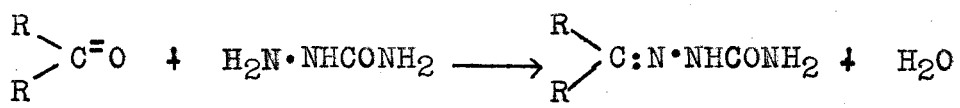
During the 8½ hours of steam distillation several liters of distillate were collected, on the top of which an oily layer formed. This upper layer proved to be active, and was separated from the water by means of a separatory funnel. After removal of the oily layer from the water, the latter was assayed and found inactive. The lack of activity might well have been due to the excessive volume, which would consist of an exceedingly dilute solution of the active principle, if it were present.

Attempts were made to fractionate the oily layer obtained from the steam distillation. The fractionation was carried out in a helice-packed column at reduced pressures (less than 1mm.) This proved to be unsatisfactory due to difficulties in fractionation, and also to thermal decomposition.

It was then decided to ascertain the identity of the constituents of the oily layer from the steam distillation without first separating it into its individual components.

Concentration of the factor from a fresh batch of cream was carried out as stated above and the oily layer subjected to a number of qualitative tests for certain classes of compounds. Attempts toward identification by determination of solubilities, and classification by this method were not carried out, because the concentrate was quite apparently a complex mixture, and such data would

have been of questionable value. However, negative tests for aldehydes and alcohols were obtained. Also, slight adsorption of bromine was noted, as well as a slight pink coloration with Schiffs' reagent on long standing. This was taken as some indication of a lower ketone or an unsaturated compound. A crystalline semicarbazone was obtained which had a melting point of 140-141 degrees. This melting point compared exactly with values given in the literature (14) for methyl vinyl ketone. The reaction for the formation of a semicarbazone from a ketone and semicarbizide is illustrated below:



The procedure followed for the preparation of the semicarbazone was that given in Shriner and Fuson (14).

It was then decided to confirm the above evidence by testing the activity of synthetic methyl vinyl ketone, with the experimental animals.

Attempts to prepare pure methyl vinyl ketone by the method of Krapiwin (12), using ethylene, acetyl chloride and aluminum chloride in petroleum ether were unsatisfactory. The ethylene was generated by the action of hot phosphoric acid on ethanol, and the ethylene passed into the reaction flask containing acetyl chloride and aluminum chloride and petroleum ether. Hydrolysis of the reaction mixture led to an uncontrolable reaction which produced a

black tarry mass. No pure methyl vinyl ketone was obtained. Using the method given by A. Wohl and A. Prill (13) fair yields of the pure compound were obtained. The procedure used was as follows: Three hundred grams of acetone, 100 grams of 35% formaldehyde solution, and 4 cc of n-sodium hydroxide were mixed and allowed to stand at room temperature for three to four hours. The reaction was complete when the mixture turned yellow to dark reddish-brown. The mixture was neutralized with 4 cc n-hydrochloric acid, and the excess acetone distilled from a water bath at 80-85°. Two to four grams of anhydrous zinc chloride were added, and the distillation continued on an oil bath until no more colorless distillate came over. The distillate consisted of methyl vinyl ketone and water. The distillate was saturated with potassium carbonate, and the ketone which separated out was removed with a separatory funnel, dried with anhydrous sodium sulfate, and distilled at reduced pressure. The boiling point was 33-4° at 130mm. The reactions involved are probably:

$$CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_3 + CH_2O \longrightarrow CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_2\text{-}OH$$

$$CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_2\text{-}CH_2\text{-}OH \xrightarrow[ZnCl_2]{dist.} CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}\overset{H}{C}\text{:}CH_2 + H_2O$$

The ketone was prepared in small amounts from time to time as needed, and in the course of this work it was found that if the amount of acetone was doubled the yield was increased appreciably. No explanation is offered,

and no attempt was made to determine the optimum quantity.

The boiling point of pure methyl vinyl ketone is given as 80 degrees, and the refractive index as 1.4086. The ketone as prepared had a boiling point of 79 degrees (uncorr.) and refractive index of 1.4053. The slightly low value for refractive index proved to be due to traces of water. When treated with suitable dehydrating agents, the value approached the accepted value.

Methyl vinyl ketone is an extremely unstable compound and polymerized readily on standing. Stabilization with hydroquinone is effective for a short time. If kept cool and at pH. of less than 7.1, it is reported to be less likely to polymerize.

When fed to experimental animals the ketone was found to be highly active in curing the stiffness previously described. In 33 experimental animals fed the ketone all were greatly benefited, and nearly all showed complete recovery. In addition the ketone relieved the condition in three days, while similar groups fed alpha tocopherol showed no change during the same period.

III. DISCUSSION

There is a very close similarity in lability between methyl vinyl ketone and the active concentrates from cream. Probably the most important evidence of the two being the same is that of physiological activity. Both the active

fraction from cream and the synthetic ketone showed high activity in curing the stiffness in the animals.

The presence of preformed methyl vinyl ketone in cream has not been definitely proven, but can be postulated as rising from the loss of one molecule of water from acetylmethylcarbinol in the following manner:

$$CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}\underset{\underset{OH}{|}}{CH}\text{-}CH_3 \longrightarrow CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH{:}CH_2 + H_2O$$

Acetylmethylcarbinol is a known constituent of cream.(6) It is also possible that methyl vinyl ketone is only the prosthetic group of a large complex molecule which was broken down by the treatment with conc. sodium hydroxide in the saponification.

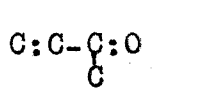
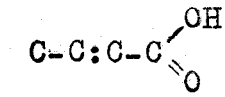
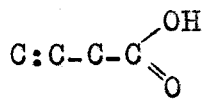
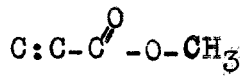
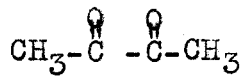
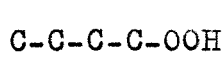
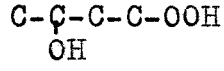
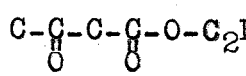
The possibility of the production of the ketone by bacterial action in raw cream and not in pasteurized cream is almost eliminated by the fact that cream pasteurized in an atmosphere of nitrogen remains active. However, if the bacteria responsible for the production of it were destroyed only by heating plus access to oxygen, the possibility would remain. At the present time there is no conclusive evidence for either case. Before the evidence for methyl vinyl ketone was obtained several ethyl esters to the fatty acids were fed in order to determine whether or not low molecular weight fatty acids showed any activity. It was found that ethyl crotonate was the only one possessing even slight activity. However, considering the formula for ethyl crotonate: $C\text{-}C{=}C\text{-}\overset{O}{\overset{\|}{C}}OET$, and for methyl vinyl ketone:

C:C.C(=O).C it can be seen that both contained the grouping C:C-C:O. Although this structure is not in the least complex, it is quite possible that a compound containing such a group is essential in guinea pig metabolism, and equally possible that the animal is unable to synthesize it. Along the same line, feeding of diacetyl: C-C(=O)-C(=O)-C produced no changes in the condition.

Table III contains a number of compounds which have the grouping C:C-C:O, or similar groupings, which might be expected to have activity, also reported are compounds which have been tested for activity.

A study of compounds possessing this and similar groups would be worthwhile. At the present time vinyl-acetic acid is being prepared, and will be tested for activity.

TABLE III

Formula	Name	Results
	Methyl vinyl ketone	very active
	Crotonic acid*	some activity
	Vinyl acetic acid	not tried
	Methylmethacrylate	some activity
	Diacetyl	no activity
	Butyric**	no activity
	Beta-hydroxybutyric	no activity
	Ethylacetoacetate	no activity

*Fed as the ethyl ester

**Higher homologues also not active

IV. SUMMARY

1. A factor necessary for guinea pig nutrition was isolated, and identified as methyl vinyl ketone.
2. A crystalline semicarbazone was obtained as a derivative with a melting point of 140-1 degrees. This is in excellent agreement with the accepted values for the semicarbazone of methyl vinyl ketone.
3. Methyl vinyl ketone was synthesized.
4. The synthetic compound proved to have activity comparing favorably with that of the active concentrates obtained from cream.
5. Synthetic methyl vinyl ketone furnished through the courtesy of E. I. Du Pont Nemours & Co. was found to have activity equal to that prepared in this laboratory.

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