A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS*

VIII. THE ISOLATION OF THE ANTISTIFFNESS FACTOR FROM CANE JUICE

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(Received for publication, April 23, 1946)

The existence of a fat-soluble dietary factor essential for guinea pig nutrition was well established through investigations by Wulzen and Bahrs (1-3) and has been confirmed by Anderson and Caldwell.1 The isolation of a highly active fraction from raw cream, capable of alleviating an induced stiffness in guinea pigs, was described by van Wagtendonk and Wulzen (4). It seems apparent from physiological studies that the factor has a regulatory effect on the phosphorus metabolism. One of the most prominent changes found was a sharp decrease in the easily hydrolyzable phosphorus fraction in the liver and kidneys during the deficiency. This fraction responded immediately to the administration of the antistiffness factor to deficient animals in that the values returned to normal after a short time of treatment (5). Similar changes were observed in the concentration of the acid-soluble phosphorus in the muscle. The concentrations of creatine phosphate and adenosine tri- and diphosphate are lower in the deficient animal (6). As a result of the deranged phosphorus metabolism other, probably secondary, changes are (1) an increase in the concentration of inorganic phosphorus and calcium in the blood, (2) an increase in calcium in the body tissues (7), and (3) an abnormal distribution of the protein nitrogen in the blood (8).

Only 3 mg. of an oil, curative in a 0.1 γ dosage, were obtained from 55 gallons of raw cream (4). Since much larger amounts of raw cream would have been needed for a successful isolation of the antistiffness factor, it was decided to test other possible sources of raw material for the presence of this factor. It was found that crude cane molasses and crude unheated cane juice were good sources of the factor, the latter being around 100 times as active. Due to the unavailability of crude cane juice, cane molasses was first used for the extraction of the factor. Later cane juice became available and was used exclusively.

* Supported by grants from the Williams-Waterman Fund of the Research Corporation and from the General Research Council of the Oregon State System of Higher Education.

We were able to extract and purify a crystalline compound which in a daily dose of 0.002 \( \gamma \) would relieve the stiffness induced by the skim milk diet in 5 days. The low level of the easily hydrolyzable phosphorus returned to normal values in the same time.

**EXPERIMENTAL**

*Method of Assay*

Guinea pigs weighing from 350 to 400 gm. were raised on the diet, as described by van Wagendonk (5). The diet consisted of skim milk powder and water, to which the necessary known vitamins and small amounts of copper and iron had been added. The syndrome developed gradually over a period of some months. Animals in which the syndrome was well developed were used for the test. The usual method of assay consisted of a manual determination of the wrist stiffness in the following way.

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 should be as straight as possible. The disengaged hand of the operator was then used to superextend the foot gently 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. The nutritionally deficient animals were very sensitive towards the treatment and manifested pain at once when the foot was forced beyond the point of easy bending. This stiffness disappeared if active fractions were administered to the animals. The results are recorded in terms of a series of arbitrary figures. A normal joint is designated as 4, a completely rigid joint as 1. Intermediate conditions are indicated by such symbols as 1.5, 2, 3, 3+, and 4−. 4P indicates that, although normal mobility has been regained, the joint is still painful under manipulation. The fractions were dissolved in cottonseed oil. In order to express activities in a quantitative way we arbitrarily defined 1 unit as follows: A solution of an active fraction in cottonseed oil contains 1 unit per cc. if, when 1 cc. is administered daily for 5 consecutive days to a sick animal, it cures the affected animal in this time, the stiffness being determined as described above.

The determination of the easily hydrolyzable phosphorus in the liver was also used as an assay. Guinea pigs were anesthetized by intraperitoneal injection of nembutal, and the liver was quickly removed and dropped into a mixture of dry ice and ether. The frozen organ was weighed and ground in a Waring blender in a measured volume of ice-cold 5 per
cent trichloroacetic acid, with 10 cc. of acid for 1 gm. of tissue. The suspension was filtered and the inorganic P in the filtrate determined according to the method of Fiske and Subbarow (9). The easily hydrolyzable P was determined by heating an aliquot for 15 minutes in a boiling water bath with N sulfuric acid, the liberated P being determined as described above. The obtained values were compared with those from the livers of deficient animals of the same age group. An increase of the easily hydrolyzable P in the livers of animals receiving the antistiffness factor was considered an indication of the activity.

**Table I**

<table>
<thead>
<tr>
<th>Activity of Other Materials</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Raw cream</td>
</tr>
<tr>
<td>Fresh kale leaves</td>
</tr>
<tr>
<td>Vacuum-dried kale (60°)</td>
</tr>
<tr>
<td>Raw potato</td>
</tr>
<tr>
<td>Bakers' yeast (Fleischmann)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Beef muscle</td>
</tr>
<tr>
<td>&quot; liver</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Alfalfa</td>
</tr>
<tr>
<td>Anaheim vacatone</td>
</tr>
<tr>
<td>Broccoli</td>
</tr>
</tbody>
</table>

* Obtained through the courtesy of Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc.
† From Distillation Products, Inc.

**Other Sources of Factor**—Various other raw materials were tested for the presence of the antistiffness factor, according to the first assay method. The results are represented in Table I. The fact that the \(\alpha\)-tocopherol phosphate-disodium salt is inactive in concentrations as high as 20 mg. again definitely establishes the fact that the described deficiency disease is not due to the absence of vitamin E from the diet.

**Isolation from Cane Juice**—The extraction procedure for cane juice was identical with that for molasses. For this reason only the method with cane juice will be given. In total 6 tons of molasses and 7 tons of cane juice were used for the extraction of the antistiffness factor.

**Extraction with Ethyl Ether**—55 gallons of crude cane juice (100 units per gm.; total 20,000,000 units) were extracted at room temperature in a
semicontinuous extraction apparatus. It was found advantageous to pass the cane juice through a stationary column of ethyl ether. In this way, emulsification, although still a serious interference, was considerably less than if the ether were to flow through the cane juice. The extraction tower was filled with wooden blocks ($2 \times 2 \times 2$ cm., preextracted with ethyl ether) which were thoroughly soaked in cane juice. The tower was then charged with 5 gallons of ethyl ether and the cane juice was passed through it at the rate of 55 gallons per 4 to 5 hours. After six extractions the tower was drained and the emulsion centrifuged to recover the ether extract. The tower was refilled with ether and the extraction continued. A 4 day extraction (four charges of ethyl ether, total twenty-four runs) was sufficient to extract all of the active material. The ether extract of each run was washed three times with 3 liters of water, dried over anhydrous sodium sulfate, and concentrated in a stream of nitrogen. Yield, 17 gm. of a dark green wax; 1,000,000 units per gm.; total 17,000,000 units.

*Distribution between Immiscible Solvents*—The wax obtained from the extraction was dissolved in 1 liter of petroleum ether (Skellysolve H) and four to five times extracted with 1 liter of 90 per cent methanol. The active substance remained in the petroleum ether layer. After drying over anhydrous sodium sulfate the solution was concentrated in a nitrogen atmosphere. Yield, 11 gm. of a green wax; 1,500,000 units per gm.; total 16,500,000 units.

*Selective Adsorption on Magnesium Oxide*—The green wax from the second step was dissolved in 500 cc. of a petroleum ether-benzene mixture (9:1). 25 to 50 gm. of magnesium oxide (adsorptive powdered magnesia, No. 2641, California Chemical Company, Newark, California) were added. The mixture was thoroughly shaken. After centrifuging, the pale yellow supernatant solution containing the active substance was again concentrated in a stream of nitrogen. Yield, 6 gm. of an orange wax; 10,000,000 units per gm.; total 60,000,000 units.3

*Molecular Sublimation*—The wax so obtained was submitted to a molecular sublimation in a simple pot still. The condensing surface was cooled with a dry ice-acetone mixture. The vacuum was maintained at 0.1 $\mu$. Some fractionation was possible. At a bath temperature of 70° a yellow inactive oil condensed at the cooled surface. At a bath temperature of 70° a yellow inactive oil condensed at the cooled surface.

The authors want to express their appreciation to Professor G. W. Gleeson from the Department of Chemical Engineering for his valuable advice in the construction of the extractor unit.

On elution of the magnesium oxide with ethanol, a fraction can be obtained which in very small dosages will increase the degree of stiffness. The removal of this fraction accounts for the increase of the total activity from 16,500,000 to 60,000,000 units in this step.
nature of 140–170° the active substance (mixed with some oil) condensed at the surface of the cold finger. The distillate was dissolved in benzene and 9 volumes of 95 per cent ethanol were added. After standing overnight in the ice box the precipitate was filtered, washed with a cold ethanol-benzene mixture (1:9), and dried in a vacuum desiccator. The major part of the oil was thus removed. The dry material was submitted to a second molecular sublimation. The now white distillate was dissolved in purified petroleum ether. The solution was cooled and the white crystalline precipitate filtered. The material thus obtained usually

**Table II**

*Stiffness Test*

<table>
<thead>
<tr>
<th>Dosage</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>4th day</th>
<th>5th day</th>
<th>6th day</th>
<th>7th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>2.5</td>
<td>2.5</td>
<td>3</td>
<td>3</td>
<td>4−</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.01</td>
<td>2.5</td>
<td>2.5</td>
<td>3.5</td>
<td>3−</td>
<td>4−</td>
<td>3+</td>
<td>4+</td>
</tr>
<tr>
<td>0.002</td>
<td>3</td>
<td>2.5</td>
<td>3−</td>
<td>2.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>2.5</td>
<td>2</td>
<td>2.5</td>
<td>2</td>
<td>2.5</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table III**

*Easily Hydrolyzable Phosphorus*

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Easily hydrolyzable P</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>ms. per 100 gm.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17.3 ± 0.5*</td>
<td>10</td>
</tr>
<tr>
<td>0.01</td>
<td>16.2 ± 0.3</td>
<td>10</td>
</tr>
<tr>
<td>0.002</td>
<td>8.5 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>4.7 ± 0.2</td>
<td>10</td>
</tr>
</tbody>
</table>

* The mean values and the standard error are given.

melted from 79–81°. Approximately fourteen recrystallizations from petroleum ether were necessary to obtain a sharp melting product. The purified material melted from 81.5–82°. It consisted of pure white leaflets. Yield, 0.1 gm.; 500,000,000 units per gm.; total 50,000,000 units.

**Activity Tests**—The activity of the crystalline compound was determined as described above. The results are given in Tables II and III.

4 2 liters of Skellysolve H were shaken five times with 100 cc. of fuming sulfuric acid, washed acid-free with distilled water, dried over Na₂SO₄, anhydrous, and distilled over sodium. The fraction boiling from 62–78° was collected and used for the recrystallizations.
The dosage of 0.002 γ can therefore be considered as the minimal dosage necessary to relieve the stiffness and to return the level of the easily hydrolyzable phosphorus to normal values.

**DISCUSSION**

From this and other previously reported investigations (4–7) it becomes apparent that the compound isolated from the cane molasses and the cane juice is able to prevent and to cure the symptoms characteristic for the deficiency. In view of the extremely small dosages required we feel justified in classifying the compound as an essential metabolite. Work towards the elucidation of its structure is in progress. It has not yet been possible to establish whether this compound is also present in the previously isolated fraction from raw cream.

**SUMMARY**

A procedure for the isolation of a crystalline factor present in molasses and raw cane juice, which cures an induced stiffness in guinea pigs, has been described. The abnormal low level of the easily hydrolyzable P in the liver of deficient animals can be returned to a normal value by the administration of minute dosages of the antistiffness factor. The smallest curative dosage is 0.002 γ.

The authors want to express their sincere thanks to Dr. Arthur G. Keller of Louisiana State University for his cooperation in obtaining large amounts of cane juice. Thanks are also due to the Pacific Molasses Company, San Francisco, California, for the generous amounts of molasses furnished and to the Aubandon Sugar Manufacturers and the United States Sugar Corporation of Clewiston, Florida, for the cane juice shipped.

**BIBLIOGRAPHY**

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