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THE OCCURRENCE OF CALCIUM CITRATE CRYSTALS IN CULTURES OF BUTYRIC ACID-FORMING BACTERIA AND THE WATER OF CRYSTALLIZATION OF CALCIUM CITRATE

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When milk or potato to which calcium carbonate has been added is fermented by certain strains of butyric acid bacteria, white granules varying in size from 0.1 to 1 mm. in diameter appear in the medium. These granules have been analyzed and found to be calcium citrate. Somewhat similar crystals have been reported by Sato[2] to occur in sweetened condensed milk. The granules are formed by both groups of anaerobic butyric acid bacteria; those that form butyl alcohol (*e. g.*, *Clostridium acetobutylicum*, Weizmann) and those that do not (*e. g.*, *B. saccharobutyricus*, von Klecki).

Experimental

Preliminary Examination.—The granules were removed by diluting the medium with water and picking out the particles with a pair of forceps. Under the microscope they appeared to be snow-white, crystalline balls from which little, blunt, prism-like needles projected in all directions. The crystals were practically insoluble in water but could be dissolved by acidifying slightly with hydrochloric acid and boiling. A small quantity of organic débris remained undissolved. The crystals gave strong positive tests for citric acid with Denigès',[3] Kunz's[3] and Naüssler's[4] reagents.

Analysis of the Crude Crystals.—The crystals were dried at 110° and analyzed, with the following results: ash, 23.0%; calcium, 17.5–18.2%; phosphorus, 0.11%. A small percentage (1.90) of the ash was insoluble in hydrochloric acid diluted with an equal volume of water.

Purification and Analysis of the Salt.—The crude salt was dissolved in dilute hydrochloric acid, the solution was filtered, neutralized with calcium hydroxide and calcium citrate precipitated by heating to boiling. The precipitate was filtered off and dried to constant weight in a desiccator and then dehydrated by heating at 110° for forty-eight hours.

Anal. Subs., 0.1348: loss (110°) 0.0164. Calcd. for $Ca_3(C_6H_5O_7)_2.4H_2O$: H_2O. 12.63. Found: 12.17.

The calcium in the anhydrous salt was as follows.

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[2] Sato, U. S. Dept. Agr. Dairy Div., World's Dairy Cong. Proc., II, 1285, **1923.**

[3] Abderhalden, "Handbuch der biologischen Arbeitsmethoden," Urban und Schwarzenburg, Berlin, **1925,** abt. I, teil 6, pp. 720, 722.

[4] Naüssler, *Chem.-Ztg.*, **38,** 937 (1914).

Anal. (ignition). Subs., 0.0538, 0.1050: CaO, 0.0182, 0.0355. Calcd. for $Ca_3(C_6H_5O_7)_2$: Ca, 24.13. Found: 24.16, 24.19.

Origin of Citric Acid.—In order to determine if the citric acid in this compound was that occurring naturally in the media or was formed by the organism in the fermentation of the sugar, a number of milk cultures were fermented. All of the flasks contained granules but the number and size varied. The granules were dissolved by adding glacial acetic acid and boiling. An equal quantity of acid was added to the control flasks. After making up to their original volume, the citric acid in the control and fermented samples was determined by Denigès'[5] method. As a check on the method, known quantities of citric acid were added to certain aliquots before analysis. Complete recovery of the added citric acid was obtained. The unfermented milk contained 0.21% of citric acid and the fermented milk from 0.04 to 0.17%. Cultures that showed a small number of granules were low in citric acid, while cultures that contained a large number of granules were correspondingly high.

These results indicate that the bacteria as such are not directly concerned with the formation of the crystals but bring about changes in the composition of the milk that favor the formation of the crystals from the calcium and citric acid normally present. The action of the bacteria was duplicated by acidifying the milk with an amount of acetic and butyric acids equivalent to that formed by the bacteria (100 cc. of 0.1 N per 100 cc. of milk). Better results were obtained by first digesting the milk with trypsin and then acidifying. Under these conditions the crystals were larger and more numerous than in the fermented milk. In the untreated milk, crystals eventually appeared but these were small in comparison to those formed in the treated samples. The size and rate of formation of the crystals appear to be dependent on the colloidal properties and hydrogen-ion concentration of the milk.

Water of Crystallization of Calcium Citrate.—In discussing the composition of the granules, it was stated that the calcium citrate was completely dehydrated at 110° and that at this temperature the theoretical water of crystallization and percentage of calcium were obtained. These results are not in agreement with the statements in Abderhalden,[6] Meyer and Jacobson,[7] Beilstein[8] and other reference works which say that calcium citrate, $Ca_3(C_6H_5O_7)_2.4H_2O$, loses 2 molecules of water between

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[5] Barthel, "Methods Used in the Examination of Milk and Dairy Products," translated by Goodwin, Macmillan and Co., London, **1910,** p. 91.

[6] Abderhalden, "Biochemische Handlexikon," Julius Springer, Berlin, **1911,** I, p. 1179.

[7] Meyer and Jacobson, "Lehrbuch der Organischen Chemie," Veit and Co., Leipzig, **1913,** 2nd ed., I, Teil 2, p. 714.

[8] Beilstein, "Handbuch der Organische Chemie," Julius Springer, Berlin, **1921,** 4th ed., III, p. 564.

110–130° and the other 2 molecules only when heated to 175–185°. These statements are apparently based on the work of Soldaini and Bertè.[9] Perhaps Soldaini and Bertè were led to use these high temperatures because the earlier workers, Heldt[10] and Kämmerer[11] had dried their salts at 200° and 210°, respectively. In a recent publication Chatterjee[12] concluded on the basis of the calcium content that the citrate when dried at 110° retained but one-half a molecule of water. As we had obtained close to the calculated values for 4 molecules of water of crystallization and calcium for the dehydrated compound by drying at 110°, it seemed necessary, therefore, to redetermine the loss of water of calcium citrate under varying conditions of pressure and temperature.

Calcium citrate was first prepared by neutralizing citric acid, using phenolphthalein, with lime water, filtering and boiling for a few minutes to precipitate the calcium citrate. After standing on the steam-bath for two to three hours, the precipitate was filtered off, washed with water, alcohol, and ether, and dried in the air for twenty-four hours. A second preparation was made by neutralizing the citric acid with sodium hydroxide, adding calcium chloride and boiling. The precipitate which formed was treated in the same manner as in the first method. This preparation was somewhat coarser than the first preparation and, as the table shows, contained less hygroscopic moisture. In all, seven preparations were made by the two methods. The loss of the water of crystallization under various conditions of temperature and pressure was determined. As a check on the results the dried samples were also analyzed for calcium. The samples were kept between watch glasses with tightly-fitting, ground-glass edges held in place by a spring clamp. The watch glasses could thus be opened during the drying and closed during the weighing. After each drying samples were taken for calcium analysis and the remainder again dried to constant weight before calculating how much sample had been taken. This procedure was found to be necessary owing to the great rapidity with which the dried salt took up water on exposure to the air. The calcium citrate was strongly ignited until the weight became constant and this weight was taken as that of the resultant calcium oxide. As a check on the result, the calcium in the calcium oxide was also determined by the permanganate method.

The data for 4 preparations are given in Table I. The amount of hygroscopic water held by the citrate varies with the fineness of division of the powder. The light, fluffy material, Preparation No. 1, contained more than twice as much water as the denser material, Preparation No. 2. A third preparation which was not washed with alcohol and ether but was air-dried for thirty-six hours contained over 12% of hygroscopic moisture.

Contrary to the statement of Soldaini and Bertè, calcium citrate may lose water of crystallization when dried in a vacuum over sulfuric acid. In eighteen hours the two preparations lost 4.36 and 4.88%, respectively. The loss is probably not a constant quantity but varies with the degree of vacuum, the physical state of the citrate and other factors.

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[9] Soldaini and Bertè, *Gazz. chim. ital.*, **29**, 489 (1899).

[10] Heldt, *Ann.*, **47**, 157 (1843).

[11] Kämmerer, *Ann.*, **148**, 294 (1868); **170**, 176 (1873).

[12] Chatterjee, *J. Phys. Chem.*, **28**, 1009 (1924).

TABLE I

LOSS OF WATER AND CONTENT OF CALCIUM IN CALCIUM CITRATE UNDER VARIOUS CONDITIONS OF DRYING

Prep. no.	Method of drying	Time of drying, hours	Wt. of sample, mg.	Loss of wt.[a]		Calcium in dry salt,[b] %
				mg.	%	
Experiment I						
1	Desiccator, 20–25°	29	650	34.5	5.30	21.23
2	Desiccator, 20–25°	29	927	17.0	1.83	21.15
1	Desiccator, vacuum, 20–25°	42	505	22.0	4.36	22.00
2	Desiccator, vacuum, 20–25°	42	605	29.5	4.88	22.22
1	Oven, 110°	18	384	31.0	8.08[c]	24.30
2	Oven, 110°	18	455	34.0	7.47[c]	24.24
1 and 2	Oven, 125°	18	474	0.0	0.00	24.20
1 and 2	Oven, 180°	12	369	0.0	0.00	24.34
Experiment II						
3	Air	..	...	..	...	20.70
4	Air	..	...	..	...	20.95
3	Desiccator, 20–25°	33	622	12.0	1.93	21.06
4	Desiccator, 20–25°	33	584	11.0	1.88	21.09
3	Oven, 110°	26	468	56.0	12.0	24.25
4	Oven, 110°	26	451	54.7	12.1	24.06
3 and 4	Oven, 125°	25	585	0.0	0.0	24.02
3 and 4	Oven, 180°	10	389	0.0	0.0	24.07

[a] Water of crystallization in $Ca_3(C_6H_5O_7)_2.4H_2O$ is 12.63%.

[b] Calcium in $Ca_3(C_6H_5O_7)_2.4H_2O$ is 21.08%, and in $Ca_3(C_6H_5O_7)_2$ is 24.13%.

[c] Loss in vacuum and at 110° calculated on desiccator-dried material is 12.1 and 12.0%, respectively.

The entire water of crystallization of these preparations was removed at 110° in eighteen hours. More than 90% of this water of crystallization was driven off during the first six hours of heating. The combined loss by drying in a vacuum over sulfuric acid and by heating at 110° is slightly lower than that required for $Ca_3(C_6H_5O_7)_2.4H_2O$ but the calcium content of the dried citrate is that required for $Ca_3(C_6H_5O_7)_2$ and shows that the two drying treatments produced the anhydrous salt. No further loss in weight or change in the percentage of calcium occurred on drying the salt at 125° and at 180°.

In the second experiment the calcium citrate was made from sodium citrate and calcium chloride in the same manner as Preparation 2 in the first experiment. The percentages of hygroscopic moisture in Preparations 3 and 4 are about the same as that in No. 2. The calcium content of the air-dried samples is a little low for $Ca_3(C_6H_5O_7)_2.4H_2O$ and agrees with the moisture determination in proving that the air-dried samples contained some hygroscopic moisture. After being dried in the desiccator over sulfuric acid the salts were found to contain the percentage of calcium required for $Ca_3(C_6H_5O_7)_2.4H_2O$. When dried at 110° both preparations lost almost the calculated water of crystallization. That the salts were completely anhydrous is shown by the figures for calcium: 24.26 and 24.06%. Further drying at 125 and 180° did not show any additional loss of weight nor did the percentage of calcium change. The data on these 4 preparations and those found for 3 others not given in Table I demonstrate that certain preparations of $Ca_3(C_6H_5O_7)_2.4H_2O$ lose all 4 molecules of water at 110°. We do not take the position, however, that all preparations of calcium citrate will become completely anhydrous at 110°. Two other preparations which were digested for a longer period of time after precipitation retained small but varying amounts of water (1.20 and 1.88%) at 110° and lost this at 180°. It is possible that the conditions under which the crystals are formed is a factor in determining the firmness with which the water of crystallization is held. The data, however, indicate clearly that no particular number of molecules of water is retained at either 110 or 125°.

Summary

Crystals of calcium citrate appear in old milk cultures (and on potato slants to which calcium carbonate has been added) after fermentation by butyric acid bacteria. The crystals are formed during evaporation of the culture and originate from the calcium and citric acid of the milk. The bacteria hasten the formation of the crystals by digesting the casein and producing a more suitable hydrogen-ion concentration. The same results can be accomplished by digesting the milk with trypsin and acidifying with acetic and butyric acids.

Statements in the literature that $Ca_3(C_6H_5O_7)_2.4H_2O$ retains 2 molecules of water at 125° are incorrect. Seven preparations of calcium citrate were found to lose all of their water of crystallization at 110°. Two preparations retained from 1 to 2% of water of crystallization at this temperature. Drying in a vacuum over sulfuric acid removed more than 35% of the water of crystallization in two instances.

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