

tering. From this point the operations were the same as in the preparation of silver oxalate, yielding finally a snow white amorphous product.

Sample.	Weight of sample.	Weight of Ag <sub>2</sub> S.	% Ag by analysis.	% Ag calc. from formula C <sub>7</sub> H <sub>7</sub> O <sub>2</sub> Ag.
I.....	0.2866	0.1390	42.22	42.32
II.....	0.2722	0.1321	42.25	...
III.....	0.2734	0.1327	42.26	...

*Analysis of Silver Anisate.*—The salt was prepared from pure anisic acid, m. p. 185°. The operations were the same as those used in the preparation of silver benzoate. The product after five crystallizations was still slightly pink.

Sample.	Weight of sample.	Weight of Ag <sub>2</sub> S.	% Ag by analysis.	% Ag calc. from formula C <sub>8</sub> H <sub>7</sub> O <sub>2</sub> Ag.
I.....	0.2438	0.1164	41.57	41.66
II.....	0.2400	0.1147	41.61	...
III.....	0.2314	0.1107	41.65	...

*Analysis of Silver Pentabromophenolate.*—A study of the different forms of this salt is being carried on by the authors. The details of preparation will be described in a future publication. The following results are given as an example of several very satisfactory analyses obtained during the course of the investigation:

Sample.	Weight of sample.	Weight of Ag <sub>2</sub> S.	% Ag by analysis.	% Ag calc. from formula C <sub>6</sub> Br <sub>5</sub> O Ag.
I.....	0.3457	0.0781	18.10	18.11
II.....	0.4492	0.0935	18.12	...
III.....	0.2806	0.0584	18.12	...

### Summary.

The cyanide-sulfide method for determining silver in organic compounds is discussed, and several typical analyses are given.

The method is shown to be a valuable one because of its applicability, speed and accuracy.

PASADENA, CALIFORNIA.

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[CONTRIBUTION FROM THE CHEMISTRY SECTION, IOWA AGRICULTURAL EXPERIMENT STATION.]

## THE OCCURRENCE AND SIGNIFICANCE OF MANNITOL IN SILAGE.

BY ARTHUR W. DOX AND G. P. PLAISANCE.

Received July 9, 1917.

### Introductory.

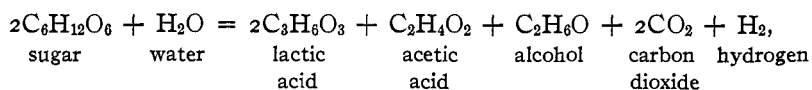
In continuation of our investigation on the fermentation phenomena that occur during the first two or three weeks after corn is put into the silo, and the products resulting from this fermentation, the writers have attempted to account in part at least for the soluble sugar which disappears but cannot be recovered in the form of volatile acid, lactic acid,

carbon dioxide and alcohol. As pointed out in previous publications,<sup>1</sup> the fundamental changes to which silage owes its keeping properties consist in the conversion of the sugar present in the juice of the fresh plant into acids which inhibit the growth of putrefactive bacteria, and carbon dioxide which expels the atmospheric oxygen and prevents the growth of mold.

#### Deficit when Fermentation Products are Balanced against Sugar Fermented.

However, the sum of the products above mentioned is invariably less than the amount of sugar that has disappeared. The following analysis, which is quite typical, will serve by way of illustration. The juice (100 g.) obtained by subjecting a sample of silage taken from the wood stave silo fourteen days after filling, to a pressure of 350 g. per sq. cm. contained 0.67 g. volatile acid, 1.50 g. lactic acid, 0.31 g. alcohol, and 1.22 g. unfermented sugar, total 3.70 g. The juice obtained under the same conditions from the fresh corn at the time of ensiling contained 5.20 g. sugar.

The deficit of 1.50 g. cannot be attributed entirely to carbon dioxide, for the following reasons: Assuming that an alcoholic fermentation first occurs and that the alcohol is subsequently oxidized to acetic acid, the amount of carbon dioxide equivalent to the acetic acid and alcohol present would be 0.76 g., leaving 0.74 g. sugar unaccounted for. However, the proportions of the fermentation products, lactic acid, acetic acid, and alcohol, correspond more closely with those represented by the Harden equation, *viz.*,



from which the equivalent amount of carbon dioxide would be about 0.73 g., leaving about 0.77 g. of the sugar unaccounted for. It is extremely improbable that in silage fermentation a greater proportion of sugar is converted into carbon dioxide than in the case of alcoholic fermentation. On account of the great number of factors involved, variations in the proportions of resulting products are to be expected and the process cannot be reduced to a simple chemical equation. We have analyses in which the deficit is still more striking on attempting to balance the equation. Furthermore, the deficit may be even greater than the analytical determinations indicate, because of a possible transformation of starch during the fermentation. It is thus apparent that other products are formed from sugar contained in the fresh plant.

<sup>1</sup> Iowa Agr. Expt. Sta., *Res. Bull.* No. 7, 10, 16.

### Other Constituents of Silage.

The literature makes mention of various other substances that have been isolated in small amounts from silage and identified. In addition to the typical products already mentioned, Russell,<sup>1</sup> of the Rothamstead Station, reports the presence of malic acid, glycerol, alanine, histidine, lysine, ornithine, pentamethylene diamine and adenine. Of these, only the first three can in any possible way be regarded as derived from sugar, the others being the well-known degradation products of protein. In all probability, the malic acid is derived from aspartic acid, one of the protein constituents, and the glycerol, although occasionally met with among the products of sugar fermentation, may also result from the saponification of fat. Manns,<sup>2</sup> at the Illinois Station, found succinic acid and mannitol in silage. Succinic acid, like the malic acid reported by Russell, is known to result from bacterial fermentation of the aspartic acid of proteins and also from the glutaminic acid from the same source, but there is considerable doubt as to whether it can be produced from sugar in a protein-free medium. Mannitol, on the other hand, is unmistakably a carbohydrate derivative and is known to be an important product in certain fermentation reactions.

### Natural Occurrence of Mannitol.

Of the various isomeric hexatomic alcohols occurring in nature, mannitol is by far the most abundant. Its presence has been demonstrated in a number of the higher plants, among which may be mentioned cactus, olive, pineapple, lilac, onion, carrot, June grass, turnip. It occurs in both higher and lower fungi. The writers have repeatedly obtained mannitol crystals from cultures of some of the common molds. In such cultures the mannitol can hardly be regarded as a fermentation product, but is rather to be considered as a normal constituent of the fungus. It is formed even when the only source of carbon in the medium is a substance not capable of fermenting into mannitol. We have isolated the substance from cultures of *Penicillium expansum* and *Aspergillus niger* on glucose which, by reason of its stereomeric structure, is incapable of yielding mannitol, and from cultures of these organisms on glycerol which contains only half the requisite number of carbon atoms. Other investigators have noted its presence in lichens and marine algae. In many cases, notably the higher fungi, the amount of mannitol increases after bruising or otherwise injuring the plant so as to allow the enzymes present to act upon some precursory substance, as trehalose. The source of the mannitol of commerce is the so-called "manna," a sweet exudation of *Fraxinus ornus*, a tree that grows in southern Europe and Asia Minor. A similar

<sup>1</sup> Russell, *J. Agr. Sci.*, 2, 392-410.

<sup>2</sup> Manns, Ill. Agr. Expt. Sta., *Bull.* 7, 190-3 (1892).

product from *Tamarix mannifera* is supposed to have been the traditional manna of the Hebrews.

### Mannitol as a Fermentation Product.

As early as 1833, Pelouze<sup>1</sup> showed that the juice of the sugar beet when fresh contained no mannitol, but after it had undergone a slimy fermentation considerable mannitol was present. Since that time considerable literature on mannitol fermentation has been published. Numerous organisms have been found capable of producing this substance, chief among which is *Bacillus mannicus*. Mannitol fermentation has been the subject of many investigations, chiefly in relation to the wine industry, where such fermentation is a disturbing factor and is responsible for much loss. According to Gayon and Dubourg,<sup>2</sup> this abnormal fermentation of wine is favored by high temperature and low acidity. Feder<sup>3</sup> found 0.8 to 1.16% mannitol in sauerkraut. The writers recently examined a sample of vinegar, which in the course of four years had developed only 0.65% acid and found a large quantity of mannitol. A patent<sup>4</sup> has even been granted for the manufacture of mannitol by the bacterial fermentation of fructose. A review of the literature will not be attempted here, further than the few citations given above which show that mannitol fermentation has long been known and has an important bearing upon alcoholic and acid fermentations. The reaction by which mannitol is produced under such conditions is a simple reduction of hexose to hexatomic alcohol, whereby two atoms of hydrogen are added to a molecule of the hexose, with the development of considerable heat.

### Experimental.

The presence of mannitol in corn silage was demonstrated by Manns<sup>5</sup> in 1892. However, Manns did not determine quantitatively the amount present, and does not state whether it occurred in sufficient quantity to be of any practical significance. Furthermore, the silage examined by Manns was in poor condition, as stated in his paper, and this fact is clearly shown by the presence of butyric and valeric acids, which together comprised 20% the total volatile acid. This would indicate an unusual and abnormal fermentation, and such silage would have a very offensive odor like rancid butter. During the twenty-five years that have elapsed since the publication of Manns' paper, the literature on silage contains no further reference whatever to the occurrence of mannitol.

We considered it desirable, therefore, to examine a number of silage samples for mannitol and determine approximately the amount present. First

<sup>1</sup> Pelouze, *Ann. chim. phys.*, [2] 47, 409 (1833).

<sup>2</sup> Gayon and Dubourg, *Ann. inst. Pasteur*, 15, 529-61 (1901).

<sup>3</sup> Feder, *Z. Nahr. Genussm.*, 22, 295 (1908).

<sup>4</sup> Guignard, British Patent No. 3261 (1913).

<sup>5</sup> *Loc. cit.*

of all, we examined samples of green corn and corn stover before ensiling and found no evidence of mannitol. On the other hand, various samples of silage that had undergone the usual fermentation in the silo almost invariably yielded the characteristic crystals of mannitol in abundance. The possibility then suggested itself that this might be a purely local phenomenon, due to the prevalence of a particular organism in this region. Accordingly, we secured samples of silage from other states in the Middle West and put them through the same treatment. We wish here to acknowledge our indebtedness to the neighboring experiment stations, for their coöperation in furnishing the desired samples.

#### Method of Isolating Mannitol.

Storer's<sup>1</sup> test for mannitol, which consists in oxidation to mannose and precipitation of the latter as the phenylhydrazone, is qualitative only, for the reason that only a part of the mannitol is converted into mannose. Since no insoluble derivatives of mannitol can be formed in aqueous or alcoholic solution, and no color reactions are known, it is necessary to isolate the substance as such and identify it by its properties. The isolation is effected by direct crystallization, taking advantage of the fact that mannitol is readily soluble in hot alcohol but only sparingly soluble in cold alcohol.

Our procedure is as follows: The silage is spread out and rapidly dried on a steam box, then ground in a power mill. One hundred grams of the dry product are placed in a continuous extraction apparatus of our own device and extracted for ten hours with about 400 cc. alcohol. The dark-colored extract is evaporated to about 100 cc. and on standing overnight a mass of mannitol crystals forms. This impure product is filtered with suction, dissolved in hot water, and filtered. In this way the resinous matter adhering to the crystals is removed and the filtrate has only a light straw color. The filtrate is evaporated to a small volume, about 0.5 cc. concentrated hydrochloric acid added to prevent the separation of magnesium lactate, and three volumes of alcohol added. The mannitol then crystallizes out in snow-white needle-shaped crystals, which give the correct melting point of 169°. The weight of the crystals in grams represents the percentage of mannitol in the dry sample. While such a method is not absolute, we have reason to believe that 85-90% of the mannitol present in the original silage is thus recovered. The mannitol was further identified by analysis in the electric combustion furnace, which gave the following results:

Calc. for  $C_6H_{14}O_6$ : C, 39.56; H, 7.69. Found: C, 39.41; H, 7.79.

#### Mannitol in Silage.

Determinations of mannitol in samples of silage from various sources were made by the above method. The following table shows the amount

<sup>1</sup> Storer, *Bull. Bussey Inst.*, 3, 98-111.

of mannitol actually isolated expressed in percentage of the dry matter:

Date, 1917.	Kind of silage.	Location of silo.	Dry matter, %.	Mannitol, % D. M.
Feb. 4	Corn	Ames, Iowa	38.1	1.94
Feb. 4	Corn	Ames, Iowa	...	1.35
June 1	Corn	Ames, Iowa	30.0	1.89
Mar. 14	Corn	Madison, Wis.	30.0	1.70
Mar. 20	Corn	Lincoln, Neb.	...	2.07
Mar. 21	Corn	Stewart, Minn.	28.0	2.51
Mar. 27	Corn	St. Paul, Minn.	31.0	1.47
Mar. 27	Corn	Urbana, Ill.	32.7	2.15
Mar. 19	Corn	Columbia, Mo.	27.3	0.52 <sup>1</sup>
June 1	Corn	Fayetteville, Ark.	20.1	None <sup>2</sup>
Mar. 20	Cane	Manhattan, Kans.	30.0	3.30
May 17	Sunflower	Bozeman, Mont.	27.1	5.61
Apr. 16	Corn and cow peas	Fayetteville, Ark.	20.0	None
Mar. 2	Sweet clover	Pontiac, Ill.	20.8	None

From the above table it is quite evident that mannitol is a normal constituent of corn silage. The average amount found in the eight samples of normal corn silage, obtained from five states was 1.88% on the dry basis. The cane silage and the sunflower silage contained mannitol greatly in excess of this amount. These two plants contain a higher percentage of sucrose than corn, whereas sweet clover, which yielded no mannitol on fermentation, contains little, if any, sucrose. It was considered probable, therefore, that sucrose, or more specifically its fructose moiety, is the parent substance from which the mannitol is formed. This explanation is in harmony with the work of Gayon and Dubourg, who found that *Bacillus mannicus* produces mannitol from invert sugar and fructose, but not from glucose, galactose, mannose, sorbose, maltose or lactose.

#### Mannitol in Experimental Silage.

It has been shown by Lamb<sup>3</sup> at this station, and by others, that corn and other plants when packed in glass jars under laboratory conditions undergo the typical silage fermentation and the resulting product is often indistinguishable from ordinary silage. This method was made use of in the present work to determine experimentally the origin of the mannitol in silage fermentation.

In some of these experiments quart Mason jars were used, in others the silage was made on a larger scale, using 20-gallon earthenware jars and adding the necessary amount of water. In all the experiments here reported the fermented product possessed the typical flavor and aroma of ordinary silage. Table II sets forth some of the typical results.

<sup>1</sup> Immature corn. Silage was abnormally high in acid.

<sup>2</sup> Silage spoiled in transit.

<sup>3</sup> Lamb. *J. Agr. Res.*, 8, 363.

TABLE II.

Material.	Sugar added.	Length of time ensiled, days.	Mannitol, % D. M.
Corn stover.....		13	None
Corn stover.....		27	None
Corn stover.....	Sucrose	13	2.12
Corn stover.....	Sucrose	30	3.04
Green corn.....		10	1.72
Corn stover.....	Glucose	30	None

Mannitol is therefore not produced by the fermentation of corn stover alone or corn stover to which glucose has been added, but the addition of sucrose results in a copious yield of mannitol. The formation of this substance is due largely, if not wholly, to the action of microorganisms rather than that of the plant enzymes, as is clearly shown by the following experiment: Two one-gallon jars were packed with stover to which sucrose and water had been added, and sterilized in an autoclave at 15 lbs. pressure for 30 minutes. They were then inoculated with a decoction from a leaf of corn silage. One jar was opened after 27 days and the other after 60 days and the amount of mannitol obtained was 3.04% and 3.87%, respectively. On the other hand, a sample of "antiseptic" silage, made with corn and ether and kept eight months, developed about half the acidity of ordinary silage but no trace of mannitol.

In Table II it was shown that corn stover yields mannitol only when sucrose is present. Table III shows the effect of adding other sugars or allied substances. In these experiments quart Mason jars were packed with 100 g. corn stover, 15 g. of the sugar to be tested and the requisite amount of water. A layer of stover was packed above this mixture in order to fill the jar, but this was discarded on opening the jar for analysis.

TABLE III.

Sugar added.	Length of time ensiled, days.	Acidity of product cc. 0.1 N per 10 cc. juice.	Reducing sugar as glucose. Mg. per 10 cc. juice.	Mannitol, g.
Fructose.....	8	36	.....	5.49
Sucrose.....	8	38	.....	3.94
	13	..	.....	2.12
Inulin.....	8	36	.....	Trace
	12	40	77.8	Trace
Glucose.....	8	35	.....	None
	12	36	180.7	None
Galactose.....	8	36	.....	None
	12	38	207.2	None
Lactose.....	8	36	.....	None
	12	36	198.2	None
Glycerol.....	8	28	.....	None

Of the above sugars only the three containing fructose yield mannitol. Fructose itself gives the largest yield, then sucrose, and finally inulin. Sucrose is readily hydrolyzed into fructose and glucose, but the hydrolysis

of inulin into fructose probably occurs only to a slight extent under these circumstances, owing to the absence of the specific enzyme.

#### Rate of Formation of Mannitol in Experimental Silage.

It has been shown in our previous paper that the fermentation of silage is practically complete in two or three weeks after filling the silo, since the characteristic products of fermentation reach their maximum in about this length of time. The deficit noted when the acids, alcohol and computed carbon dioxide are balanced against the loss of sugar would lead us to suppose that the formation of mannitol occurs simultaneously with the typical fermentation. The rate of mannitol formation was therefore studied experimentally. Quart Mason jars were packed with fresh corn that had been grown in the green-house. The corn was not as mature as that ordinarily used for silage and therefore probably contained less sucrose. It was run through a clover cutter and thoroughly mixed to insure uniformity in the contents of the various jars. The jars were sealed and the contents allowed to ferment spontaneously at room temperature. The first jar was opened on the fourth day and a jar every second day thereafter until the twenty-fourth day. Determinations of mannitol were made in each case, and in some cases acid and reducing sugar were also determined.

TABLE IV.

Age of silage, days.	Acidity of juice (10 cc.), cc. 0.1 N.	Sugar in juice (10 cc.), mg.	Mannitol, % D. M.
4	..	....	Trace
6	..	....	0.61
8	..	....	0.73
10	..	....	2.33
12	..	....	3.24
14	42	....	2.87
16	44	79.9	0.64
18	33	25.4	0.62
19	40	24.4	0.62
20	34	34.0	0.51
22	..	36.9	0.20
24	44	38.2	0.21

Plotted in a curve, the data are very striking. There is first a gradual increase in mannitol, then a sudden rise of the curve reaching the maximum on the twelfth day, after which there is a rapid fall. It is evident that the mannitol is formed simultaneously with the acid, and carbon dioxide, but unlike the acid, it then encounters some destructive agent which converts a considerable portion of it into other products, probably acid and carbon dioxide. This is not at all surprising. Many bacteria are known which ferment mannitol, and the literature on the subject is quite extensive. In fact, mannitol is used widely by bacteriologists as a source of carbon in culture media, where it is often found to be of diagnostic value in



distinguishing between species. In an actual silo the percentage of residual mannitol is usually much greater, probably because<sup>1</sup> the maximum reaches a much higher point, owing to the greater proportion of sucrose, or fructose, the parent substance of mannitol, in the more mature plant as ordinarily ensiled. Some bacteria oxidize mannitol into fructose, which if not subsequently fermented, would be included in the determination of residual reducing sugar.

#### **Possible Industrial Utilization of Mannitol from Silage.**

In the quantitative determinations of mannitol recorded in the preceding pages, the silage was first dried, then subjected to continuous extraction with alcohol. If it is desired merely to prepare mannitol on a larger scale from silage without regard to a quantitative yield, the process may be simplified. The mannitol, being readily soluble in water, exists in solution in the juice of the silage. By subjecting the silage to pressure the juice can be separated from the dry matter, the yield varying with the amount of pressure applied. In our own experiments we used a pressure of 350 kg. per sq. cm. From one kilogram of silage 65% moisture and volatile matter, we obtained 460 cc. of juice, a yield of about 70%, and consequently containing 70% of the mannitol present in the silage. By soaking the residue with water and pressing a second time, the yield could be increased considerably. On evaporating the juice to almost the consistency of a syrup and cooling, crystals of magnesium lactate separated. These were filtered off, washed with hot alcohol, and the washings combined with the filtrate. Further alcohol was then added until the filtrate contained about 75% alcohol. After standing a few hours mannitol crystallized out, which was purified in the usual way by recrystallization. From the juice obtained by the first pressing of 6670 g. of silage 30 g. of pure mannitol were obtained. The residue of silage after pressing out the juice in this manner, contains practically all of the protein, fat, starch and fiber and can be used for feeding purposes. The mother liquor from the mannitol crystals could be distilled for recovery of the alcohol and the residue combined with the pressed silage for feeding. Thus the combined residues would contain all the nutritive elements of the original silage, minus the volatile acids, and mannitol. In this way it should be possible to extract the mannitol on a large scale at very little cost.

Mannitol has not yet come into industrial use, probably because of the limited supply and the high price it commands. It yields a nitration product very similar in properties to nitroglycerin. According to Sanford,<sup>1</sup> "Nitromannite is more dangerous than nitroglycerin, as it is more sensitive to shock. It is intermediate in its shattering properties between nitroglycerin and fuminat of mercury. It is not manufactured upon the

<sup>1</sup> Sanford, "Nitro-Explosives," p. 110, D. Van Nostrand Co., 1906.

commercial scale." Prepared by the above method, mannitol should be even cheaper than glycerol, if the residues are utilized as cattle feed, and would serve as a new source for the manufacture of explosives.

#### Summary.

1. Mannitol is a normal constituent of corn silage and of silage made from other plants containing sucrose.
2. It is formed in silage fermentation by bacterial reduction of the fructose-half of the sucrose molecule.
3. It is produced in considerable amount simultaneously with the acetic acid, lactic acid, carbon dioxide and alcohol, the characteristic constituents of silage, then part of it disappears.
4. Its presence accounts in large measure for the deficit noted when the sum of these products is balanced with the fermented sugar.
5. The possible industrial utilization of mannitol from silage is pointed out.

AMES, IOWA.

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[CONTRIBUTION FROM THE CHEMISTRY SECTION, IOWA AGRICULTURAL EXPERIMENT STATION.]

### THE OCCURRENCE OF l-LEUCINE IN SWEET CLOVER SILAGE.

BY G. P. PLAISANCE.

Received July 9, 1917.

The preceding paper contains data on the occurrence of mannitol in various kinds of silage. This substance was readily isolated from silage made not only from corn but also from cane and sunflower. However, no trace of mannitol could be found in the sample of sweet clover silage examined.

Unlike the other legumes, such as alfalfa and clover, sweet clover (*Mellilotus alba*) can be ensiled without the addition of other plants to supply fermentable sugar. Sweet clover silage is at present little known. The examples examined by the writer were kindly furnished by Mr. Roy C. Bishop, County Agent of Livingston Co., Ill., who has had some experience with sweet clover silage and predicts a future for it in the Middle West.

When the samples were subjected to our usual process for the isolation of mannitol, no evidence whatever of this substance was found. Instead, a white substance crystallized in little round masses, which had altogether different properties from mannitol. On recrystallization from dilute alcohol, the product was obtained in snow-white, flat, scaly crystals. Some 18 g. of the substance were obtained from sweet clover silage by the following treatment:

The silage was first dried on a steam box, then ground in a spice mill. The dry product was extracted ten hours with 95% alcohol in a continuous