The Colorimetric Determination of Lactic Acid in Silage

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The fundamental reaction which takes place in the process of ensilage is the conversion of a proportion of the soluble carbohydrates present to lactic acid. This reaction is of such importance that when material of high protein content is being ensiled, it is desirable to add molasses to provide a readily available substrate for the lactobacilli which occur along with coliform organisms in the original grass crop. When lactic acid is formed in suitable amount it acts as a preservative for the ensiled material and serves, on account of the lowering of pH which accompanies its formation, to inhibit the activity of coliform bacteria which produce undesirable by-products.

Despite the importance of this reaction it has never been fully investigated, partly on account of the pre-occupation of many investigators with the question of quality in silage, for the determination of which the pH is a reasonable guide, and partly because the methods used for its estimation are tedious and in some cases inaccurate. For example, the method of Foreman (1920) depends upon a difference calculation which involves the experimental error of the other acid determinations, and gives a result which represents the total non-volatile acid content, of which but a part is lactic acid, a fact which has been stressed by Watson & Ferguson (1937) in their study of the method. The method of Karström & Virtanen (1937), modified from the original techniques of Hirsch-Kauffmann (1924) and Lieb & Zacherl (1932), involves the use of large amounts of material and complicated procedures for the elimination of proteins and carbohydrates.

Miller & Muntz (1938) developed a method, later modified by Barker & Summerson (1941), for the colorimetric estimation of lactic acid which depends upon the fact that lactic acid is oxidized by sulphuric acid to acetaldehyde which gives a purple-red colour with *p*-hydroxydiphenyl in the presence of metallic ions, of which copper has been found to be the most suitable (Barker & Summerson, 1941). The reaction is similar to that of Mendel & Goldscheider (1925) who, however, used *o*-dimethoxybenzene as the colour-producing reagent.

Davidson (1949) has used the Barker & Summerson technique for the determination of lactic acid in milk and milk products, demonstrating its applicability to amounts of 20–400 mg. lactic acid. In the case of these latter, and blood or minced tissue, steps have to be taken to remove the protein matter involved. Silage, however, may be looked upon as solid matter covered with, or impregnated with, lactic acid to a greater or lesser extent and the amounts of protein extractable by water from silage are relatively small. Of the 5 g. portions of fresh silage which are used in the method of analysis described below, less than 0·4 g. is protein and of this only a part is soluble in water. This small proportion may be removed from the prepared extract at the same time as the soluble carbohydrates.

The technique of Barker & Summerson, as used for the determination of lactic acid in silage, is described below.

REAGENTS

Standard lactic acid solution. Pure zinc lactate (0·0135 g.) is dissolved in 10 ml. of distilled water contained in a 1 l. standard flask. Conc. H_2SO_4 (0·5 ml.) is added and the volume made up to the mark with distilled water. This solution is stable but should be kept in a refrigerator.

p-*Hydroxydiphenyl reagent.* *p*-Hydroxydiphenyl (1·5 g.) is dissolved in 10 ml. 5% (w/v) aqueous NaOH and made up to a litre with water. This reagent is also stable but should be stored in an amber bottle.

Calcium hydroxide. The brand used is Judex.

Copper sulphate solutions. Two $CuSO_4$ solutions are required, one 0·80 M and the other 0·16 M. These are prepared from A.R. $CuSO_4.5H_2O$.

Concentrated sulphuric acid. It has been shown by Russell (1944) that nitrates and nitrites interfere with the reaction, and for this reason the N-free H_2SO_4 essential for Kjeldahl determinations is used.

METHOD

Of the representative sample of silage (about 3 kg.) brought to the laboratory for examination, about 100 g. taken from the centre of the mass is finely chopped and treated after the fashion described below. A further 100 g. portion is mixed with 100 ml. of distilled water in a beaker and set aside for 24 hr. pending a pH determination on the extract. A dry-matter determination is performed on approximately 1 kg. of the material by drying this at 80° for 24 hr.

Of the chopped material two 5 g. portions are placed in two 250 ml. beakers and 100 ml. of boiling distilled water added and the whole allowed to cool

for 5 min. Each preparation is treated in the following way. The mixture is stirred mechanically very vigorously for 5 min., during which time about 0·5 g. of solid $Ca(OH)_2$ is added to assist the subsequent filtration. The product is filtered through a hard filter paper and the first 20 ml. of filtrate are discarded. Of the filtrate 2 ml. are taken and diluted to 10 ml. with distilled water, and of this solution 1 ml. is placed in a small glass-stoppered bottle to which are added, in the following order, 8 ml. of distilled water from a burette, 1 ml. of 0·80M-$CuSO_4$ and 1 g. of $Ca(OH)_2$. A standard for comparison is prepared by using 5 ml. of standard lactic acid solution and making this up as described for the test portion, using only 4 ml. of distilled water. Similarly, a blank is prepared by using 1 ml. of water instead of the unknown. The four bottles are then stoppered and shaken for 20 min. in a small mechanical shaker, after which time the contents are filtered through small filter papers discarding the first 2 ml. of filtrate in each case. The remainder of each filtrate is collected in small test tubes. Each filtrate is treated as follows: The filtrate (1 ml.) is placed in a Pyrex tube (200 × 30 mm.) and mixed with 0·05 ml. of 0·16M-$CuSO_4$ and 6 ml. of N_2-free H_2SO_4 added from a burette with grease-free tap. The tubes are allowed to stand in boiling water for 5 min. and are then cooled by standing in a stream of running water. To each tube is added 0·1 ml. of the *p*-hydroxydiphenyl reagent and the contents shaken laterally to ensure even distribution of the insoluble reagent. The tubes are then placed in a water bath for 30 min. at 30–33°, being shaken at 10 min. intervals. Finally, the tubes and their contents are heated for 2 min. in a bath of boiling water and cooled to room temperature. Readings of the colour developments are made on an EEL (Evans Electroselenium Ltd., Harlow, Essex) photoelectric colorimeter using the standard green filter supplied by the makers and water as a zero. As the 1 ml. copper-containing portion of standard lactic acid contains 0·005 mg./1 ml. portion used, the concentration of lactic acid in the unknown is given by the formula:

% Lactic acid in fresh silage

$$= \frac{\text{Reading for unknown } (-\text{blank})}{\text{Reading for standard } (-\text{blank})} \times 0{\cdot}5,$$

and for the amount of lactic acid based on the dry matter (DM) content of the silage:

% Lactic acid

$$= \frac{\text{Reading for unknown } (-\text{blank})}{\text{Reading for standard } (-\text{blank})} \times \frac{50}{\%\text{DM content}}.$$

The results of a control experiment to test the accuracy of the method are indicated in Fig. 1, while recovery of lactic acid from mixtures of known standard and unknown varies from 96 to 102 %, and the only precaution required is absolute cleanliness as, owing to the sensitiveness of the reaction, impurities which give rise to lactic acid on heating with H_2SO_4 nullify the results. All glassware used

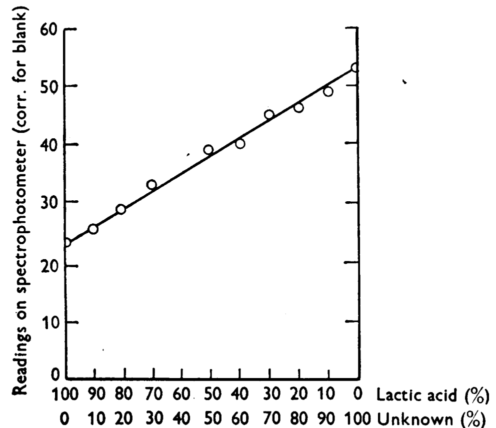

Fig. 1. Spectrophotometer readings for mixtures of standard lactic acid solution and silage extract as prepared for analysis in percentages shown (by vol.). For details of procedure see Text.

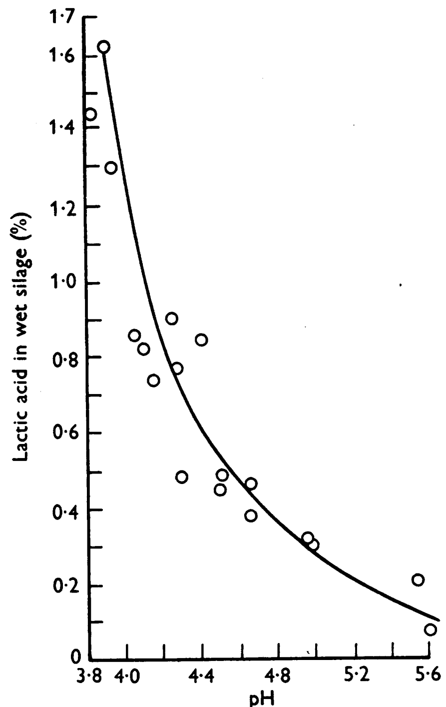

Fig. 2. Relation of lactic acid content of wet silage to pH.

is successively washed with dil. HCl, warm water with a little detergent, cold water and finally dried in an oven.

RESULTS

The silage specimens on which data are reported were from grass and grass-clover mixtures ensiled during the summer of 1950 on various farms in the north-east of Scotland, and were taken at random from specimens being analysed here.

It is seen from Fig. 2 that the lactic acid content of silage falls rapidly with rise of pH. This is in accordance with the generally held view that in silage of pH greater than 4·2 the amounts of lactic acid are small. It has not been definitely shown, however, whether or not lactic acid is formed in all silage but is subsequently destroyed in silage of high pH. An investigation into this problem is the subject of further work.

SUMMARY

The use of the procedure of Barker & Summerson (1941) for determining lactic acid is described in its relation to the estimation of this acid in silage.

It is desired to thank Mr John Baxter for his assistance in the technical side of the present work.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Davidson, J. (1949). *J. Dairy Res.* **16**, 209.
Foreman, F. W. (1920). *Biochem. J.* **14**, 451.
Hirsch-Kauffmann, H. (1924). *Hoppe-Seyl. Z.* **140**, 25.
Karström, K. & Virtanen, A. I. (1937). *Ann. Acad. Sci. Fenn.* **48**, no. 14.
Lieb, H. & Zacherl, M. (1932). *Hoppe-Seyl. Z.* **211**, 211.
Mendel, B. & Goldscheider, I. (1925). *Biochem. Z.* **164**, 163.
Miller, B. F. & Muntz, J. A. (1938). *J. biol. Chem.* **126**, 413.
Russell, J. A. (1944). *J. biol. Chem.* **156**, 463.
Watson, S. J. & Ferguson, W. S. (1937). *J. agric. Sci.* **27**, 1.

Transfructosidation in Extracts of the Tubers of *Helianthus tuberosus* L.

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In the artichoke tuber there is present a mixture of carbohydrates, of which sucrose is the component with smallest molecular weight, and inulin probably the largest (Bacon & Edelman, 1951). Since the only enzyme system which is known to produce a fructose polysaccharide *in vitro* (levansucrase; Hestrin, Avineri-Shapiro & Aschner, 1943) does so from sucrose, it seemed worth while to investigate the possibility that inulin was formed from sucrose, and with this aim in view the action of tuber extracts on sucrose was studied.

The results presented in this paper show that a reaction takes place in tuber extracts by which oligosaccharides are synthesized from sucrose at the expense of fructose residues from inulin and related polysaccharides. The name 'transfructosidation' is proposed for this type of reaction. A preliminary account was given to the Biochemical Society at Edinburgh on 21 July 1950 (Edelman & Bacon, 1950).

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METHODS

The methods were those described by Bacon & Edelman (1951) and Edelman & Bacon (1951).

Dandelion inulin (Thomas Kerfoot and Co. Ltd., Vale of Bardsley, Ashton-under-Lyne) was purified as described by Edelman & Bacon (1951). Sucrose was recrystallized one or more times from 95% aqueous ethanol. Both carbohydrates were shown by paper partition chromatography to be free from other oligosaccharides and free hexoses.

All incubations were carried out in the presence of $CHCl_3$ unless otherwise stated.

RESULTS

Initial investigation

Qualitative experiments. It was assumed that any synthetic activity in the presence of sucrose would lead first to the production of oligosaccharides behaving, on the paper partition chromatogram, like spots 2, 3 and so on (Bacon & Edelman, 1951). Since these substances were already present in the tubers, dialysis was carried out to remove them.