XIV. THE ESTIMATION OF SUCCINIC ACID.

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(Received May 17th, 1917.)

The original method employed by Pasteur for the separation of succinic acid from other acids produced during fermentation consisted in extracting the calcium salts with a mixture of alcohol and ether. The calcium succinate is insoluble and the salts of certain other acids dissolve. The method as now usually employed consists in adding to a concentrated solution of the calcium salts in water, sufficient alcohol to bring the final concentration to 85 %. The amount of succinate is estimated from the determination of the calcium in the precipitate.

The ordinary method is liable to serious errors, and probably the experience of inconstant results has led certain authors to discard it in favour of a method based on the use of some other salts such as the salts of barium. As a matter of fact however the errors to which the Pasteur method, as generally used, is liable, do not arise from any unsuitability of the calcium salt, but from other causes which are as follows:

- (1) Loss of succinic acid during concentration. Succinic acid volatilises, even from a solution which is alkaline to phenolphthalein. The loss may easily be 7 %.
- (2) The solvent action of 85 % alcohol containing traces of peptone on calcium succinate. 0.15 g. of peptone in 100 cc. of the solution may introduce an error of 10 to 15 %.
- (3) The presence of any substance which prevents the crystallisation of the calcium succinate.
- (4) Acidity of the alcohol employed. The alcohol must be neutral.
- (5) Loss through washing the precipitate.

The freshly formed precipitate of calcium salts is flocculent and in the presence of certain impurities such as peptone this condition persists. In the absence of impurities the precipitate gradually becomes crystalline. This change may be taken as an indication that the separation is quantitative. The errors referred to above may be avoided by attending to the following details.

In the case of the fermentation products studied by the writer, lactic acid is the only acid which remains to be separated from succinic acid after the volatile acids have been removed, but the method described here applies equally well to cases where other acids are present, provided the calcium salts of such acids are soluble in 85 % alcohol.

The mixture of succinic and lactic acids obtained by extracting the acidified solution of the non-volatile products of fermentation by ether, in a suitable continuous extraction apparatus, is dissolved in about 100 cc. of hot water and chalk added in excess. The solution should not be boiled as loss of succinic acid will occur by volatilisation. The neutralisation takes place slowly owing to the presence of the anhydride of lactic acid.

The final neutralisation is effected by adding a solution of calcium hydroxide after the addition of phenolphthalein. The solution is not neutralised from the start by lime water to avoid excessive dilution. The solution is kept hot during at least an hour to ensure the thorough conversion of the anhydride into calcium lactate, and the solution, distinctly pink, is filtered into a graduated flask of 200 cc. capacity. Of the filtrate 50 cc. is used to estimate the calcium corresponding to the total acids, *i.e.* succinic plus lactic and possibly other acids, the calcium being precipitated as oxalate and the oxalic acid equivalent of the calcium determined by titration with a standard solution of potassium permanganate. A suitable portion of the remaining solution is concentrated slowly to a volume of 13 cc.

This is done in a 110 cc. graduated flask in an oven kept at a little over 100°. Five or six hours are required for the concentration of 50 cc. of the solution. The author employs a series of tared 110 cc. graduated flasks and continues the evaporation until the flasks contain the required 13 g. of water. Alcohol is then added to make up the volume to 110 cc. which in the case of 96 % alcohol brings the final concentration to 85 %. The flask is well shaken and allowed to stand until the flocculent precipitate has become crystalline. This may require till next day but takes place in a few hours if the solution is free from impurity. The solution is filtered into a 100 cc. graduated flask, and after removal of the alcohol, the calcium is estimated in

this 100 cc. This calcium figure multiplied by 11/10 gives the amount of calcium corresponding to the acids of the soluble calcium salts, and the amount of succinic acid is obtained by subtracting the figure thus obtained from that previously found for the total acidity. The error of the experiment need not exceed 1 % of the weight of succinic acid present.

Should the amount of acids present be small, the estimation of the total calcium need not be made on a separate sample but after filtering the 100 cc. of the alcoholic solution the remaining 10 cc. of alcohol together with the precipitate of calcium succinate may be dissolved in water and the calcium determined. From this figure the amount of calcium corresponding to 10 cc. of the alcoholic solution is deducted, the difference representing the calcium in the precipitate¹.

The following example of the separation of a mixture of lactic and succinic acids may be given.

A mixture was made of 40 cc. of a solution of lactic acid (corresponding to 59.4 cc. N/10 lactic acid) and 30 cc. of a solution of succinic acid (corresponding to 26.25 cc. N/10 succinic acid) and the 70 cc. was extracted in a continuous apparatus by ether during nine hours. The extracted acids were treated as described above and the volume of the solution of the calcium salts made up to 200 cc. 50 cc. of this solution was brought to the boil and precipitated by oxalic acid. The calcium oxalate precipitate required for oxidation 21.1 cc. N/10 permanganate solution. The total acidity found therefore corresponds to 84.4 cc. instead of 85.6 cc. There is here an error of 1.5 %.

Another 50 cc. was evaporated to 15 cc. in a tared graduated flask and the volume made up to 110 cc. with 98 % alcohol. 100 cc. of the filtrate after removal of alcohol was precipitated by oxalic acid and yielded calcium corresponding to 13.15 cc. N/10 permanganate solution. This corresponds to 14.45 cc. for 110 cc. of the alcoholic solution. The total lactic acid is therefore 57.8 cc. N/10, which figure deducted from 84.4 gives 26.6. The amount of succinic employed was 26.25.

¹ *Note on the estimation of calcium by the permanganate method.* It is necessary for me to point out that the procedure which certain authors (Cahen and Hurtle, *Biochem. Journ.* 1916, 10, 308) have adopted in connection with the well-known method of estimating calcium oxalate by permanganate is to be strictly avoided. The correct method of procedure is to filter the calcium oxalate upon asbestos in a suitable vessel, such as the ordinary Gooch crucible, and, after washing it, to decompose it by sulphuric acid in the ordinary way. The washing takes a few seconds and there is certainly no need for the seven washings of which these authors speak. But whatever procedure is adopted for collecting and washing precipitates, there can be no justification for boiling paper with sulphuric acid solution and titrating this with permanganate. Nothing must be oxidised except the oxalic acid.

This shows that all the manipulations can be carried out with an error less than 2 %.

The actual separation of the two acids need not involve an error of more than 1 %, as will be seen from Appendix, Ex. 2 (4, 5, 6).

APPENDIX.

Experiments demonstrating the magnitude of the errors referred to in the communication.

Ex. 1. Loss of lactic and succinic acids during the evaporation of the slightly alkaline solutions of the calcium salts.

Pasteur in describing the original method indicates the necessity of slow evaporation to avoid loss of succinic acid. It seemed to the writer of interest to know whether this loss occurred even when the solution was kept alkaline throughout the evaporation.

200 cc. of a solution of calcium lactate corresponding to 55.2 cc. N/10 acid, was evaporated on the water bath in an open vessel, the solution being kept alkaline throughout by the addition from time to time of a solution of calcium hydroxide; the dried residue was dissolved in water and the solution filtered from calcium carbonate. The filtrate contained calcium corresponding to 52.75 cc. of lactic acid. This represents a loss of 2.45 cc. N/10 lactic acid, or approximately 5 %.

Under the same conditions 36.6 cc. N/10 succinic acid lost 2.85 cc. or 8 % of the succinic acid employed.

To avoid such errors the evaporation should be made as directed in this communication in a long necked graduated flask in an oven regulated so that the temperature of the air is about 110°; under these circumstances there is no loss.

Ex. 2. The effect of peptone.

Peptone may introduce a grave error. It prevents the precipitation of calcium succinate by 85 % alcohol. Unneutralised peptone will dissolve calcium succinate readily, but the experiments here described refer to peptone which has been neutralised by boiling with chalk.

Into each of six tared graduated 110 cc. flasks were introduced 10 cc. of a solution of calcium lactate corresponding to 12.95 cc. N/10 lactic acid, and 10 cc. of a solution of calcium succinate corresponding to 6.85 cc. of N/10 succinic acid.

In addition

to No. 1 was added peptone 0.170 g.

„ 2 „ „ „ 0.023

„ 3 „ „ glycerol 0.70

„ 4 „ „ glucose 0.42

Absolute alcohol was added to each to make the volume up to 110 cc.

The calcium in solution in 100 cc. of the filtrate, was determined in each case.

No.	Substance added	KMnO ₄ N/10 for 100 cc. of filtrate	Total calcium in solution	Calcium in precipitate corresponds to
1	Peptone, 0.17	13.8 cc.	15.1 cc.	4.7 cc.
2	„ 0.023	12.8	14.1	5.7
3	Glycerol, 0.70	12.2	13.4	6.4
4	Glucose, 0.42	11.75	12.9	6.9
5	Control	11.75	12.9	6.9
6	„	11.90	13.1	6.7
		Succinic acid employed		6.85

This experiment shows that glucose does not affect the precipitation. There is a small effect due to glycerol, but the amount here added is about ten times that which would be present after a fermentation even if the whole of the glycerol were allowed to remain as an impurity. The error due to this cause is therefore negligible. On the other hand even 0.023 g. of peptone in 100 cc. of the fluid, may introduce an error of 15 % upon the amount of succinic acid estimated.

SUMMARY.

By avoiding the errors described in this communication and following accurately certain details, succinic acid may be estimated in the presence of lactic acid, by the Pasteur method, with an error not greater than that of ordinary volumetric analysis.

In conclusion I beg to express my very best thanks to Professor Fernbach in whose laboratory this work was done.