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TESTS OF A WET OXIDATION AND MODIFIED VOLHARD METHOD FOR THE DETERMINATION OF CHLORIDES IN PLANT TISSUE FLUIDS

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Introduction

While the determination of the chloride content of tissues or tissue fluids has been considered in numerous papers by animal physiologists and biochemists,[1] practical experience has shown that the problem of chloride determination in plant tissue fluids is somewhat different from that in blood. It therefore seems desirable to present briefly the results of some of the tests which have been made to establish the theoretical soundness and the practicability of the method which we have used for the past three years, and which was developed as the result of several years' effort to secure the best method for use in botanical investigation.

The method which we have finally adopted involves merely (*a*) the precipitation of all the chlorides by the addition of known excess of silver nitrate solution, (*b*) the destruction of all organic compounds (and hence the freeing of all chlorine which may be in organic combination as well as the decolorization of the fluids) by digestion with concd. nitric acid at low boiling temperature, and (*c*) the determination of the excess of silver nitrate by titration with a standard solution of potassium thiocyanate, using ferric alum as an indicator.

Experimental Part

The problems presented in work on plant tissue fluids, with adequate demonstration of the theoretical correctness and the practical applicability of the methods for their solution are as follows.

The Elimination of Coloring Matter.—It is not obvious in advance that all plant pigments will be readily eliminated by any one method of treatment. Experience which many hundreds of determinations involving a very wide range of plant species has, however, shown that this is readily accomplished by boiling with the concentration of nitric acid recommended below for a few minutes to three hours.

The Removal or the Destruction of the Proteins or Other Substances which May Fix Certain Quantities of the Silver.—Experience with a wide range of plant saps has shown that substances which fix a certain quantity of the silver used in direct titration in the presence of an indicator or added

[1] A review of this literature, the most of which has appeared or has been cited in the *Journal of Biological Chemistry*, 1915–1923, seems unnecessary here.

in excess to precipitate the chlorides as silver chloride before titrating back with potassium thiocyanate (with a resulting indication of too large quantities of chlorides) may be readily altered so that they do not interfere with the determination of the chloride content by digestion on a sand-bath with concd. nitric acid.

In order to determine the efficacy of this method when applied to saps richer in protein than any of the plant tissue fluids which had been included in our series, a 1% solution of egg albumen was added to plant saps in the amount of half the volume of the sap and in the same volume as the sap. As a more rigorous test, determinations have been made on blood, on extracts of hide, and even on ground hides in aqueous suspension. All these experiments have shown, both by the visible characteristics of the solution after digestion and by the consistency of the analytical results (to be noted in the following sections) that the proteins or other substances which fix silver or interfere with titration may be readily eliminated as a source of error. The removal of proteins by precipitation need not, therefore, receive consideration. It is not to be recommended in work with plant saps since it is merely an unnecessary and time-consuming process.

The Most Suitable Relationship between the Quantity of Silver Nitrate and the Amount of Nitric Acid Employed.—When too small an amount of silver nitrate is added before digestion all of the chlorides cannot enter into combination with silver to form silver chloride, and experience shows that chlorides are lost as hydrogen chloride or chlorine on boiling with nitric acid. When the quantity of nitric acid is insufficient to free the silver nitrate previously added from the organic combination into which some of it may have previously entered, too large quantities of chlorides may be indicated,[2] since all of the silver nitrate will not be available for precipitation as silver chloride. Sufficient nitric acid should be used to destroy completely the original coloring matter and all other organic material of the sap, and to give a solution sufficiently acid for the realization of a sharp end-point in titration with potassium thiocyanate and iron alum. If this be done there will be no difficulty from this source.

A series of 12 fractions of sap to which egg albumen and sufficient sodium chloride solution had been added to increase the sodium chloride from approximately 0.0226 g. per sample to approximately 0.1178 g. per sample were digested with various amounts of nitric acid after the addition of silver nitrate equivalent to about 0.25 g. of sodium chloride (and, therefore, more than sufficient to precipitate all the chlorides present). The deviations of the individual analyses from the general average varied from —0.7

[2] In a number of experiments in which very small quantities of nitric acid were used for digestion, we have obtained indications of chloride contents abnormally high for the saps under investigation. It does not seem necessary to give constants for these or to discuss them in detail.

mg. of sodium chloride[3] to +0.5 mg. a range of analytical results of only 1.2 mg. for a range of nitric acid of 3 to 50 cc. per sample.

In a second set of experiments carried out in the same manner except that 5 cc. of approximately 1% egg albumen was added to each of 12 individual samples of 10 cc. of cotton-leaf tissue fluids, to which some sodium chloride had been added, the analyses showed deviations from the general average of —0.8 to +1.4 mg., a range of analytical results of only 2.2 mg. for volumes of nitric acid varying from 3 to 50 cc.

A third series of 10 analyses based on 10cc. samples digested with 2.4 to 20 cc. of concd. nitric acid gave analytical results deviating by not more than 0.4 mg. from the general average.

Finally, additional series analyzed with various quantities of sodium chloride and silver nitrate added before digestion and of nitric acid employed in digestion, show reasonably good recoveries, thus indicating a fairly wide allowable range of variation in quantities of silver nitrate and nitric acid employed.

We have already indicated that the amount of nitric acid used in digestion must be sufficient to destroy all organic matter and to give a sharp end-point in the titration. Experiments with quantities far in excess of this amount have failed to show any great error.

Simplification of Technique by Titration in the Presence of Silver Nitrate.—In all of our earlier work with the present method we observed the usual precaution of filtering off and carefully washing the precipitated chloride, and titrating the filtrate. Since the work of Rosanoff and Hill[4] it has been generally conceded that titration in the presence of silver chloride leads to erroneous results. Sutton[5] expressly warns against this source of error.

Filtration is, however, the most time-consuming part of the whole analytical procedure. The theoretical objection to eliminating it lies in the fact that silver chloride is more soluble than silver thiocyanate and the precipitated silver chloride as well as the excess of silver nitrate in solution reacts with the thiocyanate used in titration. Rosanoff and Hill have shown that when silver chloride is shaken with an equivalent solution of ammonium thiocyanate the reaction is so rapid as to precipitate 43% of the thiocyanate in two minutes.

Harvey[6] has shown, however, that both ferric alum and nitric acid retard the reaction of precipitated silver and thiocyanate. Whitehorn[7] has applied this principle in blood analysis, apparently with good results. Van Slyke[8] has also suggested the application of this principle, with destruction of the proteins by boiling with nitric acid, for blood.

[3] All of the analytical results are expressed in terms of sodium chloride, since it is immaterial for the purpose of the present paper whether sodium was the base with which the chloride was associated in the tissue fluids.

[4] Rosanoff and Hill, THIS JOURNAL, **29**, 269 (1907). See this paper for earlier literature.

[5] Sutton, "Systematic Handbook of Volumetric Analysis," Blakiston and Co., Philadelphia, 10th ed., **1910**, p. 143.

[6] Harvey, *Arch. Internal Med.*, **6**, 12 (1910).

[7] Whitehorn, *J. Biol. Chem.*, **45**, 449 (1921).

[8] Van Slyke, *ibid.*, **58**, 523 (1923).

The reason for the possibility of titration in the presence of silver chloride and nitric acid suggested by Harvey and by Whitehorn is that in the presence of nitric acid, silver chloride flocks out of solution in relatively large masses, and that because of the relatively small surface of these masses the reaction between the silver chloride and the potassium thiocyanate proceeds too slowly to be of importance in influencing the determination.[9]

In the method here described the pigments and proteins are destroyed and the silver chloride is precipitated by boiling with nitric acid in the presence of an excess of silver nitrate. This tends to conglomerate the silver chloride particles into masses. If the method of titration in the presence of precipitated silver chloride is legitimate under any conditions, it should be so under those of the procedure here suggested.

To test the accuracy of titration in the presence of rather large quantities of silver chloride, two series of analyses were made.

In the first a composite sample of tissue fluids, representing many species and habitats in southern Florida, was diluted with about one-third its volume of water and a series of fractions analyzed in groups of three or four after the addition of various known quantities of sodium chloride sufficient to make the chloride contents varying from 0.052 to 0.45 g. per sample. In Fractions *a* and *b* of each number the silver chloride was filtered off, while in Fractions *c* and *d* titration was carried out in the flask in which the silver chloride had been precipitated in the presence of boiling nitric acid.

The test of the influence of titration in the presence of silver chloride as against titration after this precipitate has been removed by filtration is best made by averaging the results of the two analyses made by the same method (except when one was accidentally lost by breakage) and taking the differences between the average result of titrating by the two methods. In no one of the five comparisons did the difference in chlorides indicated exceed 0.7 mg. of silver chloride per 10 cc. of sap.

In a second series of comparisons the largest difference between determinations made in the presence of and after the removal of silver chloride is 0.8 mg. per 10cc. sample. Finally, in a third group of experiments involving plant saps to which egg albumen had been added and extract of hides (used because of their large protein content) the maximum difference between the quantities of chlorides found with and without the removal of the AgCl was 0.3 mg. per sample.

The foregoing tests show conclusively *that when the silver chloride is precipitated in masses by boiling with nitric acid,* the titration may be safely carried out without the removal of the precipitate.

This reduces the method to one of extreme simplicity.

Solutions Required.—*Nitric acid;* a chloride-free, concentrated acid; 1.42. *Indicator solution;* a 40% solution of ferric ammonium sulfate. *Standard silver nitrate;* 1 cc. of a 0.1 *N* solution of silver nitrate is equivalent to 0.005848 g. of sodium chloride or to 0.003546 g. of chlorine. The absolute titer should be more accurately ascertained by checking the solution against three separate sodium chloride solutions. *Standard*

[9] Rosanoff and Hill, Ref. 4, worked with silver chloride freshly precipitated from the solution of the nitrate by hydrochloric acid. Our own experience has shown that accurate titrations cannot be made in the presence of finely precipitated silver chloride.

potassium thiocyanate; since thiocyanates are very hygroscopic a 0.1 *N* solution is standardized volumetrically against the silver nitrate by proper dilution until 1 cc. is exactly equivalent to 1 cc. of the silver nitrate standard.

Analytical Procedure.—A known volume[10] of tissue fluid is transferred to a 300cc. Pyrex florence flask. A quantity of standard silver nitrate solution more than sufficient to precipitate all of the chlorides as silver chloride is then added from a standardized pipet.[11] An approximately equal quantity of concd. nitric acid is then added from a roughly calibrated buret. The flasks are next heated, preferably on a sand-bath, at low boiling temperature, until the color is destroyed and until no solid matter remains except the white or bluish-white precipitate of silver chloride.[12] This is usually accomplished in a few minutes, but may require two hours.

After this has cooled, about 2 cc. or more of concd. iron alum solution is added as indicator, and the excess of silver nitrate is titrated in the flask[13] with standard potassium thiocyanate, until the definite salmon-red (*not yellow*) color of the ferric thiocyanate persists in spite of stirring for at least 15 seconds. The end-point should definitely appear or disappear on the addition of one drop of potassium thiocyanate or one drop of silver nitrate solutions, respectively.

Accuracy of Method.—The validity of the method has been extensively tested by the quantitative recovery of sodium chloride added to plant tissue fluids.

Tests of the consistency of analytical results are available from foregoing analyses which have been made for studying the various features of the method. Summarizing these with special reference to the problem of analytical accuracy, we may note that in a first series of 12 analyses on fractions of sap and egg albumen, the individual analyses show a deviation of

[10] For most plant tissue fluids, 5 or 10 cc. is most satisfactory. Determinations may be made on freshly expressed fluids or on those which have been preserved by sealing measured volumes in glass tubes, after the addition of a drop of formaldehyde.

[11] Experience indicates that, except in the case of plants of extremely saline regions, 10–20 cc. of standard silver nitrate is sufficient for 10 cc. of sap. In dealing with one type of material there is generally no difficulty in introducing the proper amount of silver nitrate at the beginning. In a heterogeneous series of samples in which the chloride content is not approximately known, the flasks containing the sap, silver nitrate and nitric acid are set aside for six to twelve hours. After this time additional silver nitrate is run in from a buret in small recorded quantities and any additional precipitation of silver chloride is noted. If additional precipitate is formed another portion of silver nitrate equal to the original amount is added from the buret and the flask again set aside. This process is repeated until no further precipitation of silver chloride is noted on the addition of silver nitrate.

[12] The color of the remaining solution should be a light amber.

[13] This obviates entirely errors incident to transfer of liquids or the taking of aliquot fractions.

—0.8 to +1.4 mg. from the general average of 0.1468 g., with a general average deviation of ±0.26 mg. In a second series of 12 analyses the deviations from the general average of 0.1178 g. of sodium chloride per 10cc. sample are from —0.7 to +0.5 mg., with a general average deviation of ±0.22 mg.

In a third series of analyses based on 10 samples of tissue fluids digested with various amounts of nitric acid, the individual analyses differ by not more than 0.4 mg. from the general average of 0.0454 g. per 10 cc. of fluid.

These determinations were made by filtering off the precipitated silver chloride. Titration of seven fractions of sap (containing 0.0274 g. of sodium chloride per 10 cc. to which additions of various known amounts had been made) in the presence of the silver chloride showed a maximum analytical error of 0.8 mg. These recoveries have been confirmed in a second, but smaller, experiment.

Finally, a few tests of the accuracy of the method as applied to extracts of hides and to blood, with titration in the presence of silver chloride, indicate in no case an error of analysis greater than 0.4 mg. of sodium chloride, if we accept the average value of 0.02145 g. of sodium chloride per 10 cc. of hide extract and 0.05470 g. of sodium chloride per 10 cc. of blood[14] as the chloride content of these fluids.

This work was largely carried out in the Hull Laboratories in the winters of 1919–1920 and 1920–1921. We are indebted to Dr. Fred C. Koch for advice and aid during the progress of this investigation, and for helpful criticism of the manuscript.

Summary

Tests of the theoretical soundness[15] of a method for the rapid and accurate determination of chlorides in plant tissue fluids are given.

Organic substances that introduce a source of error by fixing some of the silver added to tissue fluids to precipitate the chlorides as silver chloride and pigments which preclude the detection of end-points in titration may be effectively and conveniently eliminated as sources of error by boiling with concd. nitric acid.

When silver chloride is precipitated from solution in this way it is conglomerated into masses, as noted by Whitehorn, and titration of the excess of silver nitrate may be carried out in the presence of the precipitated silver chloride.

The method which we have developed and used extensively for plant tissue fluids gives excellent results for blood chlorides but is not recom-

[14] While accurate recoveries of chlorides can be made from blood, the method here described is not recommended for this purpose since the destruction of the hemoglobin is very time-consuming.

[15] The practicability of the method has been demonstrated by many hundreds of analyses made on a wide range of plant materials during the past three years.

mended for this purpose,[16] because of the resistance of the hemoglobin to the action of nitric acid. That it is applicable to fluids of much higher protein content than that found in plant saps is shown by the application of the method which has already been made in the analysis of hides.

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THE BECKMANN REARRANGEMENT

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When benzophenone oxime is treated with reagents such as phosphorus chloride, concd. sulfuric acid, or a mixture of acetic acid, acetic anhydride and hydrochloric acid (Beckmann's mixture), and thereupon treated with water, it is converted into benzanilide:

$$(C_6H_5)_2C{=}NOH{=}C_6H_5CONHC_6H_5 \qquad (1)$$

A phenyl group has migrated from carbon to nitrogen. The mechanism of this rearrangement has been the subject of a discussion far too voluminous to be considered here in detail. It is sufficient to summarize briefly the two theories which rest more or less upon experimental support.

According to Beckmann,[1] the rearrangement is simply a direct interchange of radicals as shown in the following scheme.

$$\begin{array}{ccc} C_6H_5{-}C{-}C_6H_5 & C_6H_5{-}C{-}OH & C_6H_5{-}C{=}O \\ \| & \| & | \\ N{-}OH & N{-}C_6H_5 & NHC_6H_5 \end{array} \qquad (2)$$

The reagents are considered to be mere catalysts, and Beckmann has given no further account of how they catalyze. This view finds its strongest support in the behavior of stereo-isomeric oximes; for in the great majority of cases, the radical which wanders from carbon to nitrogen is the one which is spatially near the hydroxyl group.

Stieglitz has given a more detailed interpretation of the process, in a series of brilliant experimental and theoretical papers extending over a period of thirty years. These investigations group a variety of rearrangements of nitrogen compounds under one comprehensive viewpoint. Here we shall deal only with Stieglitz's explanation of the oxime rearrangement.[2]

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[16] After this was written, a paper by Van Slyke appeared [*J. Biol. Chem.*, **58**, 523 (1923)] in which he has suggested for blood essentially the same method which we have used for plant saps. We still feel that, because of reasons set forth above, the method is not to be recommended for general use in blood chemistry.

[1] Beckmann, *Ber.*, **27**, 300 (1894).

[2] Stieglitz's papers are too numerous to cite individually. He has given a summary of his theories recently, THIS JOURNAL, **44**, 1293 (1922). The details quoted here were taken from manuscript notes by him, communicated to Professor C. W. Porter, and incorporated in the latter's textbook (The Carbon Compounds) just issued (Ginn and Co., **1924**).