evidence in this field to support Werner's ammonium or "coördination" hypotheses, or any modifications of them. Nor, are any facts known to substantiate "electronic" conceptions, or the existence of "electromers," in this group of compounds.[1]

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SOME OBSERVATIONS ON THE COLOR CHANGES OF THE DIPHENYLAMINE REACTION.

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The diphenylamine reaction is recognized as one of the most delicate qualitative tests for nitrate nitrogen. However, the utilization of this reaction as a microchemical test for nitrates in plant tissue presents numerous difficulties. Many substances occurring as normal constituents of the cells may diminish the intensity of the characteristic blue coloration, and in some rarer instances the coloration may be completely inhibited. But besides the interference of such substances there are grosser factors influencing the reaction, so that the results of a series of tests of the same tissue may be very inconsistent.

These latter inconsistencies directed the observations on the color changes[2] herein reported. Solutions containing only diphenylamine, nitric acid, sulfuric acid and water were used in the study of the factors controlling the production and intensity of the coloration. The factors studied were the concentration of sulfuric acid, the temperature, time and the order of mixing.

Of these factors, the concentration of the sulfuric acid is the most important. Fig. 1 shows the influence of this concentration throughout a close series from 20 to 96% sulfuric acid. The repetition of such series has always given essentially like results. Solutions were made up in 6 cc. shell vials. The total volume of solution was 5 cc., and through a single series the quantities of nitric acid and diphenylamine were constant, as, for example, in one of the best series each vial contained 0.4 mg. of diphenylamine and 0.06 mg. of nitric acid. The lower limit of blue color-

[1] Stieglitz, (THIS JOURNAL, **38**, 2053 (1916)) referring to the hydroxylammonium derivatives and to the paper by Jones, remarks "that the only electromers whose separate existence has been convincingly shown are the hydroxylamines."

[2] The chemistry of the reaction and its application to the qualitative determination of nitrates have been the objects of much previous work, to which some of the important references are as follows: Kehrman and St. Micewicz, *Ber.*, **45**, 2641 (1912); Wieland, *ibid.*, **46**, 3296 (1914); Withers and Ray, THIS JOURNAL, **33**, 708 (1911); Coron, *Ann. chim. anal.*, **16**, 211 (1911); Tillmans and Splittgerber, *Z. Nahr. Genussm.*, **25**, 417 (1913).

ation, as shown in Fig. 1, may be lowered 7% to 10%, in terms of sulfuric acid, if the sulfuric acid removed is replaced by a corresponding percentage of glacial acetic acid or potassium sulfate. This fact may indicate that the water, as such, plays a critical role in the coloration in this region of

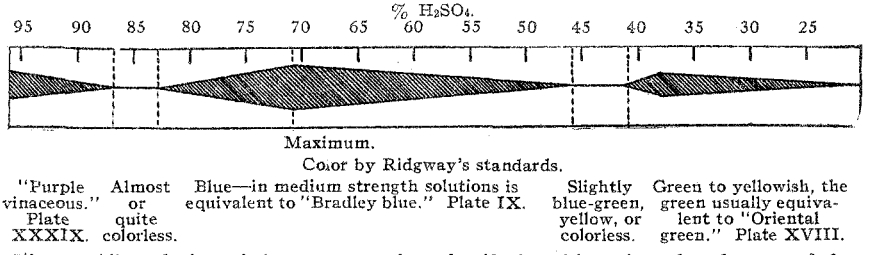

Fig. 1.—The relation of the concentration of sulfuric acid to the color changes of the diphenylamine reaction.

the series. Such a supposition would appear to be in agreement with the view that the color reaction is due to an oxidation and hydration of the diphenylamine. The 3 principle colors shown in the series of Fig. 1 are probably also caused by different degrees of oxidation and hydration of the diphenylamine molecule (cf. Kehrman and St. Micewicz).

The amount of diphenylamine can be the controlling factor, but as the limits are rather wide, this is not likely to cause difficulty in any of the formulas and methods commonly recommended. Too much diphenylamine, however, interferes with the color production.

Obviously the amount of nitrate would influence the coloration, but it is not easy to take advantage of this fact for quantitative considerations, because with any favorable concentration of sulfuric acid only slight differences of color intensity are to be noted through wide variations in the amount of nitrate present.

Variable temperatures between 20° and 50° appear to have relatively slight effect, except on the time required for the development of the maximum coloration for any given mixture.

The order of mixing is sometimes of importance for the reason that the blue color is somewhat stable, and if allowed to form under local temporary conditions of concentration during mixing, will persist after the medium has reached a general concentration (as to acid, etc.), which would have otherwise precluded the production of the resulting color.

Other series of solutions were made up as described, with the exception that each vial received an addition of a chloride, usually as potassium chloride. About 2% of potassium chloride was found to intensify the color throughout the blue region of the series, causing not only an intensification of the blue color, but also producing a lengthening of the blue region toward both the high and low concentrations of the sulfuric acid. Chlorides did not appear to affect the other colors of the series.

In consideration of the above results the writer has slightly modified the usual formulas[1] for the diphenylamine reagent as used in microchemical tests for nitrates. This reagent should be applied directly to the tissue on a glass plate or microscope slide. The tissue should be cut into thin sections or, preferably, into small rather thick pieces which may then be crushed in the reagent. The modification offers some advantage in possessing a sulfuric acid and a chloride content a little above that for maximum coloration, so that, if the reagent is used liberally the moisture of the tissue will be no more than to bring the optimum conditions for best coloration.

The modified reagent is made up of 0.05 g. of diphenylamine, 7.5 cc. of 95–96% sulfuric acid, and 2.5 cc. of 10% aqueous solution of potassium chloride. The salt is substituted for hydrochloric acid because the former evolves less free hydrogen chloride during mixing. Furthermore, potassium chloride is better than sodium chloride since the potassium hydrogen sulfate which is formed in the mixture is much more soluble in strong sulfuric acid than the sodium hydrogen sulfate would be.

When it is particularly desirable to avoid darkening of the tissue it can be accomplished at some expense of color intensity through the use of the following modification of the reagent: 0.05 g. of diphenylamine, 5.0 cc. of 95–96% sulfuric acid, 3.0 cc. of glacial acetic acid, and 2.0 cc. of 12% aqueous solution of potassium chloride.

Summary.

1. Three distinct colors in the diphenylamine reaction are noted. These colors and their intensities could be controlled through variation in the concentration of the sulfuric acid.

2. In the utilization of the diphenylamine reaction as a microchemical test for nitrate nitrogen in plant tissue, it is most important to keep the concentration of the sulfuric acid near 72% in order to obtain the best coloration.

3. Variable temperatures should be avoided throughout any series of tests, since the temperature affects the time for development of maximum coloration.

4. Modifications of the reagent and manner of making the tests are suggested in the accompanying article.

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[1] See Tunmann, "Pflanzenmikrochemie," 1913, p. 82.