PHYSIOLOGIA PLANTARUM, VOL. 19. 1966, 683–690

An Improvement in the Sensitivity of the Salkowski Reagent for Tryptamine, Tryptophan and Indoleacetic Acid

By

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(Received January 17, 1966)

Abstract

The reaction of indoles with the Salkowski reagent has been examined. It was found that the concentration of acid as well as the concentration and anionic component of the iron salt employed are critical factors in the choice of a reagent that will fail to react — or will react maximally with a given indole. Tryptamine can be reproducibly assayed with a reagent containing 0.01 *M* $Fe(NO_3)_3$ in 7.0 *M* $HClO_4$. Two ml of this reagent are added to two ml of the sample. The absorbancy is read at 450 nm after 90 minutes under uniform light conditions. Versions of this reagent can also be used for the quantitative colorimetric determination of tryptophan or indoleacetic acid.

Introduction

The quantitative colorimetric determination of indoles has most commonly been based upon their ability, originally observed by Salkowski (1885), to produce chromophores in the presence of mineral acids and inorganic oxidizing agents. Even in the most intensively studied reaction of this type, that which results in the formation of Urorosein (Herter 1908 a, 1908 b, Ellinger and Flamand 1915, Fearon and Boggust 1950, Harley-Mason and Bu'lock 1952, von Dobeneck *et al.* 1956, Houff *et al.* 1954), workers have been unable to determine the nature of the indole precursor, or to reach agreement on the structure of the pigment produced.

The modifications of this reagent suggested by Mitchell and Brunstetter (1939), Tang and Bonner (1947), and Gordon and Weber (1951), were motivated by a desire to obtain a satisfactory assay for the plant growth hormone

indoleacetic acid (IAA). That proposed by Gordon and Weber (1951) has been the most widely used, despite Platt and Thimann's (1956) statement that the production of color by IAA with this reagent is decreased to virtually nothing if the reaction is allowed to procede in the light.

The present study has been motivated by a desire to extend the use of the reagent to other indoles of interest, to explore the effect of light on the reaction, to discuss the ambiguities present in the Gordon and Weber formulation, and to investigate the factors involved in improving the sensitivity of the Salkowski reagent.

Methods and Materials

In obtaining the data for Figures 2–4, a Klett-Summerson colorimeter was employed using either a blue Corning glass filter for tryptamine and tryptophan (No. 5031) which has a transmission range of from 400 to 510 nm, or a green filter for IAA (No. 54) which has a range of from 500 to 570 nm. Measurement of the yellow color produced with the reagent with tryptamine and tryptophan at 450 nm, and the pink color produced by IAA at 535 nm using a Bausch and Lomb Spectronic-20 spectrophotometer yielded identical results. In all cases the samples assayed were contained in a total volume of 2 ml and an equal volume of reagent was added. The concentrations of reagent components was adjusted to yield the stated concentrations in the final reaction mixtures.

The data presented in Figure 1 was obtained using an E.E.L. (Evans Electroselenium Limited) colorimeter available from the manufacturer (Harlow, Essex, England). The instrument was fitted with an Ilford Filter number 604 (green) having a peak transmission at 515 nm.

The authentic indoles used were purchased from commercial sources; tryptophan and tryptamine (as Tryptamine · HCl) from Mann Research Laboratories, New York, and IAA from the California Corporation for Biochemical Research, Los Angeles, California. All of the inorganic chemicals used were reagent grade.

The colors produced were read against a water blank containing the same reagent employed in any given assay.

Results

A. *Effect of varying concentrations of inorganic oxidant*

The two inorganic oxidants most frequently used to produce Salkowski colors with indoles are the nitrite and ferric ions. The results in Figure 1 indicate that the two ions behave differently in producing colors with IAA. Even extending Fe^{3+} to mole ratios as high as 100 : 1 (Fe^{3+}/IAA) results in steadily increasing color production. As Herter (1908 a) has noted, acidification must precede the addition of nitrite.

B. *Effect of anion of ferric salt on color formation with tryptamine*

Ferric chloride is the iron salt often used as a reagent component in the production of color with IAA. However, other indoles form a wide variety

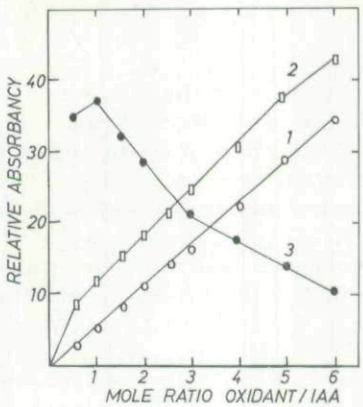

Figure 1. *Absorbancy of IAA as a function of the mole ratio of oxidant to IAA.* Measurements were made with a "604 green" 515 nm filter using an EEL colorimeter (see text for further information). 1 μmol IAA in 1 ml H_2O was added to 4 ml 60 % (by weight) $HClO_4$, then 5 ml of H_2O containing the indicated number of μ*M* of $NaNO_2$ or $FeNH_4(SO_4)_2$ rapidly added with complete mixing. The final molarity of acid in these test solutions can be calculated to be 3.7 *M*. Curves: (1) Fe^{+3}, 40 min; (2) Fe^{+3}, 20 hours; (3) NO_2, 5 min.

of colors with the Salkowski reagent (Stowe and Thimann 1954). Thus in a reagent intended to measure the indole base tryptamine (TNH_2), $FeCl_3$ becomes an undesirable salt as its aqueous solution at high concentrations is yellow, a color similar to that produced by the Salkowski reagent with this indole. The results presented in Table 1 indicate, surprisingly, that the anion supplied with the ferric salt has a marked effect upon the production of the yellow color from tryptamine.

In view of these results, and the fact that the aqueous solution of ferric nitrate is colorless, the iron salt employed in all further colorimetric estimations of TNH_2 was $Fe(NO_3)_3$ at a concentration, after addition of the test solution, of 0.005 *M*.

Table 1. *Effect of anion in ferric salt used upon the Salkowski reaction with tryptamine.* Tabulated values are absorbancies as measured in a Klett-Summerson colorimeter using a blue Corning filter (ca. 420 nm). Optical density = Klett units times 0.002. The acid used was $HClO_4$ at a final concentration in the reaction mixture of 3.5 *M*. Absorbancies in each case were measured against water blanks containing the same concentrations of acid and the corresponding ferric salt.

Iron source	Optical density observed with 60 μg/ml TNH_2 (90 minutes)	
	0.005 *M* Fe^{+3}	0.0015 *M* Fe^{+3}
Ferric ammonium sulfate	0.088	0.034
Ferric chloride	0.104	0.070
Ferric nitrate	0.160	0.078
Ferric perchlorate	0.172	0.068
Ferric sulfate	0.146	0.008
Ferric citrate	0.010	0.000
Ferric oxalate	0.014	0.010
Na-Fe-ethylenediamine tetraacetate	0.064	0.076
Ferric tartrate	0.000	0.000

C. *Effect of acid strength in the reaction mixture on color production with IAA, TNH_2, and tryptophan (TTP)*

In view of the finding of Mitchell and Brunstetter (1939) that the color production with IAA when HNO_3 and NO_2^- are employed was optimum at a final pH of 1.0, the effect of varying concentrations of acid in the final reaction mixtures of IAA, TNH_2, and TTP has been determined (Figure 2). Each compound was found to have a different optimum acid concentration: TNH_2 at 3.5 *M*, IAA at 4.0 *M*, and TTP at 4.5 *M*. This variation with acid molarity is not restricted to the reaction with ferric salts, for the nitrite reaction with IAA also peaked at 4.0 *M* $HClO_4$.

In the suggested reagent of Gordon and Weber (1951), the acid used was "35 %" $HClO_4$ in which the oxidant was dissolved, before the addition was made of the reagent to the solution to be tested for IAA content. The reader is not informed whether this is a volume or weight percentage for the $HClO_4$ used. Given a 1 ml test solution mixed with 2 ml of such a reagent, the final acid concentration would either be 2.3 *M* if the 35 % $HClO_4$ used was a weight percentage, or 3.0 *M* if the acid suggested was a volume percentage, because of the high density (1.67) of the 70 % $HClO_4$ presumably used for the dilution. Neither concentration is optimal for color production with IAA. This ambiguity has been perpetuated by all subsequent authors using the Gordon and Weber reagent, and becomes significant in light of the data presented in Figure 2.

Given a constant concentration of Fe^{3+} in the reaction mixture, the choice of acid concentration is the critical factor in the formulation of a reagent which would react maximally, or fail to react, with a given indole. Furthermore, it is not surprising to find reference to the inability of earlier reagents to react with tryptophan in the previously published modifications of the reagent (Mitchell and Brunstetter 1939, Tang and Bonner 1947, Gordon and Weber 1951) because in each case the reagent used contained mineral acids at less than a final concentration of 3.0 *M*, the concentration at which color first begins to appear in the case of tryptophan.

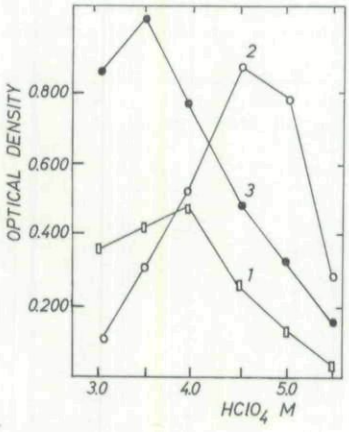

Figure 2. *Absorbancy as a function of acid strength in the final reaction mixture.* In all solutions, the final concentration of Fe as $Fe(NO_3)_3$ was 0.005 *M*. IAA measurements were made after 20 minutes, and those for TNH_2 and TTP after 90 minutes. Abscissa: Molarity $HClO_4$ in final test solution. Ordinate: Optical density, Klett units. Curves: (1) IAA 20 µg/ml, green filter; (2) TTP 60 µg/ml, blue filter; (3) TNH_2 100 µg/ml, blue filter.

D. *Effect of light on the reaction of tryptamine with the Salkowski reagent*

In view of the statements by Gordon and Weber (1951) that their reagent was stable toward light, and by Platt and Thimann (1956) that light given a reaction mixture of IAA and the same reagent, markedly inhibited the final color achieved, the effect of light on the reaction of tryptamine with perchloric acid and ferric nitrate has been examined.

When the Salkowski reaction of tryptamine was examined as a function of the age of the reagent in days after its preparation, great variability was found, but no discernable trend with time was noted. The reagent employed was stored in the laboratory in a clear glass bottle, and the reactions took place on a laboratory bench subject to any existing variation in light intensity.

In contrast to the observations of Platt and Thimann (1956) and in agreement with those of Klingmüller (1955) who both used the Gordon and Weber reagent, light causes an increase in the amount of color observed with a given concentration of test indole when the reaction mixture is formulated according to the recipe in Figure 3.

A crude examination of the wavelengths of light most effective in causing

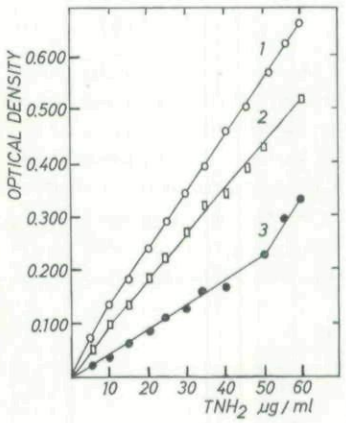

Figure 3. *Effect of light on the Salkowski reaction with tryptamine.* The reaction mixture in each case contains $HClO_4$ at a final concentration of 3.5 *M* and Fe as $Fe(NO_3)_3$ at 0.005 *M*. Measurement of color was made after 90 minutes in each case. Abscissa: TNH_2 µg/ml in the final test solution. Curves: (1) light, 29 hours; (2) dark, 20 hours + light, 9 hours; (3) dark, 29 hours.

Table 2. *The effect of differing wavelengths, but equal energies, on the color produced by Salkowski reagent with 25 µg/ml TNH_2.* The reagent used in each case is that given in Figure 3, and measurements were made after 90 minutes.

Light regions	Optical density
Dark	0.254
Blue (ca. 380–550 nm)	0.350
Green (ca. 475–600 nm)	0.306
Yellow (ca. 525–650 nm)	0.246
Red (ca. 600–720 nm)	0.330
White (Warm white fluorescent)	0.294

this increase in color is given in Table 2. The results obtained are what would be expected if light were being absorbed by the iron present in the test solution. Further investigations directed at an examination of light stimulated free radical formation might well be of use in a determination of the reaction mechanism operative in the production of the color observed.

E. *Tryptamine determination*

A Salkowski reagent containing Fe^{3+} as $Fe(NO_3)_3$ at a final concentration in the reaction mixture of 0.005 *M*, and $HClO_4$ at a final concentration of 3.5 *M* is proposed as the reagent whose properties allow the most adequate quantitative determination of tryptamine. That this reagent is useful for the practical estimation of tryptamine is indicated in Figure 4.

The production of color reaches a maximum in 60 minutes at room temperature and the colored product is stable for at least three hours when the reaction is carried out under warm white fluorescent light at an intensity of 7535 lux. There is a linear relationship between amine concentration and color produced over the range of 0 to 120 μg/ml. Duplicate assays are generally identical, with a variance of no more than three colorimetric units.

F. *Tryptophan and indoleacetic acid determination*

A reagent containing Fe^{3+} as $Fe(NO_3)_3$ at a final concentration of 0.005 *M*, and $HClO_4$ at a final concentration of 4.5 *M* would be the reagent of choice if one desired to quantitatively determine the tryptophan present in a sample lacking other indoles. Color is produced at room temperature, and reaches a maximum in 60 minutes.

While a reagent containing 4.0 *M* $HClO_4$ in the final reaction mixture yields a greater color with IAA, and is therefore the most sensitive reagent, the color produced is extremely unstable. In order to obtain a stable color

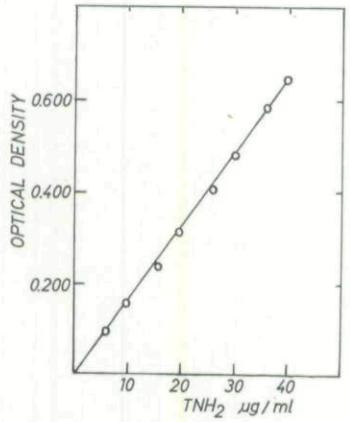

Figure 4. *The relationship between Salkowski color and tryptamine concentration.* The reagent employed is that described in Figure 3, and measurements were made after 90 minutes under warm white fluorescent light at an intensity of 7535 lux. Abscissa: TNH_2 μg/ml in the final test solution.

with IAA, the reagent of choice should include $HClO_4$ at a final concentration of 2.6 *M*.

Despite the fact that increasing concentrations of iron (Fe^{3+}) will in all cases yield increasing amounts of color, one encounters problems with solubility at very high iron salt concentrations. For this reason, it is suggested that all three versions of the reagent contain Fe^{3+} at a concentration of 0.005 *M* in the final reaction mixture.

The danger of encountering oxidizing or reducing substances in extracts which are known to interfere with the production of color with the Salkowski reagent (Gordon and Weber 1951, Platt and Thimann 1956, Siegel and Weintraub 1952) must be considered carefully, but can be minimized by making sure that extracts are as pure as possible. It should be emphasized that the reagent is not reliable in crude homogenates where such interferring substances may be present, nor can it measure individual indoles in the presence of others. A preliminary fractionation of the plant material, such as that employed by Powell (1964) is thus necessary. Use of the method in a study of the enzymological production of tryptamine is described elsewhere (Perley and Stowe 1966).

This work was initiated by B. B. Stowe while a U.S. National Science Foundation post doctoral fellow at the Department of Agricultural Biochemistry, University College of North Wales, Bangor, Caernarvonshire, North Wales. The interest and suggestions of Professor W. Charles Evans and Dr. W. R. C. Crimmin are gratefully appreciated.

The present investigation was supported by a U.S. Public Health Service research grant GM-06921 to B. B. Stowe from the National Institutes of Health, by fellowships to J. E. Perley from the Public Health Service (1-F1-GM-15, 815-O1A1) of the National Institute of General Medical Sciences, and from the Yale University Committee on Atypical Growth, supported by the American Cancer Society.

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References

von Dobeneck, H., Lehnerer, M. & Maresh, G.: Mitteilungen zur Chemie des Indols. V. Über das Urorosein. — Z. Physiol. Chem. 304: 26–34. 1956.

Ellinger, A. & Flamand, C.: Eine neue Farbstoffklasse von biochemischer Bedeutung. — *Ibid.* 62: 276–286. 1909.

Fearon, W. & Boggust, G.: Pigments derived from tryptophan. (1) Urorosein (2) Tryptochrome. — Biochem. J. 46: 62–67. 1950.

Gordon, S. & Weber, R.: Colorimetric estimation of indoleacetic acid. — Plant Physiol. 26: 192–195. 1951.

Harley-Mason, J. & Bu'lock, J.: The structure of urorosein. — Biochem. J. 51: 430–432. 1952.

Herter, C. A.: The relationship of nitrifying bacteria to the urorosein reaction of Nencki and Sieber. — J. Biol. Chem. 4: 239–251. 1908 a.

— On indoleacetic acid as the chromogen of the "Urorosein" of the urine. — *Ibid.* 4: 253–257. 1908 b.

Homer, A.: A spectroscopic examination of the color reactions of certain indole derivatives and of the urine of dogs after their administration. — *Ibid.* 22: 345–361. 1915.

Houff, W., Hinsvark, O., Wittwer, S., & Sell, H.: The nature of an oxidation product of 3-indoleacetic acid. — J. Amer. Chem. Soc. 76: 5654–5656. 1954.

Klingmüller, W.: In vitro. Studien an Heteroauxin. Thesis. — Giessen, Germany. 1955.
Mitchell, J. & Brunstetter, B.: Colorimetric methods for the quantitative estimation of indole-3-acetic acid. — Bot. Gaz. 100: 802–816. 1939.
Perley, J. E. & Stowe, B. B.: The production of tryptamine from tryptophan by Bacillus cereus (KVT). — Biochem J. 100. 1966. In press.
Platt, R. S. & Thimann, K. V.: Interference in the Salkowski assay of IAA. — Science 123: (3186) 105–106. 1956.
Powell, L. E.: Preparation of indole extracts from plants for gas chromatography and spectrophotofluorometry. — Plant Physiol. 39: 836–842. 1964.
Salkowski, E.: Ueber das Verhalten der Skatolcarbonsäure im Organismus. — Z. Physiol. Chem. 9: 23–33. 1885.
Siegel, S. & Weintraub, R.: Inactivation of indoleacetic acid by peroxidases. — Physiol. Plant. 5: 241–247. 1952.
Stowe, B. B. & Thimann, K. V.: The paper chromatography of indole compounds and some indole containing auxins of plant tissues. — Arch. Biochem. Biophys. 51: 501–516. 1954.
Tang, Y. & Bonner, J.: The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. — Arch. Biochem. 13: 11–25. 1947.