These data indicated that quantitative conversion of procarbazine hydrochloride to the azo compound occurred during the titration. No evident substitution of iodine into the phenyl ring resulted, therefore eliminating aromatic substitution as a source of interference.

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Analytical Applications of *p*-Nitrosobenzoic Acid I: Specific Colorimetric Determination of *m*-Aminophenol in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate

I. K. SHIH

Abstract □ *p*-Nitrosobenzoic acid was used in the colorimetric analysis of *m*-aminophenol. It reacted with *m*-aminophenol in sodium bicarbonate solution, producing an intensified orange–yellow color. The reaction product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. The color produced in sodium bicarbonate was stable for at least 24 hr. and exhibited a maximum absorption peak at 440 nm. Beer's law was obeyed over a wide range of *m*-aminophenol concentrations (3.2–16.0 mcg./ml.). By applying low temperature control, the reaction specificity of *m*-aminophenol with *p*-nitrosobenzoic acid can be achieved. By using this color reaction, a new method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. The method is accurate, precise, sensitive, simple, and specific. Samples of *p*-aminosalicylic acid or sodium *p*-aminosalicylate containing as little as 0.01% of *m*-aminophenol can be accurately determined. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, this color reaction of amino compounds was inhibited.

Keyphrases □ *m*-Aminophenol—colorimetric analysis (*p*-nitrosobenzoic acid) in *p*-aminosalicylic acid and sodium *p*-aminosalicylate □ *p*-Nitrosobenzoic acid—colorimetric analysis, *m*-aminophenol □ 3-Hydroxyazobenzene-4′-carboxylic acid—formation, colorimetric analysis of *m*-aminophenol with *p*-nitrosobenzoic acid □ *p*-Aminosalicylic acid, sodium *p*-aminosalicylate—colorimetric determination of *m*-aminophenol □ Colorimetry—analysis, *m*-aminophenol

In 1904, the chemical synthesis of *p*-nitrosobenzoic acid was reported by Alway (1), but further study on this compound has not appeared in the literature. The photodegradation reactions of chloramphenicol were investigated recently, and *p*-nitrosobenzoic acid was recognized as a possible intermediate in the photodegradation reactions (2). *p*-Nitrosobenzoic acid is a stable compound, decomposing at around 250° (1, 2). It possesses two potential functional groups: carboxylic acid and nitroso. The unique physicochemical properties of this compound were applied in this study to the analytical problem of *m*-aminophenol determination in *p*-aminosalicylic acid and sodium *p*-aminosalicylate.

Both the USP (3) and BP (4) require a limit test for *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate, but official methods are tedious. Although numerous analytical procedures for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate were proposed, the reported methods (5–11) were not reliable because of poor specificity. The complexity of this problem is reflected by Tatjana's work (11). He pointed out that due to the structural similarity of *m*-aminophenol and *p*-aminosalicylic acid, it was impossible to find a reagent that would react with *m*-aminophenol but not with *p*-aminosalicylic acid.

The present study was undertaken to solve this controversial *m*-aminophenol analysis by using *p*-nitrosobenzoic acid as a specific colorimetric reagent able to distinguish amino compounds of different chemical structures. Condensation between nitroso and amino compounds is a known reaction, giving rise to azo compounds (1, 12, 13). Experiments were designed to study the color reaction of *p*-nitrosobenzoic acid with a variety of amino compounds in sodium bicarbonate solution. By using the highly sensitive and specific color reaction of *m*-aminophenol with *p*-nitrosobenzoic acid, a simple and reliable method for the direct determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate was developed. *p*-Nitrosobenzoic acid was used for the first time as a potential analytical reagent.

EXPERIMENTAL

Apparatus—Recording spectrophotometers[1] were used.

Chemicals—*p*-Nitrosobenzoic acid was photosynthesized in this laboratory (2). The following chemicals were either reagent or USP grade: *m*-aminophenol[2], *o*-aminophenol[2], *p*-aminophenol[2], *p*-nitroaniline[2], sodium *p*-aminosalicylate USP[3], *p*-aminobenzoic acid[4], *p*-aminosulfonic acid[4], sodium salicylate[4], isoniazid[5], benzo-

[1] Bausch & Lomb 505 and Beckman IR-8.
[2] Eastman Organic Chemicals.
[3] May & Baker.
[4] Fisher.
[5] Hoffmann–La Roche.

Table I—Stability of *p*-Nitrosobenzoic Acid Reagent

Weeks	Molar Absorptivities—$\lambda_{max.}$, 313 nm.	$\lambda_{max.}$, 292 nm.	$\lambda_{max.}$, 223 nm.
Fresh solution	1.24×10^4	9.33×10^3	6.61×10^3
1	1.24×10^4	9.34×10^3	6.62×10^3
2	1.24×10^4	9.33×10^3	6.60×10^3
3	1.25×10^4	9.33×10^3	6.61×10^3
4	1.24×10^4	9.32×10^3	6.60×10^3
5	1.24×10^4	9.32×10^3	6.62×10^3

caine[6], sodium sulfathiazole[6], sulfisoxazole[6], sulfanilamide[6], sodium bicarbonate USP[7], ethyl acetate[4], and ethanol[4].

Preparation of *p*-Nitrosobenzoic Acid Reagent—Thirty milligrams of *p*-nitrosobenzoic acid was dissolved in 200 ml. of 1% sodium bicarbonate solution. This reagent preparation can be kept at room temperature for 5 weeks without significant deterioration.

Preparation of *m*-Aminophenol Standard Solution—*m*-Aminophenol was recrystallized from 70% hot alcohol, m.p. 123°. Forty milligrams of *m*-aminophenol was dissolved in 500 ml. of distilled water. Thus, each milliliter contained 80 mcg. of *m*-aminophenol. A freshly prepared standard solution was used.

Stability Study of *p*-Nitrosobenzoic Acid Reagent—A reagent solution containing 7.5 mcg./ml. of *p*-nitrosobenzoic acid was prepared in 1% sodium bicarbonate solution. The stability of the reagent was evaluated at room temperature by recording its absorption spectrum in a range from 210 to 360 nm. over an extended period (Table I).

Reaction of *m*-Aminophenol with *p*-Nitrosobenzoic Acid and Stability—Four milliliters of standard *m*-aminophenol solution was pipeted into a 25-ml. volumetric flask containing 10 ml. of water and 5 ml. of 5% sodium bicarbonate solution. An aliquot of 5 ml. of *p*-nitrosobenzoic acid reagent was transferred to the flask. The solution was then brought to volume with water and mixed thoroughly. Reaction was allowed to take place at room temperature (about 25°). A second solution was similarly prepared, and the flask was immersed in an ice bath maintained at 0–4°. Absorption spectra of the color solutions were recorded every 30 min. in a range from 380 to 530 nm. against a reagent blank. Absorption spectra of solutions were also checked 24 hr. after color development (Table II). To obtain the maximum accuracy of this method as later applied to the determination of *m*-aminophenol in *p*-aminosalicylic acid and sodium *p*-aminosalicylate, color reaction, unless otherwise specified, was carried out at 0–4°.

Synthesis of 3-Hydroxyazobenzene-4′-carboxylic Acid—Fifty milligrams of *p*-nitrosobenzoic acid and 100 mg. of *m*-aminophenol were dissolved in 50 ml. of 1% sodium bicarbonate solution. After standing 3 hr. at room temperature, the reaction mixture was transferred to a separator and extracted with ten 30-ml. portions of ethyl acetate to remove unreacted *m*-aminophenol. The aqueous solution was then acidified dropwise with dilute hydrochloric acid and extracted with three 50-ml. portions of ethyl acetate. Evaporation of the combined ethyl acetate extract under reduced pressure yielded 70 mg. of dark-colored product (91% yield), m.p. about 150° dec.; visible spectrum: $\epsilon = 4920$ ($\lambda_{max.}$ 440 nm. in 1% sodium bicarbonate solution), $\epsilon = 3640$ ($\lambda_{max.}$ 428 nm. in 0.1 *N* hydrochloric acid); IR spectrum (KBr): $\bar{\nu}$ 3448 cm.$^{-1}$ (OH), 1689 cm.$^{-1}$ (aryl CO_2H), 1580 cm.$^{-1}$ (aryl C=C), *etc.*

Anal.—Calc. for $C_{13}H_{10}N_2O_3$: C, 64.50; H, 4.14; N, 11.57. Found: C, 63.74; H, 4.40; N, 10.73.

Specificity of *p*-Nitrosobenzoic Acid Reagent—Several amino and phenolic compounds were tested with *p*-nitrosobenzoic acid reagent at three temperatures: 0–4, 25, and 100°. Samples were dissolved in 40 ml. of 1% sodium bicarbonate solution, and 10 ml. of reagent was added to each sample. Visual observations of color reaction are given in Table III.

Relationship between *m*-Aminophenol Concentration and Color Intensities—Standard *m*-aminophenol solutions (1–5 ml.) were pipeted into 25-ml. volumetric flasks containing 10 ml. of water, 5 ml. of 5% sodium bicarbonate solution, and 5 ml. of *p*-nitrosobenzoic acid reagent. Solutions were brought to volume with water and mixed thoroughly. The flasks were then immersed in an ice bath maintained at 0–4°. Absorbances of solutions were measured 3 hr. after color development against a reagent blank at 440 nm. For replicate study, six 4-ml. portions of standard *m*-aminophenol solutions were used (Table IV).

[6] British Drug Houses.
[7] Science Borealis.

Table II—Color Development and Color Stability

Hours	A^a	$\lambda_{max.}$, nm.	A^b	$\lambda_{max.}$, nm.
0.5	0.210	440	0.350	440
1	0.301	440	0.498	440
1.5	0.379	440	0.549	440
2	0.450	440	0.580	440
2.5	0.502	440	0.601	440
3	0.540	440	0.610	440
3.5	0.570	440	0.612	440
4	0.589	440	—	—
4.5	0.610	440	—	—
5	0.615	440	—	—
—	—	—	—	—
24	0.620	440	0.615	440

[a] Color was developed at 0–4°. [b] Color was developed at room temperature.

Interference Study—Four 50-mg. samples of sodium *p*-aminosalicylate were accurately weighed and transferred to 25-ml. volumetric flasks containing 10 ml. of water, 5 ml. of 5% sodium bicarbonate solution, and 4 ml. of standard *m*-aminophenol solution. Then 5 ml. of *p*-nitrosobenzoic acid reagent was pipeted into each sample. Solutions were brought to volume with water and mixed thoroughly. Sample flasks were then immersed in an ice bath maintained at 0–4°. After 3 hr. of color development, absorbances of sample solutions were concomitantly determined with a similarly treated standard *m*-aminophenol solution against a reagent blank at 440 nm. An interference study of isoniazid was also performed (Table V).

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate Reference Material—Four 500-mg. samples of sodium *p*-aminosalicylate were accurately weighed and transferred to 25-ml. volumetric flasks. The color development procedure was the same as previously described. The USP method (3) was also used in this study (Table VI).

Quantitation of *m*-Aminophenol in *p*-Aminosalicylic Acid and Sodium *p*-Aminosalicylate Dosage Form—Twenty tablets were weighed and ground to a fine powder. An accurately weighed portion of powder, equivalent to about 500 mg. of *p*-aminosalicylic acid, was transferred to a 25-ml. volumetric flask. The color development procedure was the same as previously described. The results are given in Table VII.

Table III—Color Reactions of *p*-Nitrosobenzoic Acid with Amino and Phenolic Compounds

Compound	Reaction Conditions and Visual Observations[c]—0–4° (0–3 hr.)	25° (0–3 hr.)	100° (0–3 min.)
m-Aminophenol[a]	OY	OY	—
o-Aminophenol[b]	sY	Y	—
p-Aminophenol[b]	sP	P	—
Sodium *p*-aminosalicylate	vsY	sY	OY
p-Aminobenzoic acid	NR	NR	sY
Benzocaine	NR	NR	sY
p-Nitroaniline	NR	NR	NR
p-Aminosulfonic acid	NR	NR	sY
Sulfanilamide	NR	NR	OY
Sodium sulfathiazole	NR	NR	Y
Sulfisoxazole	NR	NR	NR
Sodium salicylate	NR	NR	NR
Phenol	NR	NR	NR
Isoniazid	sY	sY	sY

[a] Five milliliters of standard solution, equivalent to 0.40 mg. of *m*-aminophenol, was used. [b] Ten milligrams of sample was used; for all others, a quantity of 4 g. was used. [c] OY = orange-yellow, Y = yellow, P = purple, s = slight, vs = very slight, and NR = no color reaction.

Table IV—Relationship between *m*-Aminophenol Concentrations and Absorbances

Standard Solution, ml.	*A*, 440 nm.
1	0.135
2	0.272
3	0.409
4[a]	0.544, 0.544, 0.544
—	0.546, 0.550, 0.548
5	0.670

[a] Six 4-ml. samples of *m*-aminophenol standard solution were used for replicate study.

RESULTS AND DISCUSSION

Stability of *p*-Nitrosobenzoic Acid Reagent—The stability of the reagent was evaluated by recording its UV absorption spectrum over an extended period of time. Molar absorptivities remained unchanged (Table I), indicating that the reagent preparation, when stored at room temperature, could be kept for 5 weeks without significant deterioration.

Color Reaction of *m*-Aminophenol with *p*-Nitrosobenzoic Acid—*m*-Aminophenol was reacted with *p*-nitrosobenzoic acid in sodium bicarbonate solution, producing an intensified orange-yellow color with a maximum absorption peak at 440 nm. The concentration of sodium bicarbonate, varying from 1 to 10%, did not have significant effect on the color development. The color produced was stable for at least 24 hr. (Table II). The reaction product was isolated. Based on the analytical results and supported by the known amino–nitroso condensation reaction (1, 12, 13), the product was characterized as 3-hydroxyazobenzene-4′-carboxylic acid. The maximum absorption wavelength of this compound in the visible region was shifted from 440 nm. in 1% sodium bicarbonate solution (ϵ = 4920) to 428 nm. in 0.1 *N* hydrochloric acid (ϵ = 3640). Neither absorbances nor visible spectra of both sample solutions were changed over 24 hr.

Specificity of *p*-Nitrosobenzoic Acid—In contrast to *m*-aminophenol, amounts of phenolic and amino compounds, varying from 10 mg. to 4 g., were used to react with *p*-nitrosobenzoic acid in sodium bicarbonate solution at 0–4, 25, and 100° (Table III).

Sodium *p*-aminosalicylate, *p*-aminobenzoic acid, benzocaine, *p*-nitroaniline, *p*-aminosulfonic acid, sulfonamides, sodium salicylate, and phenol, when tested at concentrations 10,000 times that of *m*-aminophenol, failed to give a color reaction with the reagent. Trace amounts of *m*-aminophenol impurity in sodium *p*-aminosalicylate (3, 4) were responsible for the slight color observed. All samples except *p*-nitroaniline, sulfisoxazole, sodium salicylate, and phenol showed various degrees of color reaction when tested at 100°. These experimental results clearly indicated that in the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, this color reaction of amino compounds was inhibited under ordinary reaction conditions. Thus, neither sodium *p*-aminosalicylate nor isoniazid would interfere with *m*-aminophenol determination (Table V). Sodium salicylate and phenol did not react to the color test due to a lack of the amino functional group. Aminophenols reacted with the reagent, yielding different color species. According to visual observations, reactivities of the three aminophenols were approximately in the following decreasing order: $m > o > p$. Thus, this color reaction may be used as a specific identity test for those compounds.

Table V—Interference Study

Composition of Synthetic Mixture, mg. MAP[a]	PASNa[b]	INH[c]	Recovery of MAP, % PASNa	INH
0.320	50.0	—	99.9	—
0.320	50.0	—	99.9	—
0.320	50.0	—	100.1	—
0.320	50.0	—	100.2	—
0.320	—	5.0	—	100.2
0.320	—	5.0	—	100.4
0.320	—	5.0	—	100.6
0.320	—	5.0	—	100.1
Average			100.0	100.3
SD			±0.14	±0.22

[a] Four milliliters of standard solution, equivalent to 0.08 mg. of *m*-aminophenol (MAP), was used. [b] PASNa = sodium *p*-aminosalicylate. [c] INH = isoniazid.

Table VI—Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate Reference Material

m-Aminophenol Found, % Method A[a]	Method B[b]	USP Method[c]
0.012	0.042	0.01
0.012	0.042	n.r.[d]
0.012	0.042	n.r.
0.012	0.042	n.r.

[a] Color was developed at 0–4°. [b] Color was developed at room temperature. [c] A quantity of 690 mg. of sodium *p*-aminosalicylate was used according to the method. [d] n.r. = negative result.

Beer's Law and Replicate Study of *m*-Aminophenol—A linear relationship between absorbances and *m*-aminophenol concentrations was obtained (Table IV). Replicate studies showed good agreement of individual results (Table IV).

Experimental Conditions for Precise Determination of *m*-Aminophenol in Sodium *p*-Aminosalicylate and *p*-Aminosalicylic Acid—Since the method was primarily designed for quantitation of small quantities of *m*-aminophenol in sodium *p*-aminosalicylate and *p*-aminosalicylic acid, the control of the reaction temperature at 0–4° is essential to minimize possible degradation of *p*-aminosalicylic acid into *m*-aminophenol during color development. Since absorbances of sample solutions were concomitantly determined with the reference sample of *m*-aminophenol, 3 hr. of color development would be sufficient.

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate—Sodium *p*-aminosalicylate USP was analyzed for the *m*-aminophenol content. Results of 0.012 and 0.042% was obtained when the color of samples was developed at 0–4 and 25°, respectively. Degradation of sodium *p*-aminosalicylate at higher temperature may account for this discrepancy. Thus, for precise *m*-aminophenol determination, low temperature control (0–4°) must be applied. The *m*-aminophenol content of sodium *p*-aminosalicylate was also determined by the USP method (3). The amount of *m*-aminophenol was calculated from the following equation: % *m*-aminophenol = $(A - 0.320)/1.16$, where *A* is the absorbance of sample solution measured at 420–435 nm. Three negative values and one positive result were obtained (Table VI). In view of the photometric error and poor reproducibility of the method, negative values probably resulted from the sample containing *m*-aminophenol in the neighborhood of 0.01%. In a qualitative sense, the official method was in agreement with the proposed method.

For routine control purpose of *m*-aminophenol, a simple procedure is suggested: Use a calculated amount of *m*-aminophenol, equivalent to the maximum tolerance as specified in the official monographs. Develop color from an accurately weighed sample of sodium *p*-aminosalicylate (or *p*-aminosalicylic acid) and *m*-aminophenol standard under the same conditions using *p*-nitrosobenzoic acid reagent. The color developed from the sample should not be deeper than that of the *m*-aminophenol standard.

Quantitation of *m*-Aminophenol in Sodium *p*-Aminosalicylate and *p*-Aminosalicylic Acid Dosage Form—Commercial products of sodium *p*-aminosalicylate and *p*-aminosalicylic acid tablets were

Table VII—Quantitation of *m*-Aminophenol in *p*-Aminosalicylate and Sodium *p*-Aminosalicylate Dosage Forms

	m-Aminophenol Found, %[a] Product A[b]	Product B[c]	Product C[d]
	0.018	0.041	0.030
	0.018	0.041	0.032
	0.018	0.041	0.031
	0.018	0.042	0.031
Average =	0.018	0.041	0.031

[a] Percent *m*-aminophenol was calculated on the basis of declared amount of *p*-aminosalicylate. [b] Effervescent *p*-aminosalicylate tablet. [c] Buffered *p*-aminosalicylate–isoniazid tablet. [d] Sugar-coated sodium *p*-aminosalicylate tablet.

analyzed for *m*-aminophenol content. The results are given in Table VII.

CONCLUSIONS

1. Condensation reaction between *p*-nitrosobenzoic acid and a variety of amino compounds in sodium bicarbonate solution was studied. *m*-Aminophenol reacted with *p*-nitrosobenzoic acid, producing an intensified, stable orange–yellow dye. The product was isolated and characterized as 3-hydroxyazobenzene-4′-carboxylic acid. In the presence of deactivating groups such as CO_2R, SO_2R, and NO_2, the color reaction of amino compounds was inhibited. Thus, sodium *p*-aminosalicylate, *p*-aminobenzoic acid, benzocaine, *p*-aminosulfonic acid, sulfonamides, *p*-nitroaniline, *etc.*, when tested at concentrations 10,000 times that of *m*-aminophenol, failed to give the color reaction.

2. All aminophenols reacted with *p*-nitrosobenzoic acid, producing discernible colors. The relative rates of color development were approximately in the following decreasing order: $m > o > p$. This color reaction thus can be used for specific identity tests for the three isomeric aminophenols.

3. By using *p*-nitrosobenzoic acid as a specific color-producing agent for *m*-aminophenol, a sensitive and reliable method for the direct determination of *m*-aminophenol in sodium *p*-aminosalicylate or in *p*-aminosalicylic acid was developed. Samples of sodium *p*-aminosalicylate and *o*-aminosalicylic acid containing as little as 0.010% of *m*-aminophenol could be accurately determined.

4. *p*-Nitrosobenzoic acid was used for the first time as a potential colorimetric reagent. Other possible applications of this new reagent are worth investigating in the future.

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Elimination of Choline Interference in Microbiological Assay of Inositol in Pharmaceutical Products

LEWIS WALENROD and REUBEN LEVINTON

Abstract □ A method for the assay of inositol in the presence of choline is described which eliminates interference by the latter. By using *Schizosaccharomyces pombe* as the test organism, results comparable to those obtained with *Saccharomyces carlsbergensis* were obtained without the need for separation of the interfering compound.

Keyphrases □ Inositol—microbiological assay in the presence of choline without interference, using *Schizosaccharomyces pombe* as test organism □ *Schizosaccharomyces pombe*—used as test organism to eliminate interference in inositol analysis, compared to *Saccharomyces carlsbergensis* □ Choline—eliminated as interference in nositol microbiological assay

The most widely used method of assay for inositol in the United States, proposed by Atkins *et al.* (1) and Jurist and Foy (2), employs *Saccharomyces carlsbergensis* as the test organism. In pharmaceutical preparations, inositol and choline are often used in combination as lipotropic agents; choline produces significant inhibition of the response of *S. carlsbergensis* to inositol (3).

Various methods have been suggested to eliminate the effect of this interference. It was found that passing dilute aqueous solutions through columns of a cation exchanger[1] produced interference-free eluates. Taylor and McKibbin (3) compensated for the interference by adding choline to the standard tubes. However, both of these procedures involve additional manipulations which are time consuming as well as potential sources of error.

This paper describes a method, routinely used in these laboratories, which permits the direct assay of inositol in the presence of choline without the need for separative or compensatory processes. The assay is based on the method of Norris and Darbre (4).

EXPERIMENTAL

The test organism used was a strain of *Schizosaccharomyces pombe* (ATCC 16491)[2]. The yeast is maintained by monthly transfer on malt agar slants consisting of 10 g. malt extract (Difco), 0.2 g. yeast extract (Difco), and 1.8 g. agar/100 ml. distilled water. Slants are incubated at room temperature for 24 hr. and then stored under refrigeration.

[1] Folin Decalso, The Permutit Co., New York, N. Y..

[2] Obtained from F. W. Norris, Department of Biochemistry, University of Birmingham, Birmingham, England. It is currently available from the American Type Culture Collection, Rockville, MD 20852