that the rates of dealdolization can be studied by this method. However, in conjunction with imide analysis (1) for choosing the necessary amount of sample to be assayed, the method is applicable in those cases where the stoichiometry of Schemes I and II holds.

The procedures stated can be applied only in the absence of imide hydrolysis. The method depends on titration of excess base by $HClO_4$, and any acidic function produced would decrease the titratable free hydroxylamine. However, the amount of hydrolyzed imide can be compensated by prior potentiometric titration of the sample to be analyzed by methods given previously (9) and equivalent DMAE added. Under these conditions the amount of imide (1) plus the difference between the carboxylic acid content and the liberated ammonia determined by potentiometry (9) would have to be used to determine the amount of mixture to be analyzed.

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Schiff Base Formation in the Development of a Spectrophotometric Assay for Sulfonamides

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The reaction between aromatic amines and aromatic aldehydes was investigated as the basis of a spectrophotometric assay for sulfonamides. Those properties of the equilibrium reaction which affect the absorptimetric properties of the system were studied to evaluate the method. The effects of pH, ionic strength, concentration of aldehyde, solvent system, and temperature were determined. The method was applied to the determination of sulfanilamide, sulfadimethoxine, and succinylsulfathiazole, and comparative analyses were performed with the method of Bratton and Marshall. The low absorbance method of precision spectrophotometry was employed in the determination of microgram quantities of sulfanilamide.

THE OBJECTIVE of this investigation was to evaluate a spectrophotometric method of analysis for sulfonamides based on the formation of chromophoric Schiff bases (*N*-alkyl aldimines). The reaction between various aldehydes and sulfonamides has been reported previously by Butler and Ingle (1) and Castle (2), who prepared crystalline Schiff base derivatives of sulfonamides and utilized the crystalline properties of these derivatives as a means of qualitative identification. An assay procedure based on this method also has been reported for *p*-aminosalicylic acid and isoniazid (3).

In the equilibrium reaction, which occurs in the Schiff base formation (Fig. 1), a carbinolamine intermediate is formed upon the reaction of amine and aldehyde. A second equilibrium reaction subsequently takes place in which the carbinolamine intermediate forms the Schiff base and water. These reactions are known to be dependent upon the hydronium ion concentration, and the effects of pH on the equilibrium and kinetic properties of Schiff base formation have been studied (4).

In this study the effects of temperature, pH, ionic strength, solvent, and concentration of reactants on the spectral properties of Schiff bases were determined to ascertain which factors required careful control. The method was applied to the analysis of several sulfonamides, and the precision, accuracy, and sensitivity were evaluated. A comparative study of this method was made with the method of Bratton and Marshall (5).

EXPERIMENTAL AND RESULTS

Reagents.—Vanillin U.S.P.[1] and salicylaldehyde[2] were employed as the chromogenic reagents.

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[2] Eastman Organic Chemicals, Rochester N. Y

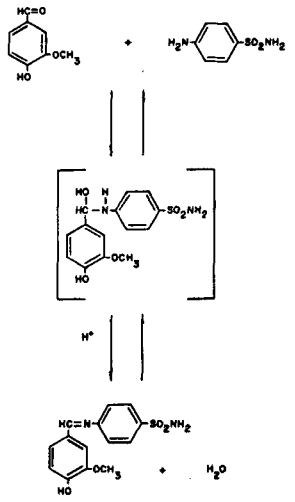

Fig. 1.—The formation of a Schiff base from vanillin and sulfanilamide.

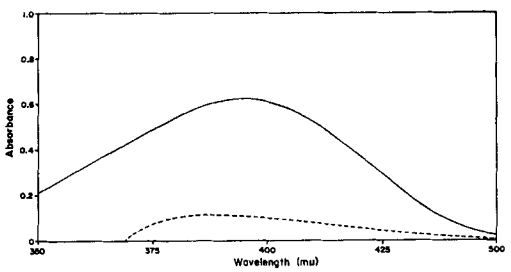

Fig. 2.—Spectral-absorbance curves for Schiff bases of sulfanilamide. Key: ——, vanillin Schiff base, - - -, salicylaldehyde Schiff base.

Sulfanilamide N.F.[3] was used to study the absorptimetric properties of the reaction; it was employed also as the sample drug in the sulfanilamide assays. Tablets used for analysis were sulfadimethoxine[4] and succinylsulfathiazole.[5] Each product was labeled as containing 0.5 Gm. of sulfonamide per tablet. For the preparation of standard curves, U.S.P. sulfanilamide reference standard[6] was used; for sulfadimethoxine and succinylsulfathiazole, powdered samples obtained from the manufacturers were employed.

Determination of Absorbance Values.—The instrument used throughout this study was the Beckman model B spectrophotometer equipped with a Raytheon voltage stabilizer. All solutions were determined against a reagent blank in Pyrex 1-cm. rectangular cells. The optical cells containing the absorptimetric solutions were permitted to remain in the instrument for at least 10 min. prior to making readings to insure an equilibrium temperature. The relative concentrations of aldehyde and sulfonamide employed in the preparation of the solutions differed, depending on the absorbance intensity desired. The aldehyde reagent was prepared as alcoholic or hydroalcoholic solutions, and the sulfonamides were dissolved in aqueous solutions of HCl or H_2SO_4 with the aid of gentle heat. All sulfonamides were dried according to the official procedures before they were used.

Spectral Characteristics.—Schiff base compounds of sulfanilamide were prepared using vanillin, salicylaldehyde, benzaldehyde, and *p*-nitrobenzaldehyde as chromogenic reagents. Solutions of these Schiff bases were 0.02 *M* in aldehyde, 0.1 *M* in HCl, and contained 5 mcg. of sulfanilamide per milliliter of solution. Spectral absorbance curves then were determined, and the curves obtained with vanillin and salicylaldehyde are shown in Fig. 2. The Schiff bases of benzaldehyde and *p*-nitrobenzaldehyde did not absorb significantly at these concentrations. Since vanillin gave the most intense absorbance, it was used as the chromogenic reagent in all subsequent determinations. The wavelength of maximum absorbance occurred at 390 mμ, and all other absorptimetric measurements of the vanillin–sulfonamide systems were made at this wavelength. Neither free vanillin nor the free sulfonamides absorb significantly at this wavelength.

Effect of Temperature.—For the study of the effect of temperature, a solution was prepared as follows. Ten milliliters of sulfanilamide solution (1 mg./ml.) in 5 *N* H_2SO_4 was taken, and sufficient distilled water was added to make 50 ml. of solution. The absorbance values then were determined at various temperatures. A plot of temperature *versus* absorbance is shown in Fig. 3.

Since this portion of the study showed that absorbance readings varied with temperature, it was necessary to establish some means of temperature control for the spectrophotometric method in this study. In previous work where temperature affected absorbance values (6), it had been reported that for solutions prepared at room temperature, a constant temperature was obtained within several minutes after the absorption cells were placed in the cell holder of the spectrophotometer. The criterion for constant temperature was the attainment of a constant absorbance reading for a specified period of time. In this study, such constant absorbance readings were obtained with the Beckman model B spectrophotometer within 5 min. for solutions prepared at room temperature.

Stability of Absorbance System.—The absorptimetric system attained a constant absorbance value immediately upon admixture of the reagents, and

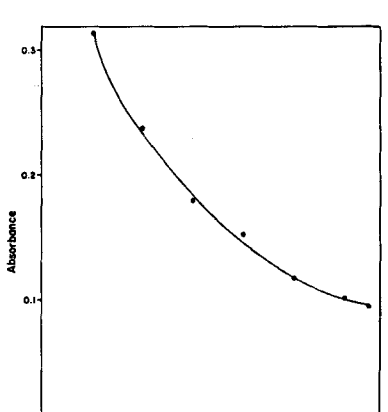

Fig. 3.—Effect of temperature on absorbance.

[3] Mallinckrodt Chemical Works, New York, N. Y.
[4] Madribon. Hoffmann-LaRoche, Inc., Nutley, N. J.
[5] Sulfasuxidine. Merck, Sharp and Dohme, Philadelphia, Pa.
[6] U.S.P. Reference Standards, New York, N. Y.

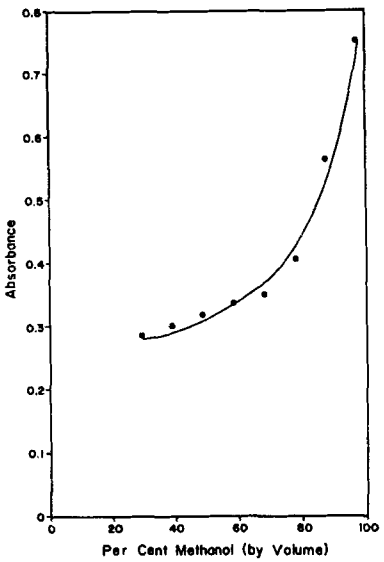

Fig. 4.—Absorbance as a function of methanol concentration.

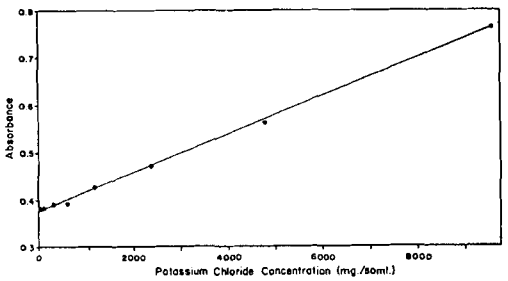

Fig. 5.—Effect of potassium chloride concentration on absorbance.

the absorbance remained constant over a period of 24 hr. for a given temperature.

Effect of Concentration of Aldehyde.—The pronounced equilibrium nature of the reaction dictates that if the concentration of aldehyde is increased while the concentration of sulfonamide is held constant, the concentration of Schiff base will increase. Even after a relatively high molar ratio of vanillin to sulfonamide (25:1) was present, the absorbance still increased linearly as a function of increasing vanillin concentration, all other conditions remaining constant. This behavior is indicative of the low equilibrium constants characteristic of Schiff base formation (4).

Effect of Solvent System.—It was observed that the absorbance values were dependent to a marked degree on the proportion of aqueous–nonaqueous solvent present in the system. For a system which consisted of 10 mg. of vanillin per milliliter and 0.01 mg. of sulfanilamide per milliliter, the absorbance varied with the methanol concentration, as shown in Fig. 4. The system was approximately 1 *N* in H_2SO_4.

Effect of Increased Ionic Strength.—Solutions were prepared which consisted of sulfanilamide, 0.5 mg./ml., and vanillin, 1 mg./ml. The solvent system was 0.1 *N* HCl. Increasing quantities of KCl[1] were added to each solution. A plot of salt concentration *versus* absorbance appears in Fig. 5.

Effect of pH.—Since low pH values were to be investigated, an HCl buffer system was used in the study of the effect of pH. Potassium chloride was used in conjunction with the acid to provide a constant ionic strength of 1.0. The Beckman model H-2 line operated pH meter was used to determine the pH values of the solutions. The buffers used in the standardization of the pH meter were the HCl–KCl buffer solutions described in the U.S.P. Buffer solutions of pH 1.1 and 1.8 were used. The pH meter was standardized with the buffer of pH closest to the pH of the solution being measured. Before determining the pH of the solution, the standardization of the pH meter was checked against the other standard buffer solution. The absorptimetric solutions were prepared by dissolving exactly 50 mg. of sulfanilamide and 50 mg. of vanillin in the various buffers, and the resulting solutions were quantitatively transferred to a 50-ml. volumetric flask. The appropriate buffer then was added to volume. The absorbance and pH of each solution was determined, and a plot of absorbance as a function of pH was prepared, as shown in Fig. 6.

Determination of Sulfanilamide in Low Concentrations.—Sulfanilamide N.F. was determined in micrograms per milliliter concentrations using both the ordinary method and the low absorbance method of precision spectrophotometry (7, 8). In preparing the standard solutions to be used for determining

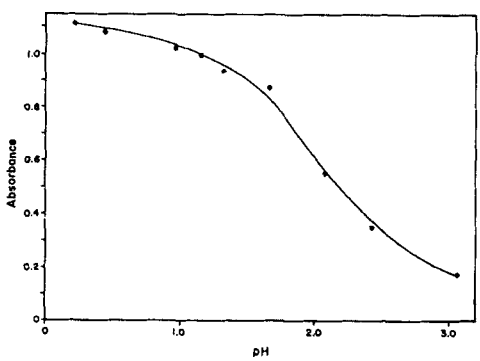

Fig. 6.—Absorbance as a function of pH at constant ionic strength.

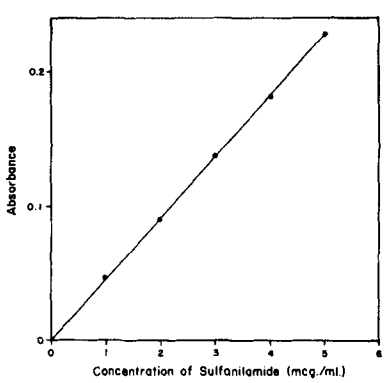

Fig. 7.—Calibration curve for the determination of sulfanilamide in low concentration using the ordinary method of spectrophotometry.

[1] Mallinckrodt, analytical reagent.

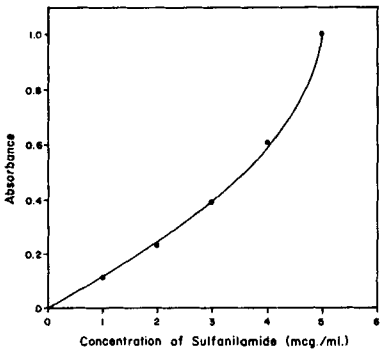

Fig. 8.—Calibration curve for the determination of sulfanilamide in low concentration using the low absorbance method of precision spectrophotometry.

TABLE I.—DETERMINATION OF LOW CONCENTRATIONS OF SULFANILAMIDE

Determination No.	Concn., mcg./ml. Sample A Ordinary Method	Sample A Precision Method	Sample B Ordinary Method	Sample B Precision Method
1	1.04	1.00	3.42	3.44
2	1.06	1.00	3.47	3.48
3	1.02	1.00	3.47	3.48
4	1.04	1.00	3.52	3.49
5	1.00	0.98	3.45	3.45

TABLE II.—PRECISION OBTAINED IN THE DETERMINATIONS OF SULFANILAMIDE N.F. AT LOW CONCENTRATIONS

	Sample A Ordinary Method	Sample A Precision Method	Sample B Ordinary Method	Sample B Precision Method
Mean, mcg./ml.	1.03	1.00	3.47	3.47
Variance	0.0005	0.0001	0.0014	0.0005
S.D.	0.0224	0.0100	0.0374	0.0224
Relative S.D.	2.17%	1.00%	1.08%	0.65%

the calibration curves, the following reagents were used: (*a*) acidic ethanol—45 ml. of HCl was added to sufficient U.S.P. alcohol to give a total volume of 1000 ml.; (*b*) vanillin reagent solution—3% vanillin in acidic ethanol; (*c*) U.S.P. sulfanilamide reference standard solution—1 Gm. of U.S.P. sulfanilamide reference standard per liter in acidic ethanol.

Suitable dilutions of the sulfanilamide reference standard solution were made so that 1 ml., when added to 9 ml. of vanillin reagent solution, supplied the desired concentration of sulfanilamide for the various standard solutions. Absorbance values for the preparation of standard curves were determined by the ordinary method and by the low absorbance method of precision spectrophotometry (7, 8). In this precision method, the 100% transmittance is set, as usual, with a reagent blank. The 0% transmittance, however, is set by use of a solution of the substance being measured, with a concentration that is somewhat greater than that of the most concentrated solution to be measured (8). Therefore, for obtaining the absorbance values by the precision method, the 0% transmittance was set using a reference solution which was 6 mcg./ml. in sulfanilamide. The calibration curves for the ordinary method and the precision method appear in Figs. 7 and 8, respectively. Figure 8 shows that the low absorbance method of precision spectrophotometry provides results in which the relationship between absorbance and concentration is not linear (8). However, this method does provide an expanded absorbance scale, and low absorbance readings thus are avoided. Dilutions of the N.F. sample of sulfanilamide were prepared in the same manner as the reference standard solutions. Two different concentrations were determined, and the concentrations were obtained from the calibration curves. Sample *A* in each determination represented 1 mcg. of sulfanilamide per milliliter, and sample *B* represented 3.5 mcg./ml. The concentrations of sulfanilamide for each determination are given in Table I. The precision data for both methods appear in Table II.

Comparisons of the Schiff Base Method with the Method of Bratton and Marshall.—The method of analysis for sulfonamides by the method of Schiff base formation was carried out for various sulfonamides, and comparative analyses were performed using the colorimetric procedure of Bratton and Marshall. Assays were performed on sulfanilamide, sulfadimethoxine tablets, and succinylsulfathiazole tablets. In the determination by the Schiff base formation method, sufficiently high concentrations of sulfonamide were employed so that an alcoholic solvent system was not required. In the preparation of the standard curve for sulfanilamide, 250 mg. of U.S.P. sulfanilamide reference standard was dissolved in 0.1 *N* HCl and quantitatively transferred to a 100-ml. volumetric flask. HCl (0.1 *N*) was added to volume. Volumes of this solution ranging from 1 to 6 ml. were placed into 25-ml. volumetric flasks, and 1 ml. of 2.5% vanillin in methanol was added. The solutions were brought to volume with 0.1 *N* HCl. The absorbance values were determined, and a calibration curve was prepared. The absorptimetric solutions of the sulfanilamide sample were prepared in the same way, and the values for the per cent of sulfanilamide in the samples were determined from the concentration values obtained from the calibration curve.

Similar procedures were followed for the determinations of the tablets of the other sulfonamides. With succinylsulfathiazole, saponification with

TABLE III.—DETERMINATION OF SULFANILAMIDE SULFADIMETHOXINE TABLETS, AND SUCCINYLSULFATHIAZOLE TABLETS BY THE METHOD OF SCHIFF BASE FORMATION AND THE METHOD OF BRATTON AND MARSHALL

	Schiff Base Method Mean % of Labeled Amt.	Schiff Base Method Relative S.D., %	Bratton-Marshall Method Mean % of Labeled Amt.	Bratton-Marshall Method Relative S.D., %
Sulfanilamide N.F. (Mallinckrodt)	99.56	0.85	99.75	1.00
Sulfadimethoxine tablets (Roche)	97.44	1.50	99.14	0.77
Succinylsulfathiazole tablets U.S.P. (Merck, Sharp and Dohme)	99.76	0.41	99.40	0.33

sodium hydroxide as outlined in the N.F. XI was required to form the primary amine. The commercially available sulfadimethoxine tablets used were colored with a yellow dye; but it was found that for the dilutions used, the absorbance from the color was not sufficient to interfere with the spectrophotometric assays by either method.

For the analysis by the method of Bratton and Marshall, the procedure followed was that outlined by Dux and Rosenblum (9).

Four determinations by each method were performed for each sulfonamide. The mean per cent of labeled amount and the relative standard deviation (10) for each sulfonamide are shown in Table III for both methods.

DISCUSSION

The results of this investigation indicate that the formation of Schiff bases can be utilized as a suitable assay procedure for sulfonamides. As in most analytical methods, provisions must be made for the control of temperature, solvent system, pH, ionic strength, and concentration of reactants.

Since the Schiff base formation reaction is known to be exothermic (11), the influence of temperature on absorbance, as observed in Fig. 3, was as expected. Figure 3 shows that analyses based on this reaction must be carried out at a controlled temperature. Solutions prepared at room temperature achieved a constant temperature within 5 min. after being placed in the spectrophotometer sample chamber. However, it should be pointed out that for assays based on previously prepared calibration curves, it is necessary to ascertain that the temperature of the standard solutions used in obtaining the calibration curve and the temperature of the solutions being assayed are the same. A constant temperature cell holder mounted in the spectrophotometer also can be used to maintain a constant temperature.

As the result of the formation of 1 mole of water per mole of Schiff base produced, maximum Schiff base formation can be expected in nonaqueous solvents. Figure 4 indicates that maximum absorbance and consequently maximum sensitivity can be obtained in nonaqueous solvent systems. Sensitivity of the method can also be increased by increasing the concentration of aldehyde present in the system, since high aldehyde concentration drives the reaction in the direction of Schiff base formation.

Ionic strength and pH are additional factors which require control in an assay procedure based on Schiff base formation. Figure 5 shows that absorbance is greatly increased at high salt concentrations. This phenomenon is perhaps due to the ionic attraction provided by the salt for polar water molecules. The absorbance is also influenced by hydrogen ion concentration. The study of pH at constant ionic strength (Fig. 6) indicates that maximum sensitivity can be achieved at pH values below 1.5. Moreover, the method is less sensitive to small variations in pH in this range.

A favorable characteristic of the system is that the absorbance of the Schiff base is stable and does not fade over a 24-hr. period. This is an advantage over the colorimetric method of Bratton and Marshall. In the latter method, absorbance readings must be made within 15 min. after color development, due to precipitation of the azo dyes formed in the method (9).

In determinations of low concentrations of sulfanilamide, it was found that the method was sensitive to sulfanilamide concentrations of approximately 0.5 mcg./ml. (3×10^{-6} *M*) when suitable solvent system and aldehyde concentration were employed. In low absorbance determinations, the precision method of spectrophotometry provided a means of avoiding low absorbance values; but even with the ordinary method, good analytical results were obtained.

The comparative analyses performed with the diazotization method of Bratton and Marshall seem to indicate that the results obtained by the two methods compare favorably. The method of analysis of sulfonamides based on Schiff base formation does not involve diazotization. Thus, it eliminates the need for freshly prepared sodium nitrite solutions and ammonium sulfamate solutions required with the method of Bratton and Marshall. Control of pH and ionic strength are required in both methods (9).

The method of Schiff base formation provides a rapid and sensitive procedure for sulfonamide analysis. Analysis over a wide concentration range is possible by adjusting the water content of the solvent system and the concentration of aldehyde. Beer's law was obeyed at all concentration ranges and under all conditions studied.

Subsequent work will investigate the applicability of this technique in analysis of sulfonamide in biological materials and of sulfonamide mixtures in conjunction with paper and thin-layer chromatography.

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