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Quantitative Method for Determination of Indole, Tryptophan, and Anthranilic Acid in the Same Aliquot

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Procedures for the determination of indole and tryptophan have in most instances employed modified Ehrlich and Hopkins-Cole methods (1–8). Generally these methods require solvent partition of the different indole derivatives and yield unstable color products, and therefore are somewhat cumbersome when a large number of determinations is required. Spies and Chambers (9) measured tryptophan without interference by indole but made no attempt to quantitate indole.

The difference in reactivity of indole derivatives (10) suggested that under controlled reaction conditions indole and tryptophan might be determined in the same aliquot.

Whereas the methods for indole and tryptophan have been primarily dependent on a reaction with an aldehyde, the determination of anthranilic acid has depended on diazotization and a coupling reaction (11, 12). When the latter method was applied to the determination of anthranilic acid in the complex bacterial medium of Kuiken *et al.* (13), erroneous results were obtained; the color intensity increased continuously for several hours with an occasional formation of anomalous colors.

In the present investigation it was found that indole and anthranilic acid yielded distinct and differently colored products on reaction with *p*-dimethylaminobenzaldehyde (PDAB) in 0.5 *N* sulfuric acid; tryptophan formed no color under these conditions. Furthermore, an increase in the acidity of the test solution followed by the addition of nitrite yielded a color, the intensity of which was proportional to tryptophan concentration. This observation permitted the development of a simple method for the determination of anthranilic acid, indole, and tryptophan in the same

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aliquot. Parts of this method were applied to metabolic studies in microorganisms (14, 15).

MATERIALS AND METHODS

Reagents

PDAB solution: 60 mg of *p*-dimethylaminobenzaldehyde per milliliter of 3 *N* H_2SO_4.

$NaNO_2$ solution, 0.1% aqueous.[2]

30 *N* H_2SO_4.

Procedure

One-milliliter aliquots of the PDAB solution were added to matched colorimeter tubes containing 5 ml of aqueous solutions of the substance(s) to be analyzed. Absorbancy was measured after 20 min at 420 mμ (anthranilic acid) and at 550 mμ (indole).[3] Three milliliters of 30 *N* H_2SO_4 was added with thorough mixing to the previously chilled (ca. 0°C) test solutions.[4,5] After 1 hr, the tryptophan color was developed by the addition, with thorough mixing of 0.1 ml of the $NaNO_2$ solution. After 30 min, absorbancy at 580 mμ was measured (tryptophan).

Generally, two sets of solutions were used for plotting the standard curves: set (*a*) contained both tryptophan and anthranilic acid at three levels each; standard curves were plotted from readings at 420 mμ (anthranilic acid standard) and at 580 mμ (tryptophan standard). Set (*b*) contained a constant concentration of tryptophan and four levels of indole and were read at 550 mμ (indole standard), at 420 mμ (see corrections for indole interference with anthranilic acid determination), and at 580 mμ (see graphic method for elimination of indole interference in tryptophan determination).

RESULTS

Absorption Spectra

The absorption spectra of the colored solutions resulting when PDAB was added to solutions containing indole, anthranilic acid, and trypto-

[2] The optimal nitrite concentration was found to vary due to presence of other medium constituents and would have to be determined for each system used.

[3] All measurements, unless otherwise specified, were made with a Lumetron model 401A photometer.

[4] The yellow color due to anthranilic acid faded immediately; some pink indole color remained.

[5] In the presence of light, the tryptophan color developed slowly and color intensity increased for a long time. The addition of nitrite speeded up this reaction; the final color intensity was the same in solutions exposed to light and those kept in the dark.

phan indicated that the colored compound formed due to anthranilic acid did not absorb at the wavelength used for indole determination (550 mμ) (Fig. 1). Thus it appeared that anthranilic acid would not interfere with

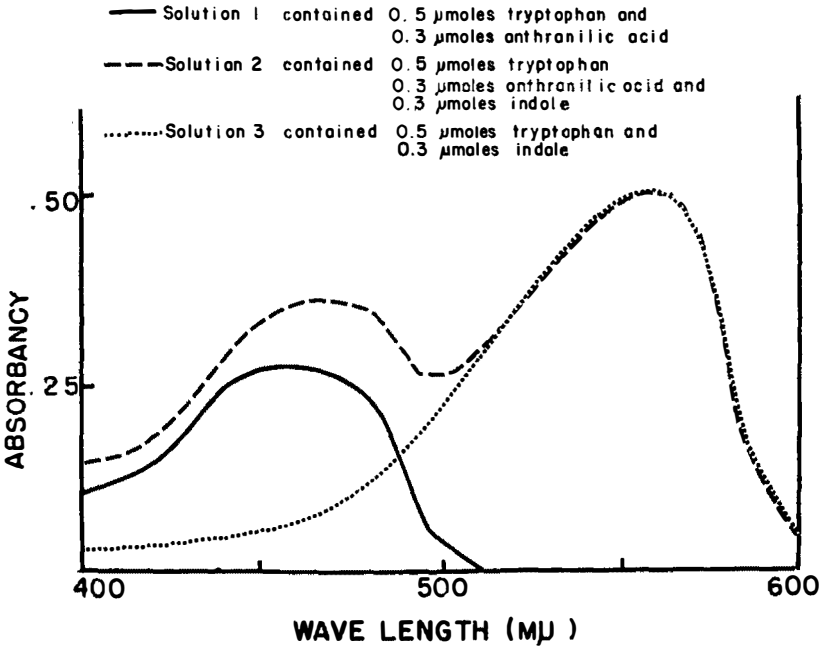

FIG. 1. Absorption spectra obtained with a Cary recording spectrophotometer model 11 MS. Volume of each solution was 5 ml. Color was developed with 1 ml of PDAB solution (60 mg PDAB/ml of 3 N H_2SO_4).

TABLE 1

STABILITY OF ANTHRANILIC ACID COLOR WITH TIME

Tabulated values are absorbancies at 420 mμ as measured with a Lumetron model 401A photometer. Color was developed by addition of 1 ml of PDAB solution (60 mg of PDAB/ml of 3 N H_2SO_4) to tubes containing 5 ml of the aqueous solution of tryptophan, anthranilic acid, and indole.

Tryptophan μmole	Anthranilic acid, μmole	Indole, μmole	Time for color development prior to reading, hr	
			1.5	27
0.3	0.3	—	0.299	0.295
0.5	0.3	—	0.309	0.305
—	0.3	—	0.290	0.288
0.5	0.3	0.3	0.292[a]	0.302[a]

[a] Values corrected by subtracting the absorbancy due to indole color.

indole determination. However, the indole color does absorb slightly at the wavelength used for the anthranilic acid determination (420 mμ), and would therefore interfere with anthranilic acid determination. Since the absorption of both compounds was additive, a mere subtraction of the absorption at 420 mμ due to indole permitted the determination of anthranilic acid (Table 1). Tryptophan did not form color in weak acid and, hence, would not interfere with the determination of either indole or anthranilic acid.

Anthranilic Acid Determination

The intensity of the anthranilic acid color (yellow) was proportional to the anthranilic acid concentration (Fig. 2). The curve is linear to an

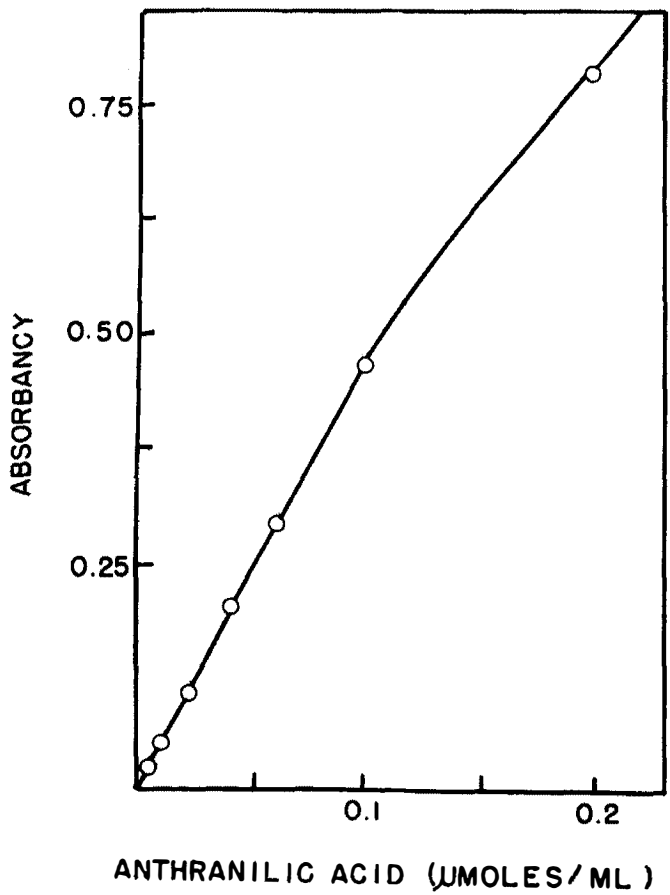

FIG. 2. Absorbancy as a function of anthranilic acid concentration. Measurements were made with a 420 mμ filter in a Lumetron model 401A photometer.

absorbancy of 0.35. However, reproducible results were obtained with anthranilic acid concentrations yielding absorbancies as high as 0.75. The color was stable over a period of 27 hr (Table 1) and was not affected by ordinary fluorescent light.

Indole Determination

The absorbancy of the colored product formed from indole as a function of concentration is shown in Fig. 3. The color is stable for several

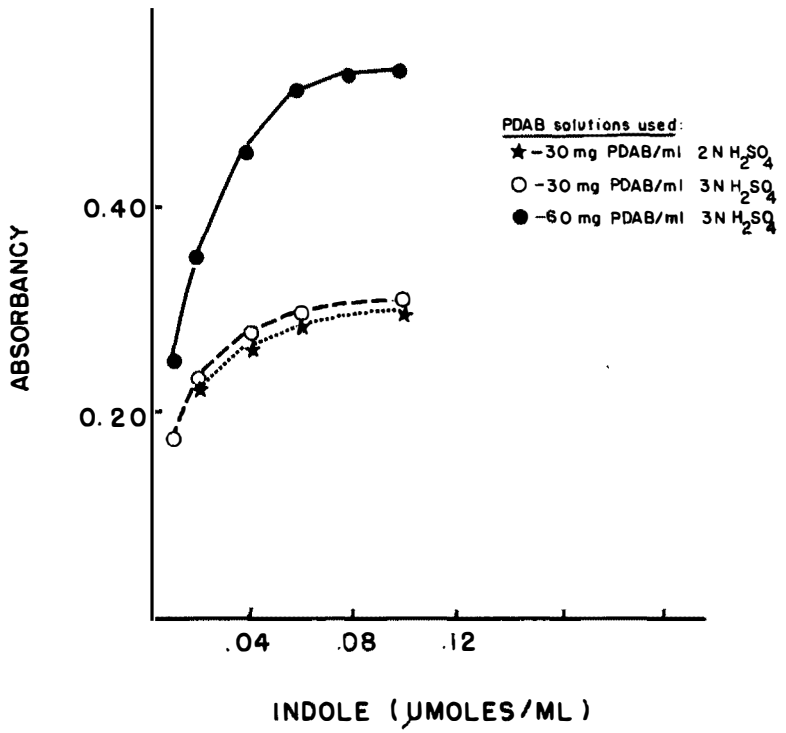

FIG. 3. Absorbancy of indole color as a function of PDAB concentration. Measurements were made with a 550 mμ filter in a Lumetron model 401A photometer.

hours and is highly reproducible. Ordinary laboratory illumination (fluorescent light) does not affect its intensity.

The study of the reaction between indole and PDAB to form the color complex indicated an equilibrium reaction: $K = 17$ molar^{-1} (14). This suggested that an increase in PDAB concentration may shift the equilibrium of the reaction in the direction of the formation of the color complex. A twofold increase in the PDAB concentration resulted in a marked increase in the intensity of the color of indole solutions. In addi-

tion there was a significant increase in the range of indole concentrations that could be measured by the colorimetric method (Fig. 3). The color formed under these conditions was stable. In order to prevent the precipitation of the slightly soluble PDAB, the higher acid concentration (3 *N*) of the PDAB solution was chosen. Since a large increase in acidity of the solutions caused the indole color to fade fairly rapidly, the use of much larger concentrations of PDAB would be impractical.

The heating of aqueous solutions of indole during color development increased the intensity of the color. Subsequent cooling caused a drop in the intensity of the color. To avoid erroneous results due to fluctuations of temperature and of PDAB concentrations, a standard curve should be plotted for every set of indole determinations.

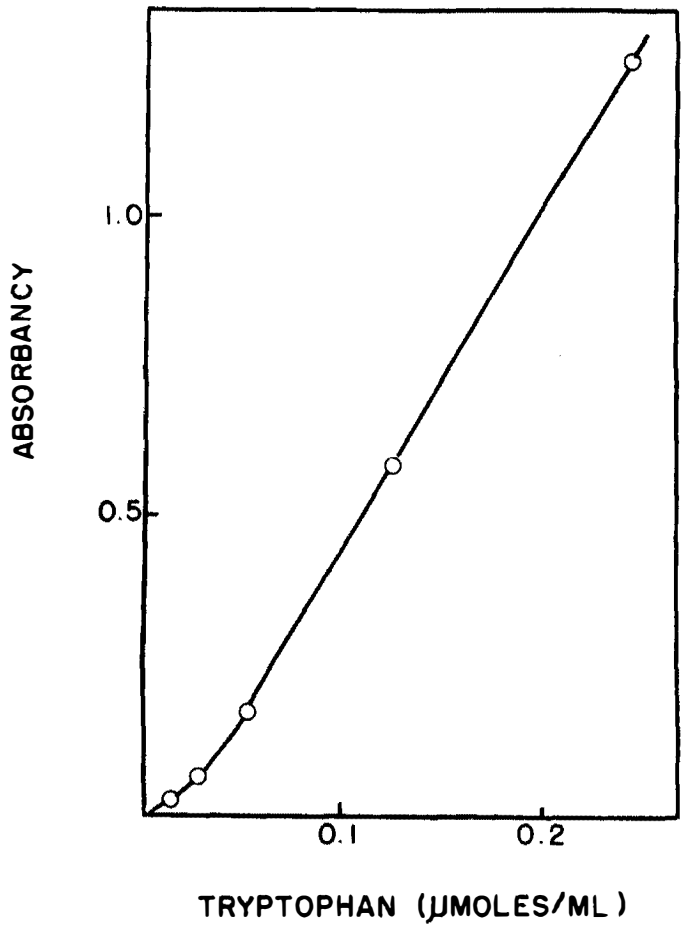

FIG. 4. Absorbancy as a function of tryptophan concentration. Measured at 580 mμ in a Beckman DU spectrophotometer.

Tryptophan Determination

The absorbancy of the tryptophan color (blue) as a function of concentration is shown in Fig. 4. This color is also stable for several hours. The curve is linear (except for an initial small segment) to an absorbancy of at least 2.0 in the Beckman DU spectrophotometer. In the determination of tryptophan, solutions kept in the dark throughout the procedure give readings about 2% higher than do those exposed to light. Spies and Chambers (16) reported a light-catalyzed destruction of tryptophan in strong acid solution. With our method no such effect is observed; tryptophan and indole are complexed with PDAB before the addition of concentrated acid, and this reaction may protect tryptophan from light-catalyzed destruction.

Thorough chilling of the solution before the addition of the concentrated sulfuric acid is essential. Failure to prechill leads to a marked decrease in the density of tryptophan color as well as to erratic results. Tryptophan determinations were affected neither by temperature fluctuations nor by slight differences in PDAB concentrations.

TABLE 2

INTERFERENCE OF INDOLE WITH TRYPTOPHAN COLOR DEVELOPMENT

One-milliliter aliquots of PDAB solution (60 mg of PDAB/ml in 3 *N* H_2SO_4) were added to tubes containing 5-ml samples. Color was developed by addition of 3 ml of 30 *N* H_2SO_4 and 0.1 ml of 0.05% sodium nitrite. Tabulated values are absorbancies at 580 mμ as measured with a Lumetron model 401A photometer.

Tryptophan, μmole	Indole, μmole	Observed	Corrected[a]	Error[b]
0.50	—	0.358		
0.30	—	0.195		
0.10	—	0.033		
—	0.30	0.093		
—	0.20	0.055		
—	0.10	0.024		
—	0.01	0.005		
0.50	0.30	0.463	0.370	0.012
0.30	0.30	0.306	0.213	0.018
0.10	0.30	0.148	0.055	0.022
0.30	0.20	0.262	0.207	0.012
0.50	0.10	0.394	0.370	0.012
0.30	0.10	0.230	0.206	0.011
0.10	0.10	0.069	0.045	0.012
0.30	0.01	0.197	0.192	−0.003

[a] Values for solutions containing both indole and tryptophan were corrected by subtracting the absorbancy of a solution containing indole alone at the same concentration.

[b] Difference between the corrected value and the absorbancy of a solution containing tryptophan alone at the same level.

A. *Effect of Nitrite Concentration.* The optimal nitrite concentration must be determined for each system used. In the presence of constituents other than tryptophan, more nitrite is required for development of the maximum color intensity than is needed when a pure solution of tryptophan is assayed. Presumably part of the nitrous acid is consumed in side reactions such as those with amino groups and reducing sugars. It is possible to choose a nitrite level that gives approximately equal and maximal color intensity both in medium containing amino acids and glucose and in water. This minimizes changes in color intensity due to changes in the concentration of constituents other than tryptophan during bacterial growth. Too high levels of nitrite cause the appearance of a green color, and on further addition of nitrite a yellow precipitate is formed.

B. *Attempts to Correct for Indole Color in Tryptophan Determination.* Preliminary results indicated that tryptophan does not interfere with the indole or anthranilic acid determination. On the other hand, indole produces some color under the conditions used for the tryptophan assay. The following attempts were made to correct tryptophan assays for the presence of indole:

1. Subtraction of absorbancy. The absorption due to indole color in tryptophan determination could not be corrected by mere subtraction. This indicated that under conditions for tryptophan determinations there is some sort of interaction in solutions containing both tryptophan and indole which cannot be represented by a simple addition of the individual colors when present alone (Table 2).

This unaccountable error in tryptophan determination was less pronounced with smaller amounts of PDAB (30 mg/tube) and prior to this observation was attributed to errors in experimental techniques. Additional experiments indicated that this error tended to increase with indole concentration. Attempts to find a wavelength at which indole would not interfere have failed.

2. Graphic method (extrapolation to zero indole concentration). The magnitude of the absorbancy of the solutions containing both indole and tryptophan was found to be directly proportional to the indole concentration when tryptophan concentration was kept constant. Furthermore, several experiments indicated that, for a series of solutions each with a specific tryptophan concentration but varying indole concentration, a family of parallel lines was obtained. Extrapolation of these lines to zero concentration of indole should yield the corrected absorbancy for each of the specific tryptophan concentrations. These results suggested that such a plot may be used for the determination of tryptophan in the presence of interfering concentrations of indole.

This method of treating the data was experimentally tested in the following way: Two standard curves were determined by measuring the absorbancy of the colors developed in two series of solutions containing 0.10 μmole/ml and 0.06 μmole/ml of tryptophan, respectively, and varying amounts of indole. Two test solutions were prepared containing 0.02 μmole/ml of tryptophan but different amounts of indole. The absorbancies of the colors developed during the tryptophan determination with these test solutions were plotted on the same graph as the standard curve (Fig. 5). A line drawn through either of these points and parallel to the standard curves intersected the ordinate at a point that cor-

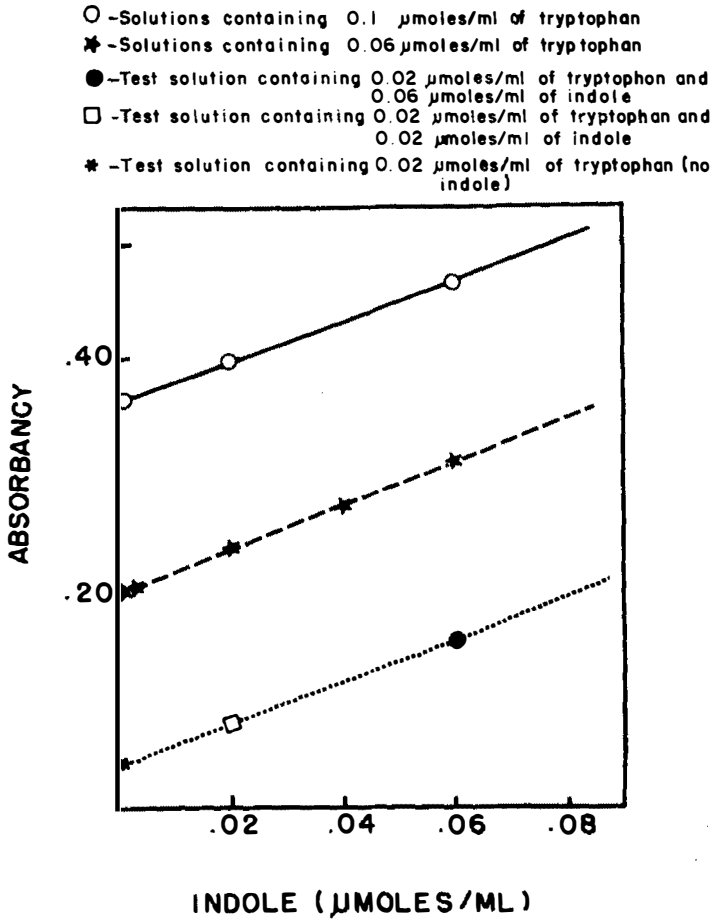

FIG. 5. Absorbancy of solutions containing constant concentrations of tryptophan as a function of indole concentration. Measurements were made with a 580 mμ filter in a Lumetron model 401A photometer.

responded to the absorbancy of a similar solution containing 0.02 μmole/ml of tryptophan and no indole. The data of additional experiments plotted in a similar manner indicated that the error due to the presence of indole color in tryptophan determinations can be corrected accurately.

Determination of Anthranilic Acid, Indole and Tryptophan in the Same Samples

The procedure for the successive determination of anthranilic acid, indole, and tryptophan in the same samples was essentially the same as the one described for the individual compounds (See "Materials and Methods"). Results (Table 3) indicated: (*a*) tryptophan did not interfere with anthranilic acid and indole determinations, (*b*) anthranilic acid did not interfere with either indole or tryptophan determinations, (*c*) indole interference during anthranilic acid determination was eliminated

TABLE 3

INTERACTION AMONG ANTHRANILIC ACID, INDOLE, AND TRYPTOPHAN DURING THEIR DETERMINATION

Tabulated values are absorbancies at 550 mμ (indole), 420 mμ (anthranilic acid), and 580 mμ (tryptophan) as measured with a Lumetron model 401A photometer. Colors due to indole and anthranilic acid were developed by addition of 1 ml of PDAB solution (60 mg of PDAB/ml of 3 *N* H_2SO_4) to colorimeter tubes containing 5 ml of aqueous solutions of the compounds to be determined. Tryptophan color was developed by addition of 3 ml of 30 *N* H_2SO_4 followed by 0.1 ml of 0.1% $NaNO_2$.

Part 1. Effect of anthranilic acid and indole on color development under conditions used for tryptophan determination

Tryptophan, μmole	Indole, μmole	Anthranilic acid, μmole	Absorbancy at 580 mμ	
			Observed	Corrected[a]
0.3	—	—	0.286	(0.286)
0.3	0.3	0.3	0.361	0.290
0.2	—	—	0.170	(0.170)
0.2	0.3	0.3	0.238	0.165
0.1	—	—	0.060	(0.060)
0.1	0.2	0.3	0.102	0.060
0.1	0.1	0.3	0.068	0.060
—	0.3	—	0.022	0.000
—	0.2	—	0.000	0.000
—	—	1.00	0.000	(0.000)
—	—	0.30	0.000	(0.000)
—	—	0.10	0.000	(0.000)
—	—	0.025	0.000	(0.000)

[a] Values corrected by graphic extrapolation to zero indole concentration except those figures within parentheses.

TABLE 3 (*Continued*)

Part 2. Effect of tryptophan and anthranilic acid on color development under conditions used for indole determination

Indole, μmole	Tryptophan, μmole	Anthranilic acid, μmole	Absorbancy at 550 mμ
0.3	—	—	0.505
0.3	0.5	0.2	0.480
0.3	0.5	0.1	0.482
0.3	0.5	0.05	0.490
0.3	0.3	0.3	0.512
0.3	0.2	0.3	0.503
0.2	—	—	0.453
0.2	0.5	0.1	0.433
0.2	0.1	0.3	0.444
0.1	—	—	0.337
0.1	0.1	0.3	0.340
0.05	—	—	0.229
0.025	—	—	0.139
—	1.00	—	0.000
—	0.1	—	0.005
		1.00	0.004
		0.025	0.001

Part 3. Effect of tryptophan and indole on color development under conditions used for anthranilic acid determination

Anthranilic acid, μmole	Tryptophan, μmole	Indole, μmole	Absorbancy at 420 mμ	
			Observed	Corrected[b]
1.0	—	—	0.780	(0.780)
0.5	—	—	0.460	(0.460)
0.3	—	—	0.289	(0.289)
0.3	0.3	0.3	0.357	0.290
0.3	0.2	0.3	0.355	0.288
0.3	0.1	0.2	0.340	0.282
0.3	0.1	0.1	0.330	0.295
0.2	—	—	0.197	(0.197)
0.2	0.5	0.3	0.265	0.198
0.1	—	—	0.104	(0.104)
0.1	0.5	0.3	0.167	0.100
0.1	0.5	0.2	0.156	0.098
0.05	—	—	0.050	(0.050)
0.05	0.5	0.3	0.119	0.052
—	1.0	—	0.000	(0.000)
—	—	0.3	0.067	0.000
—	—	0.2	0.058	0.000
—	—	0.1	0.035	0.000
—	—	0.05	0.024	0.000

[b] Values corrected by subtraction of the absorbancy due to indole concentration except those figures within parentheses.

by a subtraction of its absorbancy at 420 mμ, and (*d*) indole interference during tryptophan determination was eliminated by a graphic extrapolation to zero indole concentration as specified before.

Interfering Substances: 1. Glucose, autoclaved in the presence of L. arabinosus medium. The instability and poor reproducibility of the color were traced to the presence of glucose during autoclaving. In a single experiment autoclaving caused a lowering of 85% in the absorbancy of indole and of 32% in absorbancy of tryptophan, as compared with unautoclaved medium. Glucose added after autoclaving does not affect the results for either compound. A similar effect was observed by Patton and Hill (17), who showed that the presence of glucose during autoclaving caused a significant decrease in tryptophan as determined microbiologically.

2. Iodide ions. Small amounts of iodide (25 mg KI in the standard assay) caused a slow and irreversible fading of the ordinarily stable indole color (absorbancy of 0.242 dropped to 0.151 in 1 hr). The blue color of the normally developed tryptophan solutions was altered to a yellow green when small amounts of potassium iodide were added. The blue color of normal intensity reappeared upon the addition of sodium thiosulfate (0.1 *N*).

3. Thiosulfate. Two drops of 0.1 *N* sodium thiosulfate solution caused the immediate and nearly complete disappearance of the indole color (absorbancy of 0.295 dropped to 0.045). Thiosulfate had no detrimental effect on the tryptophan color.

4. Indole derivatives. Skatole reacts with PDAB in both dilute (0.5 *N*) and concentrated (11 *N*) acid; it was found to interfere with indole and to a lesser extent with tryptophan determination. On the other hand, indoleacetic acid forms color only in concentrated acid and hence interferes with tryptophan, but not with indole, determination (10).

SUMMARY

The Ehrlich method for the determination of indole-like compounds was expanded by the appropriate control of acidity and oxidation to differentiate between anthranilic acid, indole, and tryptophan. This method afforded a convenient quantitative determination of these three compounds in the same aliquot. This determination depends upon the reactions of anthranilic acid and indole with *p*-dimethylaminobenzaldehyde (PDAB) in 0.5 *N* acid to form colored products. The formation of a colored derivative of tryptophan with PDAB requires the presence of much higher acid concentration (ca. 11 *N*) and an oxidizing agent. The color formation does not appear to be affected by the presence of constituents of a synthetic *Lactobacillus* medium.

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