INDOLE REACTIONS IN BACTERIA

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Received for publication January 30, 1958

Kitasato's (1889) discovery that *Escherichia coli* could be distinguished from *Klebsiella* species (*Aerobacter aerogenes*) by virtue of its ability to produce indole initiated the detection of indole production as an accepted analytical tool for the bacteriologist. The use of *p*-dimethylaminobenzaldehyde for this purpose, first applied by Böhme (1906), modified by Kovacs (1928), and most recently refined by Gadebusch and Gabriel (1956), has become the accepted method for the detection of this metabolite in cultures. Thus, Edwards and Ewing (1955) and Kauffmann (1954) recommend the use of the simple Kovacs' test (*p*-DAB, direct). The *Manual of Microbiological Methods* (1957) indicates Goré's (1921) modification is more specific than the aforementioned Kovacs' test.

Although the relationship between tryptophan and indole was obvious to investigators as early as 1912 (Zipfel), the majority of workers were unable to enhance indole production by the addition of tryptophan to the medium (Kulp, 1925). Wood *et al.* (1947) introduced tryptophan as an integral part of the medium for the quantitative estimation of tryptophanase. Since *p*-dimethylaminobenzaldehyde reacts not only with indole but also with α and β substituted pyrroles, investigators advised distillation of cultures to separate the more volatile indole from related metabolites (Fellers and Clough, 1925; Goré, 1921). Wood *et al.* (1947) extracted indole with toluene prior to its treatment with *p*-dimethylaminobenzaldehyde. Gallop and Seifter (1958, *unpublished data*) discovered recently that alkaline hydroxylamine reacts specifically with unsubstituted pyrroles and indole to form products which yield colored complexes in acidic media. In view of the lack of specificity of the widely used direct *p*-dimethylaminobenzaldehyde test, a study comparing that determination with another modified Ehrlich's test (extracted *p*-dimethylaminobenzaldehyde) and with the hydroxylamine reaction was made.

MATERIALS AND METHODS

Organisms. One hundred and seventy-two organisms were studied. All but seven of these microorganisms were isolated during routine bacteriological analysis of clinical material. The others were obtained from the American Type Culture Collection. The identity of all organisms was established according to the tenets of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957) and, when necessary, confirmed by immunochemical methods.

Media. All organisms were maintained on Eugon (BBL) agar slants, kept refrigerated after initial incubation at 37 C. Transfers were made to Eugon (BBL) broths which were incubated at 37 C for 18 hr. Such cultures served as inocula for all other media tested. Transfers were usually made with sterile Pasteur pipettes which delivered 0.05 ml per drop.

The substrate originally employed for the growth of and indole production by the organisms was trypticase nitrate broth (BBL), designated in this study as medium A. Media B and C contained, in addition to the ingredients of medium A, 0.5 g L-tryptophan, while medium C was further modified by the addition of 0.01 g pyridoxal per 100 ml. Medium D (in per cent) was composed as follows: glucose, 0.1 g; K_2HPO_4, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; casein hydrolyzate (Mallinckrodt), 0.01 g; yeast extract (Difco), 0.01 g; agar (Difco), 0.01 g; L-tryptophan, 0.5 g; pH 7.2 $\pm$ 0.1. In medium E the ingredients of medium D were supplemented with NH_4Cl, 0.02 g and $NaNO_3$, 0.02 g per 100 ml.

Anaerobic cultures were set up with medium B in small ground-glass stoppered bottles. These were filled, after dry sterilization, with sterile culture medium, boiled to remove excess air, inoculated after cooling, and filled with additional recently boiled and cooled medium to the rim of the bottle, care being taken to avoid introduction of air. The ground-glass stopper

was then jammed into place. The bottles were incubated at 37 C and indole determinations performed at various time intervals covering several days, a new bottle being assayed each time.

Reagents. The reagent for the direct *p*-dimethylaminobenzaldehyde test was prepared according to the method of Gadebusch and Gabriel (1956). The quantitative and qualitative *p*-dimethylaminobenzaldehyde reagents were prepared according to Wood *et al.* (1947); the hydroxylamine hydrochloride reagents were made up as directed by Gallop and Seifter (1958, *unpublished data*) as follows: NaOH, 4 N; $NH_2OH \cdot HCl$, 0.25 N; H_2SO_4, 4 N.

Procedures. (1) Direct *p*-dimethylaminobenzaldehyde:—To 1.0 ml of an 18 hr or older broth culture, 0.5 ml of the reagent is added. The color developed is recorded immediately and after 5 min. A spot test modification of this test was also employed. To 4 to 5 drops of the culture in the depression of a porcelain or plastic plate, 2 drops of the reagent are added and the color recorded immediately and after 5 min.

(2) *p*-Dimethylaminobenzaldehyde method with extracted indole:—(a) Quantitative procedure: To 1 ml aliquot of the supernatant of an 18 hr or older broth culture, obtained by centrifugation for 10 min at 2000 rpm, 1 ml of toluene is added and the two are mixed gently. Five-tenths ml of the toluene layer is removed to a clean test tube. One ml *p*-dimethlyaminobenzaldehyde in ethyl alcohol (95 per cent) is added, followed by 8.5 ml of acid alcohol. The tube is allowed to stand at room temperature and then its color is compared with a reagent blank at 540 mμ. Cultures or standards in excess of 15 μg were diluted with uninoculated broth to bring them into the 1 to 15 μg range.

(b) Spot test procedure: To 4 to 5 drops of culture medium in which the organism has grown for at least 18 hr, an equal number of drops of toluene is applied in the depression of a spot plate. Slight agitation with the tip of a Pasteur pipette is followed by the transfer of some of the toluene layer (2 to 3 drops) to an adjacent clean depression. One or two drops of the *p*-dimethylaminobenzaldehyde reagent are now added as well as 5 to 6 drops of acid alcohol. Color development is recorded immediately and after 20 min.

(3) Hydroxylamine method:—(a) Quantitative procedure: one ml of an 18 hr or older broth culture supernatant, obtained by centrifugation at 2000 rpm for 10 min, and 1 ml of NaOH are mixed; 2 ml of the hydroxylamine reagent are added. This mixture is allowed to stand for 15 min. Five ml sulfuric acid are then added and the color developed read against a reagent blank at 530 mμ.

(b) Spot test procedure: This test was performed in a porcelain plate depression using drops per ml of the quantitative procedure, without the waiting period.

Uninoculated control media and indole standards accompanied all determinations. All spectrophotometric determinations were carried out in a B & L Spectronic 20 spectrophotometer.

Compounds related to indole, obtained through the generosity of Drs. F. Millich and E. Becker, Department of Chemistry, The Polytechnic Institute of Brooklyn, Brooklyn, New York, are listed in table 1. Since these compounds were available in minute quantities only, 1 or 2 crystals were dissolved in 0.5 ml of medium A and the various spot tests were performed.

RESULTS

The specificity of the reagents used in this study was investigated by comparing the ability of each to react with indole derivatives, as indicated in table 1. The Gallop-Seifter reagent displayed the greatest specificity of all the reagents tested; it developed color only with indole and 5-methyl indole, and color development was independent of the time of reaction. The extracted *p*-dimethylaminobenzaldehyde reagent reacted immediately only with indole and 5-methyl indole, but yielded positive results with the other compounds within 20 min. With the direct *p*-dimethylaminobenzaldehyde reagent all 17 substituted compounds developed color complexes indistinguishable from that of the unsubstituted indole. This color became apparent within 5 min after mixing the reactants.

Table 2 reflects the application of the several indole spot tests to a variety of bacteria representing alleged indole producers.

The direct *p*-dimethylaminobenzaldehyde test is not specific for indole, whereas, as employed here, the extracted *p*-dimethylaminobenzaldehyde and hydroxylamine tests were specific. The results with both these tests parallel one another closely, although 3 *Proteus mirabilis* and 3 additional *Klebsiella* sp. gave positive results only with the extracted *p*-dimethylaminobenzalde-

TABLE 1

Spot tests with indole derivatives

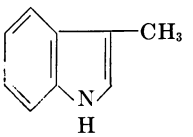
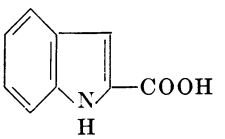
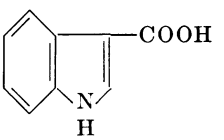
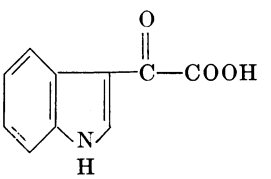
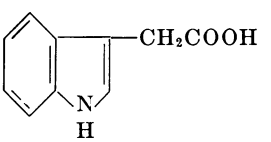
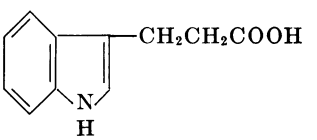
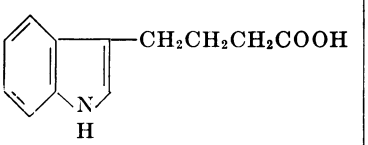
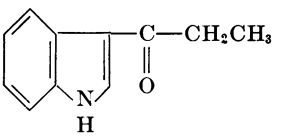
Compound	Formula	Methods*					
		p-DAB Direct		*p*-DAB Extracted		Hydroxylamine	
		Immediate	5 min	Immediate	20 min	Immediate	20 min
3-Methyl indole		−	+	−	+	−	−
Indole-2-carboxylic acid		−	+	−	+	−	−
Indole-3-carboxylic acid		−	+	−	+	−	−
Indole-3-glyoxilic acid		−	+	−	+	−	−
Indole-3-acetic acid		−	+	−	+	−	−
Indole-3-propionic acid		−	+	−	+	−	−
Indole-3-butyric acid		−	+	−	+	−	−
Ethyl, indole-3-ketone		−	+	−	+	−	−

TABLE 1 (continued)

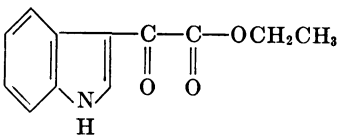
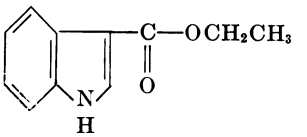
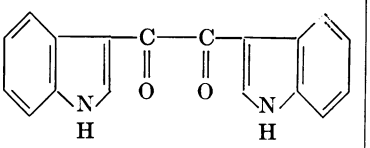
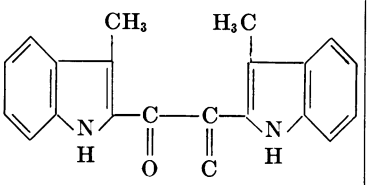
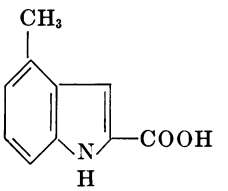
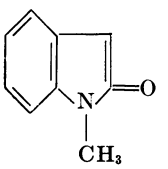
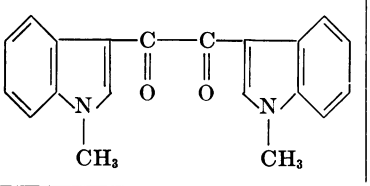
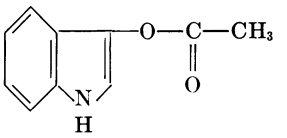
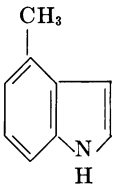
Compound	Formula	Methods					
		p-DAB Direct		*p*-DAB Extracted		Hydroxylamine	
		Immediate	5 min	Immediate	20 min	Immediate	20 min
Indole-3-glyoxilic acid, ethyl ester		−	+	−	+	−	−
Indole-2-carboxylic acid, ethyl ester		−	+	−	+	−	−
Di-indole-3,3′-diketone		−	+	−	+	−	−
Di-3-methyl indole-2, 2′-diketone		−	+	−	+	−	−
5-Methyl indole-2-carboxylic acid		−	+	−	+	−	−
1-Methyl indole-2-one		−	+	−	+	−	−
Di-1-methyl indole-3, 3′-diketone		−	+	−	+	−	−

TABLE 1 (continued)

Compound	Formula	Methods					
		p-DAB Direct		*p*-DAB Extracted		Hydroxylamine	
		Immediate	5 min	Immediate	20 min	Immediate	20 min
3-Acetoxyl indole		−	+	−	+	−	−
5-Methyl indole		+	+	+	+	+	+

* *p*-DAB = *p*-dimethylaminobenzaldehyde; + = positive reaction; − = negative reaction.

TABLE 2

Application of indole spot tests to bacterial broth cultures

Organism	No. of Sp. Representatives Studied	Method*			
		p-DAB Direct		*p*-DAB Extracted	Hydroxylamine
		Immediate	5 min	20 min	20 min
Escherichia coli	35	35+	35+	35+	35+
Klebsiella sp.	50	22+, 28−	50+	7+, 43−	4+, 46−
Proteus vulgaris	14	14+	14+	14+	14+
Proteus mirabilis	22	10+, 12−	22+	3+, 19−	22−
Escherichia freundii	6	1+, 5−	6+	1+, 5−	1+, 5−
Pseudomonas aeruginosa	8	3+, 5−	8+	1+, 7−	1+, 7−
Alkalescens-dispar group 2	5	5+	5+	5+	5+
Proteus morganii	2	1+, 1−	2+	2+	2+

* *p*-DAB = *p*-dimethylaminobenzaldehyde; + = positive reaction; − = negative reaction.

hyde test. However, it can be seen from figures 1 and 2 that the sensitivity of the extracted *p*-dimethylaminobenzaldehyde test extends to lower limits than does the hydroxylamine test and the additional positives obtained with the former test may very well mirror the presence of indole in low concentrations.

A number of indole-negative bacteria were examined in the same manner. These included 6 species of the genus *Salmonella*, *Serratia marcescens*, *Shigella newcastle*, *Alcaligenes faecalis*, *Staphylococcus aureus*, viridans streptococci, and *Streptococcus faecalis*. Negative results were obtained with the hydroxylamine and the extracted *p*-dimethylaminobenzaldehyde methods, while the direct *p*-dimethylaminobenzaldehyde test was positive within 5 min. *Vibrio metschnikovii* ATCC no. 7708, *Sarcina lutea* ATCC no. 9341, *Veillonella parvula* ATCC no. 10790, *Flavobacterium suaveoles* ATCC no. 958 *Pasteurella multocida* ATCC no. 6530, *Achromobacter lipolyticum* ATCC no. 11367, and *Providencia* sp. ATCC no. 12013 were added to this study on the basis of their reported tryptophanase activity.

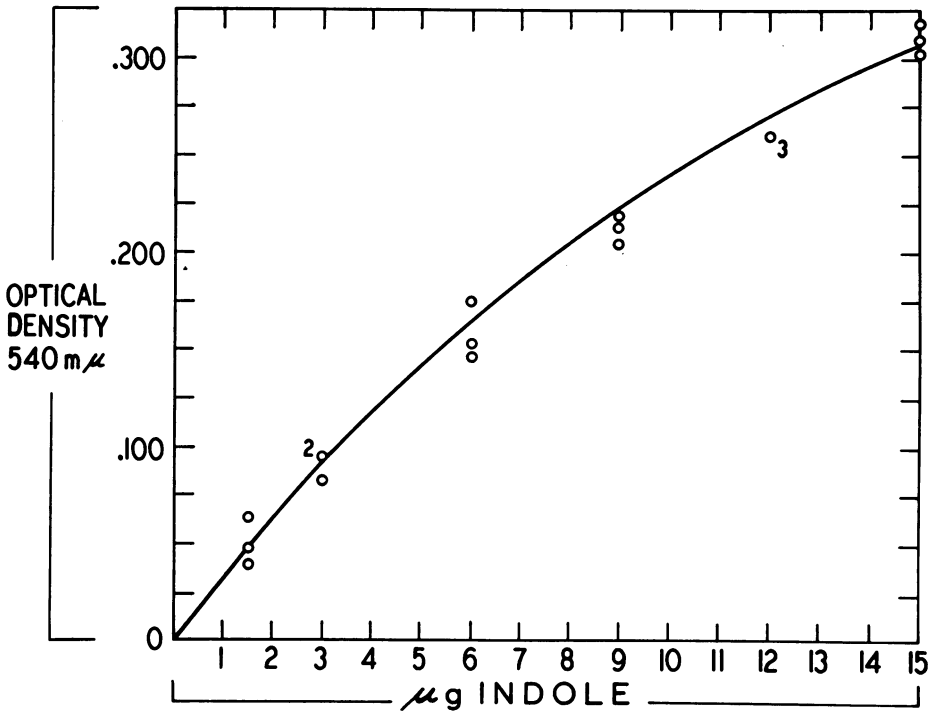


Figure 1. Typical calibration curve for the quantitative determination of indole with *p*-dimethylaminobenzaldehyde.

Only the *Providencia* sp. gave positive results with all methods. The others represent, at best, indole-negative variants of originally indole-positive strains, though all reacted with directly applied *p*-dimethylaminobenzaldehyde.

Known concentrations of indole added to a solution of tryptophan or medium A were recovered quantitatively by the extracted *p*-dimethylaminobenzaldehyde method and the hydroxylamine reaction. Similarly, quantitative indole determinations performed by both methods on culture supernatants of 10 strains of *E. coli* were in close agreement.

The qualitative application of both tests to anaerobically grown *E. coli* established the need for oxygen for the elaboration of tryptophanase as postulated by Happold and Hoyle (1935) and Frye (1955). The positive direct *p*-dimethylaminobenzaldehyde reaction may indicate the presence of indole-3-acetic acid suspected by Frye (1955).

A variety of media was tested in order to find substrates which yielded reproducible results with respect to indole production. The results obtained are recorded in table 3. It is apparent that medium B, described previously, gave the highest production of indole. The synthetic medium D showed higher values than medium B with 3 cultures but proved inferior to it with the remainder. Neither the addition of inorganic nitrogen salts to the medium in order to spare tryptophan for indole production, nor the incorporation of pyridoxal improved the yields of indole.

Although figures 1 and 2 represent typical calibration curves, it was of advantage to include several standards of varying indole concentrations in the appropriate medium with each series of quantitative determinations.

DISCUSSION

The number of reagents suggested as a means for the detection of microbial indole production is great indeed. However, the meticulous work of several investigators (Kulp, 1925; Zipfel, 1912; Frieber, 1921; Malone and Goré, 1921) has shown that *p*-dimethylaminobenzaldehyde is superior to most reagents employed. Concern with the specificity of the formation of the *p*-dimethylaminobenzaldehyde-indole complex was

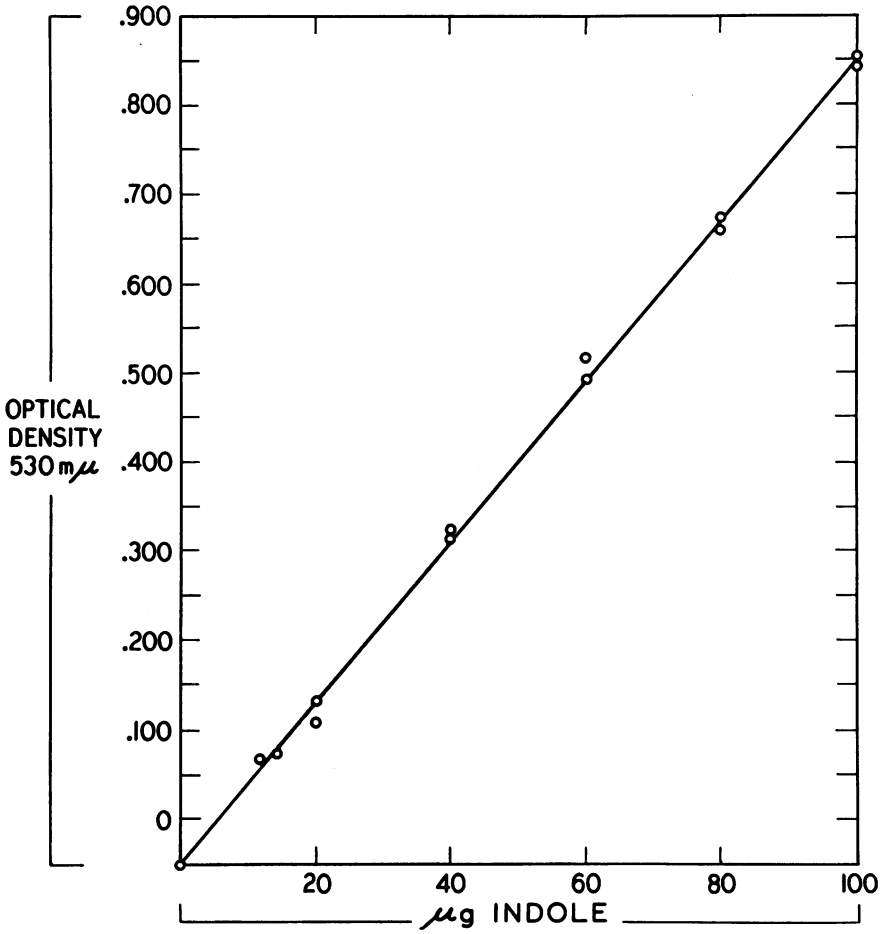


Figure 2. Typical calibration curve for the quantitative determination of indole with hydroxylamine.

expressed as early as 1912 by Zipfel. He showed, despite Frieber's (1921) later claims to the contrary, that several substituted pyrroles and other nitrogen containing compounds such as phenylmethyl pyrazolone, acetylglucosamine, heme, urobilinogen, and skatole reacted with the aldehyde to produce a complex similar or identical with the rosindole of the indole-*p*-dimethylaminobenzaldehyde condensation. These observations heralded the discoveries of Fischer and Orth (1934), Sidgwick and Taylor (1937), and Mauzerall and Granick (1956) who demonstrated the capacity of pyrrolenines (formula 1), the tautomers of pyrrole, to condense with *p*-dimethylaminobenzaldehyde as shown in formula 2. Compound A, in an acid medium, rearranges (formula 3) into the quinoidal red-violet compound familiar to the bacteriologist.

The derivatives of pyrrole show the same ability to undergo this condensation so long as α, β unsubstituted unsaturation can occur.

Zipfel (1912) also was aware that false positive reactions may be encountered with any number of peptone media. These aldehyde condensation products, probably in the nature of Schiff's bases (Feigl, 1954), could be differentiated from the pyrrole condensates by their development of a blue color with nitrite.

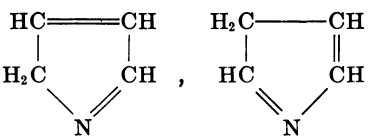
To increase the specificity of the Ehrlich aldehyde reaction for indole, Malone and Goré (1921) and Goré (1921) made use of the volatility of indole. Essentially, they added the reagents to the cotton plug and by immersion of the culture tube into gently boiling water effected a crude but adequate distillation as confirmed by Kulp (1925). In addition to distillation, Kulp (1925) indicated extraction with a variety of organic solvents to separate indole from tryp-

TABLE 3

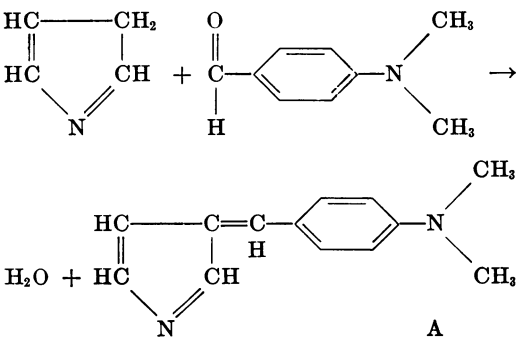
Indole production on various media

Escherichia coli Strain No.	μg Indole per ml Medium after 24 hr Incubation				
	Medium				
	A	B	C	D	E
1	23	23	22	35	31
2	8	15	7	23	19
3	13	30	12	19	12
4	8	8	8	0	0
5	19	40	20	24	23
6	14	21	20	22	20
7	16	70	21	30	22
8	13	32	15	17	16
9	46	100	47	30	31
10	11	41	18	0	0
11	36	46	31	33	33
12	41	74	45	22	17
15	0	6	0	0	0
16	0	9	0	0	0
17	7	11	7	7	5
18	7	32	10	11	8
19	27	100	33	15	14

Indole was determined by the quantitative extracted *p*-dimethylaminobenzaldehyde reaction on the supernatants of cultures.

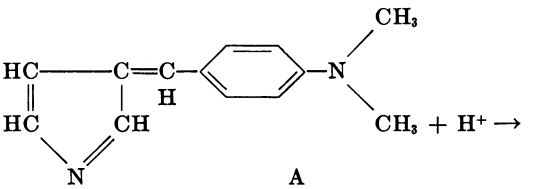


Formula 1

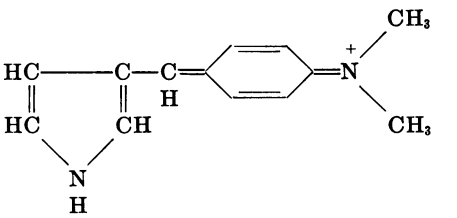


Formula 2

tophan, peptides, or protein mixtures. Wood and his coworkers (1947) extracted indole with toluene from a mixture of indole, pyridoxal phosphate, tryptophan, and tryptophanase. This procedure is used in the present investigation.



A



Formula 3

The chemistry of the hydroxylamine reaction awaits further elucidation (Gallop and Seifter, 1958, *unpublished data*). It offers definite advantages for use in microbiological classification. It is positive only with pyrroles and indoles unsubstituted in the α and β position. Interference by closely related metabolic intermediates is eliminated. The qualitative reaction may be carried out directly on the medium without prior centrifugation or extraction. It is readily adapted for the quantitation of indole in culture supernatants.

One wonders why the earlier, thorough investigations should have been ignored in favoring the simple but inaccurate direct *p*-dimethylaminobenzaldehyde method as the method of choice for detecting indole in bacterial cultures. The hydroxylamine method and the extracted *p*-dimethylaminobenzaldehyde technique compare most favorably as qualitative determinants of indole. Undoubtedly, the Goré method (1921) and the Gnezda oxalic acid reaction as described by Holman and Gonzales (1923) are more specific than the Kovacs' test (1928). Experiments, not described here, indicate that exposure to air or oxygen hasten the color development between indole or its derivatives and the *p*-dimethylaminobenzaldehyde, in the form of the direct or Kovacs' reagent, obviating its use with a spot plate method and making the determination in test tubes dependent upon the surface to air ratio.

The modified *p*-dimethylaminobenzaldehyde and hydroxylamine methods could be employed safely with media containing tryptophan, as the free amino acid, in considerable concentration. Addition of tryptophan to a medium always

increased the amount of indole. This yield was not enhanced by the addition of pyridoxal or inorganic nitrogen. It is not clear why growth in a synthetic medium gave cell yields comparable to those in complex media but did not result in similar indole production, although the carbohydrate content was kept low to insure tryptophanase production (Zipfel, 1912).

The use of tryptophan enriched media coupled with sensitive indole tests enables the bacteriologist to classify organisms with greater assurance relative to their indole producing capacity. On a standardized substrate, indole-positive organisms, which show only slight dissimilarities in other physiological activities, may be differentiated on the basis of quantitating their indole production.

ACKNOWLEDGMENT

The authors are grateful to Dr. James I. Berkman, Chief, Department of Laboratories, The Long Island Jewish Hospital, for his support and encouragement, and to Dr. Paul Gallop, Medical Research Laboratory, Department of Medicine, The Long Island Jewish Hospital, and Dr. S. Seifter, Department of Biochemistry, Albert Einstein College of Medicine, for suggesting this investigation and their advice.

SUMMARY

Two new efficient qualitative spot tests for the detection of indole in microbial cultures are described. Both methods can be employed in the quantitative estimation of indole on supernatants of cultures, grown in a medium to which tryptophan may be added with impunity.

The superiority of both methods to the widely accepted direct *p*-dimethylaminobenzaldehyde method is discussed.

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