INDOL AND SKATOL DETERMINATION IN BACTERIAL CULTURES

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The indol test has always been unsatisfactory from a bacteriological standpoint. The variability of the media employed, the diversity of methods, the inaccuracies of the tests and the dissimilar strains of the organisms used, all have tended to vitiate laboratory results. Various investigators have recognized the unreliable nature of indol tests in the characterization of bacterial species and have proposed not only many improvements to the original methods but have devised several new ones. The literature on the subject of indol and skatol detection and determination is voluminous and pertinent references, only, can be included in this paper. A brief summary of some of the more important contributions relating to the several methods most commonly used in bacteriological laboratories will, however, next be given.

THE EHRLICH ALDEHYDE REACTION FOR INDOL AND SKATOL

This test depends upon the production of a pink or red color with or without heat on the addition of para-dimethylaminobenzaldehyde to the acidified test solution. This red coloration is soluble in chloroform or amylic alcohol. The test was first proposed by Ehrlich in 1901. He showed that skatol gave under similar treatment a violet-blue color. Schmidt (1903) checked these color reactions and concluded that both indol and skatol could be accurately detected by the Ehrlich test. The test was applied to bacteriology soon after by Haenen (1905). Steensma (1906) found that upon the addition of dilute potassium nitrite the red color of indol was not changed, whereas the violet-

blue of skatol became deep blue and was soluble in chloroform. Deniges (1908) attempted to substitute cinnamic aldehyde and also vanillin for the more expensive Ehrlich reagent. This he did with varying success. He found that benzene, which he used to extract the indol, usually contained some substance which if present interfered with the color reaction. Boehme (1906) added potassium persulfate to the completed Ehrlich test for the purpose of intensifying the color. He also pointed out the application of the method to the study of bacteria. Baudisch (1915) called this improved Ehrlich-Boehme test very satisfactory for indol and skatol. Kligler (1914), Lewis (1915), and Crossonini (1910) all recommend the test as sensitive and accurate and more suitable for indol detection in bacterial cultures than the older Salkowski test with which they compared it. Weehuizen (1908) used the test in his investigation on the indol in flowers and found it gave very satisfactory results when applied to the distillate. He used potassium nitrite to intensify the colors. He found phloroglucinol gave tests identical with indol and that distillation was necessary to obtain accurate results. Blumenthal (1909) found that small amounts of skatol if present would be masked by the indol and vice versa, giving intermediate colors. He found the delicacy of the test to range from 1:1,000,000 to 1:5,000,000. He, as well as Homer (1915) suggested the use of spectroscopic methods in the identification of indol and skatol in the Ehrlich color tests. Herter (1905), also Herter and Foster (1905, 1906) determined indol quantitatively in mixtures by a combination of the Herter (beta-naphtholquinone sodiummonosulfonate) and Ehrlich tests using a double distillation process. Zoller (1920) rejected the Ehrlich method for indol in favor of the nitroso indol test performed on the distillate. His objections in the light of our present knowledge regarding the test cannot be considered valid. This investigator furnished a valuable contribution to our knowledge of indol determination by his work upon volatility, distillation and hydrogen ion concentration. The reaction of the Ehrlich reagent with indolacetic and indol propionic acids has been studied by Hopkins and Cole (1903), Herter (1908), and Salkowski (1919). A red color is formed

somewhat similar to the indol coloration though insoluble in chloroform. Among those who have used and recommended the test are Lewis (1915), Jordan (1919), Chen and Rettger (1920), Wenner and Rettger (1919), Van Loghem and Van Loghem-Pouv (1912), Zipfel (1912), Frieber (1921), Kolle and Wassermann (1913), Norton and Sawyer (1921), Barthel (1921), Cannon (1916), Horowitz (1916), and Rivas (1912),Goré (1921) and Levine and Linton (1924).

According to the Manual of Methods prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists (1923), the Ehrlich test is performed as follows:

> The reagent is a 2 per cent solution of paradimethylaminobenzaldehyde in 95 per cent alcohol. One cubic centimeter of this reagent is added to the culture, then drop by drop concentrated hydrochloric acid is added until a red zone appears between the alcohol and the peptone solution. Not more than 0.5 cc. of the acid is required. On standing the zone deepens and widens. The red color is soluble in chloroform and the test may be confirmed by shaking the culture with chloroform to see if the pigment dissolves. If it proves soluble the test is considered positive.

STEENSMA OR VANILLIN TEST FOR INDOL AND SKATOL

In 1906 Steensma proposed the substitution of vanillin for the more expensive para-dimethylaminobenzaldehyde which is used in the Ehrlich test. Weak indol solutions gave with a few drops of a 5 per cent alcoholic solution of vanillin an orange red color unchanged by sodium nitrite while skatol yielded a red violet color changed by this reagent to blue violet. The presence of skatol masks the red coloration due to indol. Deniges (1908) also made use of vanillin as well as cinnamic aldehyde to replace Ehrlich's reagent with satisfactory results. Weehuisen used this test in conjunction with Ehrlich's in his investigations on the indol in flowers. Blumenthal (1909) stated that while the vanillin and protocatechuic aldehyde tests were fairly satisfactory where indol alone was present, they proved inaccurate in mixtures of indol and skatol. Nelson (1916) made a thorough study of the Steensma vanillin test and compared the color reactions obtained

by the use of numerous alcohols, aldehydes and acids and the solubility of these pigments in various solvents. The nature of these color bodies is not known. Although he recommends hydrochloric or sulfuric acids, most of the acids are suitable for making these tests. Indol produces a deep orange color insoluble in chloroform while skatol gives a deep red to violet color soluble in chloroform. Although Nelson usually obtained very good results with this test, occasionally they varied greatly and were not satisfactory. Zoller (1920) scored the Steensma vanillin test and called it unreliable on account of the large number of interfering substances and the ease with which the colors become masked. Frieber (1921) indicated that this test gave positive results with indol, alpha-methyl indol and beta-methyl indol (skatol). The test is used in some laboratories to a considerable extent, principally to check the results obtained by other methods.

The committee on Bacteriological Technic of the Society of American Bacteriologists (1923) recommend the following procedure in carrying out the vanillin test for indol:

> To 5 cc. of the culture add 5 drops of a 5 per cent solution of vanillin in 95 per cent alcohol and 2 cc. of concentrated sulfuric acid. Indol gives a clear orange by this test which reaches its greatest depth in two or three minutes. Tryptophane on the other hand, gives a reddish violet which develops more slowly and deepens on standing or heating.

THE SALKOWSKI NITROSO-INDOL TEST (CHOLERA RED REACTION)

Salkowski (1883) proposed the use of concentrated sulfuric or nitric acid together with a very dilute solution of potassium nitrite. In the presence of indol a pink ring or coloration between the acid layer and the culture is formed. The color changes to blue green upon the addition of potassium hydroxide. Large amounts of indol produce a reddish brown precipitate. Where the culture already contains nitrites as in the case of *V. cholerae* the addition of nitrites is unnecessary. This was based upon the color reactions of nitrites formerly suggested by Baeyer (1870) and by Nencki (1875). Kitasato (1889) applied the test to bacteriology, while Nonotte and Demanche (1908) improved the

method of applying the test and attempted to make it quantitative. Zoller (1920) pointed out the necessity of distilling the substance which was being tested and indicated the inaccuracies of making indol tests directly upon the original cultures. He showed that skatol carbonic acid interfered with the test and added several refinements such as a direct distillation from a slightly alkaline solution and the application of the test to the distillate in a quantitative manner. He obtained very satisfactory results and considered the delicacy of the reaction to be about 1:500,000. In order to concentrate the color he extracted it with isopropyl or isoamyl alcohol. Tobey (1906) claimed that some slight differences are present in the color reactions obtained in the "Cholera red" and nitroso-indol tests. Baudisch (1915), Telle and Huber (1911), Crossonini (1910) and others have investigated this test but with the exception of Zoller and possibly Tobey most investigators have shown a preference for the Ehrlich test. Chen and Rettger (1920), Kligler (1913), Nelson (1916) Zipfel (1912), Wenner and Rettger (1919), Frieber (1921), and others have compared the Salkowski with the Ehrlich test for indol and all agree that the former is less delicate and less accurate than the latter. Frieber (1921) has demonstrated that indolacetic acid gives positive results with the Salkowski test, which is therefore not altogether reliable.

According to the Committee on Bacteriological Technic of the Society of American Bacteriologists in the Manual of Methods (1923), the Salkowski test is performed as follows:

Mix 5 cc. of the culture with about one-third its volume of 1:1 sulfuric acid. Then add on the surface a small amount of a 0.02 per cent solution of sodium nitrite. A positive reaction is indicated by a pink zone between the acidified culture fluid and the nitrite solution.

DIMETHYLANILIN TEST FOR SKATOL

To 5 cc. of the solution to be tested for skatol add a few drops of fresh dimethylanilin and shake vigorously. Add about 4 cc. of concentrated sulfuric acid to form a layer at the bottom. A violet ring is formed in dilutions of 1:1,000,000 or more. The color is soluble in chloroform. Indol does not interfere.

The United States Bureau of Chemistry in testing for the presence of skatol in decomposed foods also recommends this test. Clough (1922) modified the test by using hydrochloric acid in place of sulfuric acid and heating to bring out the pink color. The dimethylanilin should be recently redistilled else an interfering substance may obscure the color reaction. By this modified test a 1:5,000,000 dilution of skatol may easily be detected.

THE HERTER β-NAPHTHO-QUINONE SODIUMMONOSULFONATE TEST

Herter in 1905, and Herter and Foster (1905, 1906) proposed the use of β-naphtho-quinone sodiummonosulfonate for the detection and determination of indol. A double distillation is carried out first from the alkaline mixture and then on the acidified distillate. The addition of the reagent followed by potassium hydroxide in excess produces a blue condensation product of indol. The addition of acid changes this color to yellow. Now if this acid solution is redistilled, skatol if present, passes into the distillate and indol remains behind and may therefore be separated quantitatively. Gorter and DeGraeff (1908) claimed the method would give trustworthy results if a double distillation were carried out. They found ammonia interfered with the color reaction and that it must be eliminated before making the test. The delicacy is given as 1:8,000,000. Nelson (1916), also Zoller (1920) both compared this test with the other common indol tests but found that although it gave fairly dependable results, the reagent was almost impossible to obtain and that the test solutions of it deteriorated very rapidly. The test requires a long time to complete and they claim that ammonia and phenols interfere with the reaction. However Bergheim (1917) highly recommends the test particularly for the determination of indol and skatol in feces. A double distillation is necessary though the results are quantitative and skatol and ammonia do not interfere.

KONTO FORMALDEHYDE TEST

Konto in 1906 reported that formaldehyde added to the acidified distillate to be tested for indol yields a violet red color if

indol is present. Skatol under similar conditions gives a yellow or brown color. The delicacy is 1:600,000. Nelson (1916) also Clough (1922) did not find this test to be entirely trustworthy and therefore used other more suitable tests.

LEGAL NITROPRUSSIDE TEST

The Legal test (cited by Salkowski, 1883, also Hawk, 1918) for indol depends upon the formation of a blue color upon adding a few drops of a freshly prepared solution of sodium nitroprusside and making the resulting mixture alkaline with potassium hydroxide. If the mixture is now acidified with glacial acetic acid the violet color becomes blue. Deniges (1908) used the test with fair results and improved its technique somewhat by using an excess of acetic acid in the barely alkaline test mixture. The reaction is not as sensitive as the Ehrlich test. Frieber (1921) showed that indolacetic acid does not interfere with this test.

THE GNEZDA OXALIC ACID TEST

Gnezda (1899) found that oxalic acid, either in the solid form or in concentrated solution, takes on a red color with indol or with indol vapor. Morelli (1908), also Pittaluga (1908) applied the test to bacterial cultures. Blotting paper soaked in oxalic acid solution suspended over the test culture reacts very sensitively to indol. Zipfel (1912) found the test to give satisfactory results though he apparently prefers the Ehrlich test. Freund (1922), and also Baudet (1913), have reported satisfactory results with this test. Very recently Holman and Gonzales (1923) have fully recommended the oxalic acid reaction and claim it is reliable, simple, practical, and delicate. The action depends upon the volatility of indol and may be used in agar cultures as well as in liquid ones.

THE ESCALLON AND SICRE FURFURAL TEST

Escallon and Sicre (1906) recommended the use of furfural in testing for the presence of indol. The culture is first extracted with chloroform, the latter driven off by heat and the residue

taken up with a few drops of alcohol. The reagent is made by adding 1 gm. glucose to 55 cc. of hydrochloric acid, warming to boiling and making up to 100 cc. When the alcoholic residue is warmed with 3 cc. of the above furfural reagent, if indol is present a reddish orange color is produced.

THE DAKIN GLYOXYLIC ACID TEST

Dakin (1906) proposed the use of glyoxylic acid as a test for indol, skatol and tryptophan. To 1 cc. of the solution to be tested 1 cc. of the dilute glyoxylic acid solution is added and about 2.5 cc. of concentrated sulfuric acid. With indol a red to brown color forms at the zone of contact, while with skatol a deep red to violet coloration results. Homer (1914) studied the effect of formic acid, glyoxylic acid and glyoxal on the tryptophan derivatives and found that intensely colored substances were often formed in the presence of oxidizing agents.

THE CRISAFULLI PYRROL REACTION (PINE WOOD TEST)

Crisafulli (1895), Rohmann (1908), Hawk (1918) and others describe the following test for indol. Moisten a pine splinter with concentrated hydrochloric acid and insert it into the material under examination. If indol is present in appreciable quantity the wood becomes a cherry-red color.

SASAKI METHYL ALCOHOL TEST FOR SKATOL

Sasaki (1909) suggested the addition of 3 or 4 drops of methyl alcohol and an amount of concentrated sulfuric acid equal to the test solution. The acid must contain a trace of a ferric salt and the alcohol must be free from acetone. Skatol gives a reddish violet color whereas indol produces no color.

THE BAUDISCH OR NITROMETHANE TEST

Baudisch (1915) reported the following test for indol: A few drops of nitromethane followed by a little dilute potassium hydroxide are added to the test culture and heated. Cool and add one cubic centimeter of amyl alcohol, shake well and while

shaking add hydrochloric acid in excess. After the amyl alcohol has separated heat the mixture almost to boiling. In the presence of indol the amyl alcohol layer becomes red or rose-colored.

OTHER TESTS FOR INDOL AND SKATOL

Besides the several tests already described many others are mentioned in the literature on the subject. Most of them are really modifications of the aldehyde color reaction and make use of such substances as pyruvic aldehyde, glyceric acid and aldehyde, protocatechuic aldehyde and heliotropinpiperonal. Since most of them have no extensive use in the laboratory and are in general neither as sensitive nor as accurate as some of the better known and extensively used tests for indol and skatol, this discussion for the most part will be limited to the latter.

DISCUSSION OF THE TESTS

All the foregoing tests, with the exception of the glyoxylic acid of Dakin and the nitromethane test of Baudisch, were tried out by us experimentally in the laboratory using for most of the tests pure indol and skatol, as well as bacterial cultures and decomposed salmon. The Konto formaldehyde, Gnezda oxalic acid,[1] Escallon and Sicre furfural, pine wood, Sasaki methyl alcohol, pyruvic aldehyde and glyceric acid and aldehyde tests for indol or skatol proved to be unsatisfactory. These tests require fairly large amounts of indol or skatol to be at all reliable and even so, the color reactions were neither clear cut nor constant.

In the second list of tests, classed as fairly satisfactory though not entirely so, and for some reason or another not recommended for accurate work, are the Legal nitroprusside, Herter β-naphtho-quinone sodium monosulfonate, and the Salkowski nitrosoindol tests. Providing these tests are made on the distillate rather than on the culture direct, much better tests will be obtained. In the case of the Salkowski and Herter tests this pro-

[1] The excellent paper of Holman and Gonzales (1923) did not appear until after our laboratory work had been completed, hence in using this test we were not able to use the refinements suggested by these authors.

cedure is absolutely necessary on account of interfering substances such as indol-acetic acid, indol-propionic acid, ammonia, and alcohols and aldehydes, and other metabolic products which may be present in bacterial cultures. The indol acetic and indol propionic acids give a color reaction similar to indol, but since they are only very slightly volatile in water vapor, these products will be left behind. The Herter β-naphtho-quinone monosulfonate test for indol is useful in separating quantitatively indol from skatol, but the procedure is involved and the reagent exceedingly difficult to obtain.[2] The Salkowski test is not a delicate reaction and small amounts of indol may be entirely overlooked. A red coloration may indicate either indol, indol-acetic acid or indol-propionic acid, or all of them, if the test is made on the culture direct without distillation. Zoller (1920) has shown that the test is much more delicate and accurate when made on the distillate. He even obtained very satisfactory quantitative results. The nitroprusside test while accurate for considerable amounts of indol is not delicate enough for even ordinary routine indol tests.

The Ehrlich para dimethylaminobenzaldehyde reaction[3] was found to be by far the most accurate, reliable and simple test for indol or skatol when unmixed. When mixed together in a culture the color ranges from red to deep violet, according to the respective amounts present. For skatol alone the dimethylanilin test has been found to give very satisfactory results. In testing for skatol this should be made the primary test and the results should be checked by the Ehrlich paradimethylaminobenzaldehyde and Steensma vanillin tests. A dilution of skatol of 1:5,000,000 may be detected by the dimethylaniline test. It should be used on the distillate only.

Next to the Ehrlich test for indol and the dimethylanilin test

[2] We were unable to purchase this reagent in the U. S. Dr. O. Bergheim of the Jefferson College, Philadelphia, kindly furnished us with a liberal sample of this substance.

[3] The Gore (1921) modification of the Ehrlich test did not come to our attention until our study had been completed. Since this test depends upon the volatility of indol it should yield results comparable with the distillation test as described in this paper.

for skatol, the Steensma vanillin test for indol and skatol gave the most satisfactory results. It was found to be fully as delicate as the Ehrlich test though not as reliable. Interfering substances are occasionally present which mask or obscure the color reactions even when the tests are performed on the distillate. Experiments showed that hydrochloric acid could be substituted for sulfuric acid retaining at the same time the sensitiveness of the test but eliminating the charring effect of the concentrated sulfuric acid on the organic substances usually present. In an attempt to improve upon the use of vanillin as an indicator of indol and skatol a number of substituted vanillins were used. Methyl-vanillin, monobrom-vanillin and benzidine-mono-vanillin were tested out for both indol and skatol using both hydrochloric and sulfuric acids. It was found that these substituted vanillins proved less sensitive than vanillin itself. With skatol the violet blue product formed was entirely soluble in $CHCl_3$ while the orange product of indol was only very slightly soluble. In this way it is possible to effect a separation of indol and skatol. The following procedure gave very satisfactory results.

MODIFIED STEENSMA VANILLIN TEST

Make the test whenever possible upon the distillate (direct or steam). To 5 cc. of the solution being tested add 5 drops of 5 per cent solution of vanillin in 95 per cent alcohol, 2.5 cc. concentrated hydrochloric acid and mix. Indol gives a clear orange color, while skatol gives a violet to violet blue color soluble in chloroform. The orange color produced by indol is insoluble in this reagent. Delicacy for indol and skatol was found to be about 1:3,000,000. The application of heat hastens the reaction. Only definite positive results should be recorded.

THE MODIFIED EHRLICH PARADIMETHYLAMINOBENZALDEHYDE TEST

After much preliminary work using the various tests under a great variety of conditions, it was decided to make a detailed study of the Ehrlich reaction and if possible to eliminate errors, increase its delicacy, standardize the technic of the test and make it as nearly quantitative as possible.

It was found that discordant results were often due to varia-

tions in the color and thickness of the glass in the test tubes. Some of these tubes had a bluish green color which served to mask in part the faint pink color produced by small amounts of indol. Since it was attempted to record amounts of indol as small as 0.2 micromilligram of indol in the 5 cc. of test solution (1:25,000,000)

TABLE 1

Influence of the Ehrlich reagent on the color of the indol test

INDOL	EHRLICH REAGENT	COLOR PRODUCED
p.p.m.	*cc.*	
0.2	0.5*	Pure pink
0.2	0.75	Pink with slight yellow
0.2	1.0	Pronounced yellow
0.4	0.5*	Pure pink
0.4	0.75	Nearly pure pink
0.4	1.0	Pink with slight yellow
0.4	1.25	Pink with more yellow
0.4	1.5	Pink with still more yellow
0.4	1.75	Pronounced yellow
0.4	2.00	Pronounced yellow
0.8	0.5*	Pure pink
0.8	0.75	Deeper pure pink
0.8	1.0	Pink with trace of yellow
0.8	1.25	Pink with slight yellow
0.8	1.5	Pink with considerable yellow
0.8	1.75	Pink with strong yellow
0.8	2.0	Yellowish pink
2.0	0.5,* 0.75, 1.0	Pure pink
2.0	1.25	Pink with trace of yellow
2.0	1.5	Pink with slight yellow
2.0	2.0	Pink with considerable yellow

* Five-tenths cubic centimeter of the Ehrlich reagent was the amount selected as the optimum for small amounts of indol.

it was absolutely necessary to use tubes of uniform bore, thickness and color. Thin walled, colorless, uniform tubes were used in all these tests.

Paradimethylaminobenzaldehyde dissolved in alcohol has a yellow color; this, also, was found to interfere with the tests for indol and skatol, particularly when the latter are present in very

small amounts. Different amounts of indol in 5 cc. of water were treated with varying amounts of the reagent and the colors produced were noted. Ten drops of concentrated hydrochloric acid were used in each test, the color being extracted with chloroform.

Table 1 shows that as the amount of the Ehrlich reagent is increased in the test, the greater is the interference of the yellow color, this being particularly true where dilute concentrations are present. From these data and from other tests conducted at this time it was concluded that 0.5 cc. of the reagent (paradimethylaminobenzaldehyde) represented the optimum amount for small amounts of indol, 2.0 micromilligrams (0.4 p.p.m.) or less.

It was found that concentrated hydrochloric acid partially destroys the interfering yellow color of the Ehrlich reagent, though the ten drops of the former usually used in the test, has little effect. Amounts of concentrated hydrochloric acid varying from 0.5 to 2 cc. were used in experimental tests. The result was that as the acid concentration increased the percentage of color destroyed increased also. However a point was reached at 2 cc. hydrochloric acid where the usual pink color of the completed test changed very quickly to orange. This destruction of the pink color takes place especially rapidly if the tubes are heated. Since the orange color is objectionable, it was decided, considering all the factors entering into the test, that 1 cc. concentrated hydrochloric acid gave optimum results because at this concentration of acid a considerable percentage of the objectionable yellow color of the reagent was destroyed without affecting the final reading of the test.

Several experiments were carried out to ascertain the effect of heating on the indol test as it had been found that the color developed much more rapidly when heat was applied. However, the time of heating was found to have a decided influence upon the final test. It appeared that the hydrochloric acid partially destroyed the pink indol color if the tubes were heated for more than thirty seconds. After repeating several times an experiment to determine just how much heating should be given, it was found that an exposure of twenty seconds in boiling water

gave optimum intensity of color without any harmful effect of the hydrochloric acid being apparent. The color which developed in 20 seconds in boiling water was approximately equal in intensity to that produced after standing for twenty to twenty-five minutes at room temperature. It is therefore a saving of time to apply heat to the test tubes before making the final reading. In this connection it should be stated that before adding chloroform to extract the color, it is necessary that the test solution be below the boiling point of chloroform, e.g., 61.2°C.

DISTILLATION OF INDOL FROM CULTURES AND FROM AQUEOUS SOLUTIONS

Indol, skatol and some other products of decomposition are volatile with steam. In this investigation it was necessary to determine the percentage recovery of indol in the distillate. Zoller (1920) determined that a slightly alkaline reaction in the culture was best (between pH 8 and 10.5). He used a single direct distillation and found that nearly all the recoverable indol was recovered in the first 75 cc. distilled, the total volume being 100 cc. Several investigators have shown that only a part of the indol added to a solution or solid suspension is recovered by either direct or steam distillation.

In order to determine the effect of varying hydrogen ion concentrations upon the recovery of indol in the distillate, varying amounts (5 to 50 cc. of a 10 per cent solution) of both sodium hydroxide and hydrochloric acid were added to the regular distillation flasks in addition to 12 micromilligrams of pure indol and 500 cc. of water. The material was then steam distilled, 500 cc. of the distillate being collected and tested for indol. The average per cent recovery of indol of the nine distillations where the alkali was used was 46.4 per cent, the amount of alkali making little difference in the results. On the other hand the average per cent recovery of the five acid distillations was only 26.7 per cent. In none of these cases was the recovery very satisfactory yet the alkaline solutions yielded nearly twice as much indol in the distillate as the acid solutions.

Reducing the volume of the solution distilled for indol appeared

to increase the percentage recovery. In a series of distillations where the residual volume in the flask was varied from 310 to 65 cc. the percentage recovery increased from 26.6 to 53.3 per cent.

Little difference was noted as to whether steam or direct distillation was the more satisfactory. Since the concentration could be controlled and little bumping or frothing occurred, steam distillation was used in this work. Another reason for using steam distillation is because scorching of proteins forms either indol or an indol-like substance which gives some of the color reactions of indol. As a result of a large number of tests it was determined that the length of time of the distillation had little or no bearing upon the percentage of indol recovered in the distillate. When known amounts of indol were added to per-

TABLE 2

Recovery of indol from bacterial cultures by steam distillation

DISTILLATE		TEST SOLUTION (5 CC.) DILUTED TO*	TOTAL AMOUNT OF INDOL
Number	Cubic centimeters		
		cc.	*p.p.m.*
1	100	40	2.56
2	100	10	0.12
3	100	10	0.02
4	100	5	0.01

* It was necessary to dilute the test solution to this volume because the red color due to indol was too intense for accurate comparison with standards.

fectly fresh, chopped up salmon in a flask with 500 cc. of water and distilled with steam, the recovery of indol varied from 47 to 65 per cent.

In order to determine the percentage recovery of indol in the first 100 cc. of the distillate, a culture tube containing 10 cc. of Dunham's peptone solution inoculated with an organism from pink salmon which was known to produce indol, was incubated for one week and then distilled with steam. The distillate was collected in four 100 cc. portions and the amount of indol in each portion determined separately. The results are given in table 2.

These data indicate that it is unnecessary to distil more than 100 cc. when a 10 cc. culture is used for the test. Ninety-four

per cent of the total indol recovered was obtained in the first 100 cc. of distillate.

Another series of six experiments to determine the volatility of indol with steam from protein substances was performed. Decomposed salmon, known to contain considerable amounts of indol was used. Two hundred grams of the ground up flesh macerated in 200 cc. of water were steam distilled and the distillate collected in 100 cc. portions until about 10 such portions had been collected. The indol was then extracted with ethyl ether and estimated colorimetrically according to the modified Ehrlich technic. The average per cent of indol recovered in the first 500 cc. distilled was 82.7. The per cent collected in the first 100 cc. portion of the distillate collected was only 23.8. The indol in flesh appears therefore to be rather firmly held and it is only with difficulty entirely removed. Such a condition is analagous to the determination of ammonia in water and in certain organic substances. However, it is possible by using a fixed arbitrary technic, to obtain fairly comparable results. The Ehrlich test gives an accurate indication of the amount of indol or skatol extracted from the distillate; since, however only 80 or 90 per cent of the total indol is actually recovered the results obtained by the method are not absolute. Furthermore, it is impracticable to recover all the indol present in a culture or other substance and it is believed that the distillation of a certain amount of the material being tested and the collection of a definite amount of distillate offers the best method possible for the quantitative determination of indol and skatol in cultures or other substances. In the case of peptone, tryptophan or trypsinized protein solutions, the indol and skatol are more readily liberated than from the complex proteins such as are present in flesh or plants, and for this reason a higher per cent recovery might be expected where culture media contain these relatively simple substances.

Still another experiment to determine the recovery of indol in the distillate was carried out using Limburger cheese, a substance which contains much indol. One hundred grams of cheese with 100 cc. of water were distilled with steam and three, five

hundred cc. portions of distillate were collected. Each portion was extracted two or three times with ethyl ether and the indol present in each separate extraction determined. The results are given in table 3. This table further confirms previous experiments where fish flesh and culture solutions were used. Although the greater part of the indol is found in the first 500 cc. of the distillate, considerable amounts are also found in the second and third 500 cc. portions.

TABLE 3

Recovery of indol from Limburger cheese by steam distillation

DISTILLATION NUMBER	SUCCESSIVE EXTRACTION	TEST SOLUTION (1 CC.) DILUTED TO*	TOTAL AMOUNT OF INDOL
		cc.	*p.p.m.*
1	A	300	180.0
	B	20	16.0
	C	2	1.6
2	A	300	90.0
	B	20	6.0
3	A	30	48.0
	B	5	6.0

* The intensity of the red color due to indol made it necessary to dilute the test solution in order to accurately compare the color with that of the standards.

EXTRACTION OF INDOL FROM THE DISTILLATE

Referring to table 3, it is evident that one ether extraction in a separatory funnel serves to remove nine-tenths or more of the indol present in the distillate. This was found to be true regardless of whether the indol was distilled from flesh, bacterial cultures, or cheese. Petroleum ether has been recommended for the extraction of indol but in this investigation it was found to be inferior to ethyl ether. The petroleum ether contains impurities unless well purified, which seriously interfere with the indol color reactions, particularly in the vanillin test.

Upon vigorously shaking the distillate containing indol with ethyl ether, a persistent emulsion often resulted making it difficult to separate the ether layer from the aqueous layer. It was found

that c.p. HCl dispersed the emulsion and simplified the separation of the layers. Commercial acid is not satisfactory because of impurities which interfere with a clear color test. The addition of two cubic centimeters of the c.p. hydrochloric acid was found to give very satisfactory results in dispersing the emulsion while not making it too difficult to neutralize subsequently with sodium hydroxide. Although only approximately 50 per cent of the ether used in the first extraction separates from the water, practically all of the recoverable indol is obtained in the first extraction.

During bacterial decomposition substances other than indol and skatol which are volatile with steam, may interfere with the color tests for indol and skatol. Since phenols and cresols and certain other substances which might be present in a decomposing mixture, are soluble in dilute alkali, the acidified ether extract of the distillate was washed with 5 cc. of 2.5 per cent NaOH to remove such substances. Of course it was then necessary to rewash the ether extract with 5 cc. of dilute HCl to remove the alkali. Repeated experiments showed that unless this was done the color reactions were sometimes obscured.

EVAPORATION OF THE ETHER EXTRACT

After the ether extractions have been washed with alkali and acid they must be evaporated over a small volume of water. Usually 10 cc. is used unless it is desired to apply several different tests for indol and skatol. Ordinarily where the Ehrlich test is used for indol and the dimethylanilin test for skatol, 10 cc. of test solution is sufficient. It was found that there was always some loss of indol upon evaporation, regardless of the method used. Spontaneous evaporation was compared with both hot water immersion and water bath with aspiration. Some difference was noted in the several methods and for the sake of speed, the application of heat with aspiration appears desirable. Needless to say the same technic should be employed in every test. For the sake of uniformity, heating on the water bath until most of the ether has been evaporated and removing the last traces by aspiration is recommended.

COLOR TESTS ON THE WATER TEST SOLUTION

After the ether has been entirely removed from the flasks containing the test solutions, these are ready to be subdivided and the actual color tests made. Two 5 cc. portions are placed in thin walled, colorless glass test tubes; one of these is used for the test and the other held in reserve. The necessary reagents are added from burettes, the tube placed in boiling water for twenty seconds, plunged into ice or cold water until cool and the chloroform is then added to extract the color due to the indol or skatol. After thorough shaking the tubes are allowed to stand at room temperature until compared with the standards. Unnecessary delay at this point should be avoided as the color changes somewhat and decreases in intensity after some hours. The standards are prepared from standard indol and skatol solutions prepared in exactly the same way. Such solutions were conveniently prepared from stock alcoholic solutions containing 80 mgm. indol or skatol per 100 cc. These stock solutions when tightly stoppered retained their full strength for several months at least. On the other hand a word of caution is necessary in the case of dilute aqueous solutions of indol, for it was found that such solutions deteriorated very rapidly and if used at all had to be freshly prepared each day. If the color is found to be too intensely red to read accurately, it is desirable to add a further measured volume of chloroform until a clear pink color is obtained in 1 cc. of the extract. It is also possible to dilute the reserve test solution with water and then apply the test to the aliquot. Both methods are equally satisfactory as proved by laboratory tests. The amount of indol present in the 5 cc. test solution to be satisfactorily estimated, should not exceed about 10 micromilligrams.

TECHNIC RECOMMENDED FOR INDOL AND SKATOL DETERMINATION IN BACTERIAL CULTURES

Materials

1. Dunham's pepton solution: 10 grams pepton; 5 grams NaCl: 1000 cc. tap water. Reaction neutral (pH 7.0).

Tube 10 cc. per tube and sterilize. Since pepton occasionally contains indol, checks should always be made on the media.

2. Concentrated C. P. HCl.
3. Ethyl ether U. S. P.
4. NaOH (2.5 per cent solution) for washing ether extract in separatory funnel.
5. Dilute HCl (10 cc. concentrated C. P. HCl in 200 cc. H_2O) for washing ether extract.
6. Paradimethylaminobenzaldehyde (2 grams in 100 cc. 95 per cent alcohol).
7. $CHCl_3$—U. S. P. For extracting the indol or skatol.
8. Indol color standards (containing 0.5, 1 and 5 and 10 micromilligrams indol in 5 cc. H_2O). One micromilligram in 5 cc. is equal to a dilution of 1:5,000,000 or 0.2 part per million.
9. HCl for final indol test (600 cc. concentrated C. P. HCl plus 200 cc. distilled H_2O).
10. Distilled water.

Method

Incubate bacteria to be tested for indol production in Dunham's peptone solution, preferably at 37.5°C. for five days. Transfer contents of culture tube to 250 cc. Fry flask and wash with 40 cc. H_2O. A current of steam is passed through the culture and 100 cc. of distillate collected. If direct distillation is carried out then make up to 100 cc. and distil 75 cc. Acidify with 2 cc. concentrated HCl (2) and extract once with 50 cc. ethyl ether in a 300 cc. separatory funnel and separate ether layer. Then wash ether extract in same separatory funnel with 5 cc. of 2.5 per cent NaOH (4) followed by 5 cc. dilute HCl (5), separating the ether layer in each case. Usually these successive washings with alkali and acid are unnecessary, though they appear to remove phenols and other interfering substances. Add 10 cc. H_2O and carefully evaporate the ether on water bath. Divide the water remaining into two 5 cc. portions and test for indol as follows:

Add to one 5 cc. portion in a small colorless test tube 0.5 cc. paradimethylaminobenzaldehyde (6), then 1 cc. HCl (9). Place in boiling water for twenty seconds, shake vigorously, then place tube in ice water for one-half minute and extract with 1 cc. $CHCl_3$ (7). Compare extracted red color with that of indol standards prepared in exactly the

same manner. This test is accurate to 1:25,000,000. It is also a good though arbitrary quantitative method.

Skatol standards are not usually prepared unless the qualitative test is positive, as it is so rarely found. Skatol is indicated in this modified Ehrlich test by a pale blue color in the chloroform extract, which becomes deeper upon standing. The regular dimethylanilin test for skatol has already been described and may be applied to the reserve 5 cc. portion of the test solution.

After perfecting the technic of the indol and skatol tests as applied to bacterial cultures, several hundred determinations were made using many species of microörganisms derived from a variety of sources. Other experiments to ascertain the effect of the brand of pepton upon the speed of indol formation were also made. The several American peptones and the one imported pepton tested for indol content gave entirely negative results, though all gave positive tests for tryptophan. Witte's pepton gave by far the deepest color in the tryptophan test as well as developing more quickly than the other peptones. Slightly higher results for indol as well as more rapid formation was found using Witte's pepton, but the results were sufficiently uniform to recommend any of the domestic peptones for indol determinations. The work of Fellers, Shostrom, and Clark (1924) on hydrogen sulfide production by bacteria, showed that the several commercial peptones varied greatly as regards the formation of hydrogen sulfide. Tilley (1921) has shown this is also true in regard to indol formation. A sample of tryptophan digest submitted by a domestic firm was found to be entirely suitable for use in indol determinations. The use of such tryptic digests materially decreases the time necessary to incubate the culture. Maximum indol liberation may take place after as short a time as twenty-four hours, though for most peptones and bacteria a longer period of time appears desirable. It is known that after several days certain bacterial cultures containing indol, gradually lose their indol content. It was found that the maximum of indol liberation occurred in from forty-eight to ninety-six hours in Dunham's pepton solution, whereas a week or more was sometimes required where casein, salmon flesh or beef were

used. Table 4 contains data on the progressive accumulation of indol in cooked salmon media.

In this experiment the incubation temperature was 37°C., duplicate tubes being withdrawn at each 48 hour test period. The fact that separate tubes were used for the tests aids in explaining the variations obtained in the results. Nevertheless with the exception of culture 30-A, the results show a rather constant increase in indol content up to eight days. The blank determinations for indol upon the medium alone gave entirely negative results.

In order to determine the progressive liberation of skatol by bacteria, a skatol-liberating anaerobic clostridium isolated by one of the authors (1922), was inoculated in a 2 liter flask of Dunham's solution (pH 7) and incubated under anaerobic conditions at 35°C.

TABLE 4

Progressive accumulation of indol in bacterial cultures on cooked salmon media*

INDOL						BLANK ON MEDIA ALONE
Culture 26-A	Culture 26-B	Culture 9-A	Culture 9-B	Culture 30-A	Culture 30-B	
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	
0.02	0.02	0.3	0.16	1.6	0.02	0
0.12	0.36	0.88	0.72	0.12	0.12	0
Lost	0.88	1.0	1.0	8.0	13.0	0
0.6	4.0	3.6	3.6	4.8	Lost	0

* All organisms were lactose fermenters of the colon-aerogenes group.

for thirty days, test portions of 10 cc. being withdrawn from time to time. These portions were distilled and skatol determined by the dimethylanilin test. No skatol was liberated in twenty-four hours, 20 parts per million was formed in 48 hours; 28 parts per million in seventy-two hours; and no further increase was noted even after thirty days. A repetition of the experiment gave very similar results except that an increase in skatol content in the culture from 20 to 32 parts per million occurred between the ninety-six and hundred forty-four hour testing periods.

In the course of salmon spoilage investigations, a large number of cultures of bacteria from both raw and canned salmon were examined by the modified Ehrlich test for their ability to liberate indol. Of 249 cultures obtained from raw salmon, 70 were found

to liberate indol and 3 skatol, whereas in 57 cultures (nearly all spore-forming bacteria) obtained from canned salmon only 9 liberated indol and none, skatol. Twenty-six additional cultures isolated from canned crab-meat, clams and fruit (some of which was spoiled), showed 6 cultures positive for indol and none for skatol.

A large number of known species of bacteria and other microorganisms were carefully cultured in Dunham's solution, incubated from five to seven days at 30°C., and finally tested for indol and skatol by the modified Ehrlich and other tests. One hundred eleven cultures representing 55 species were thus tested. The Ehrlich test as usually made directly upon the bacterial culture was compared with the test as performed upon the distillate. The vanillin (Steensma), Salkowski, and dimethylanilin tests were also made direct upon each culture. In the Ehrlich technic, attention was paid to the development of the pink color both in the untreated culture and in the distillate. The solubility of the pink color in chloroform was also noted in all tests made either upon the culture direct or upon the distillate. The vanillin test was performed in the usual way upon the untreated culture using hydrochloric acid. The Salkowski and dimethylanilin tests were made in the regular way also, though the former was performed only upon the untreated cultures and not upon the distillate. The results are given in table 5.

Comparing first the results obtained in table 5 by performing the Ehrlich test upon the culture direct and upon the distillate, it was found that all except four of the indol positive cultures could be detected by making the test directly upon the culture. On the other hand three slightly positive cultures obtained by the direct test were found to be negative upon testing the distillate. When large amounts of indol or skatol were present it could usually be detected by all the methods employed, though it was demonstrated to the authors' satisfaction that small amounts of indol were often overlooked when the Ehrlich test was made directly upon the culture. In about the same degree, the Salkowski and Steensma tests failed to detect small quantities of indol or skatol when the untreated culture was used for the

TABLE 5

The indol and skatol reactions of various organisms

SPECIES	NUMBER OF STRAINS TESTED	EHRLICH TEST DIRECT ON CULTURE		EHRLICH TEST MADE ON DISTILLATE		VANILLIN TEST DIRECT	SALKOWSKI TEST DIRECT
		Pink color formed	Pink color extracted by $CHCl_3$	Pink color formed	Micromilligrams indol in $CHCl_3$ extract		
Staph. pyogenes	2	−	−	+ sl.	0	−	−
Sar. lutea	2	−	−	−	0	+	−
Sar. ventriculi	1	+	+ v. sl.	−	0	++	−
Bact. enteritidis	2	+ v. sl.	−	+ v. sl.	0	−	1 −; 1 + sl.
Bact. dysenteriae Shiga	2	−	−	−	0	−	−
Bact. dysenteriae Flexner	2	+++	++	+	5	+++	1 −; 1 + sl.
Bact. paratyphosum A	4	−	−	−	0	−	−
Bact. paratyphosum B	3	++	−	++	2	+	1 +; 2 −
Bact. paratyphosum B	3	−	−	−	0	−	2 −; 1 +
Cl. skatol n. sp	2	+++	+ blue	+	20	++	++ violet
Bact. typhosum	4	−	−	−	0	−	−
Bact. suipestifer	2	−	−	+	0	−	−
Bact. capsulatum	1	+++	−	−	0	+	+ v. sl.
Vibrio comma	1	++	++	+	13	+++	++
Bact. communior	2	+++	++	+++	20	+++	+
Bact. lactis viscosum	2	+ sl.	−	−	0	−	−
Lactob. acidi lactici	2	+ sl.	−	+ sl.	0	+	−
Bact. coli	3	+++	+	+++	15	+++	++
Bact. cloacae	2	+ sl.	−	+	0	+	+
Bact. cloacae	1	++	−	+	2	+	−
Bact. aerogenes	3	++	−	+	1.5	++	2 ++; 1 −
Bact. aerogenes	1	+	−	+ sl.	0	+ sl.	−
Bact. alkaligenes	2	−	−	−	0	−	−
Erwinia carotovora	2	+	+ sl.	+ sl.	0	+	+ v. sl.
Ps. aurescens	2	−	−	−	0	+ sl.	−

Ps. fluorescens	2	+	−	++	0	+	−
P. vulgaris	2	++	−	++	10	++	++
P. vulgaris	1	+	−	+	0	+ sl.	+ sl.
Cl. sporogenes	2	−	−	−	0	+ v. sl.	1 +; 1 −
Cl. botulinum A	2	+ sl.	−	−	0	−	−
Human feces culture	6	+++	++	++	25	+++	++
Guinea pig feces culture	6	+++	++	++	12	+++	+
Halophilic bacteria sp. ?	2	+	−	+ sl.	0	+ sl.	−
B. pseudotetanicus	1	+ sl.	−	+ sl.	0	+ sl.	−
B. fusiformis	1	−	−	−	0	−	−
B. mycoides	3	+ sl.	−	−	0	−	−
B. centrosporus	1	−	−	−	0	−	−
B. megatherium	2	+	−	−	0	+ sl.	−
Thermobacterium sp.?	1	−	−	−	0	+	−
Thermobact. thermodifferens	1	−	−	−	0	−	−
Thermobact. aerothermophilus	1	+ sl.	−	+ sl.	0	+	−
B. terminalis	1	+ sl.	−	+ v. sl.	0	+	−
Erythrob. prodigiosus	1	−	−	−	0	+ v. sl.	−
Thiobact. janiculatus	1	+	−	−	0	−	−
Sar. lactis acidi	1	−	−	−	0	+ v. sl.	−
B. cereus	2	+ sl.	−	+ sl.	0	+ v. sl.	−
B. mesentericus	2	+ sl.	−	−	0	+ sl.	−
B. circulans	1	+	−	+	0	+	−
B. cohaerens	1	−	−	+	0	+ sl.	−
B. albolactis	1	+ sl.	−	−	0	+ sl.	−
B. subtilis	2	+	+ sl.	++	0	++	1 −; 1 + sl.
B. flexus	1	−	−	−	0	+	−
B. vulgatus	2	+	−	−	0	+ sl.	−
Sacch. ellipsoideus	1	+ sl.	−	−	0	−	−
Saccharomyces sp.?	1	−	−	−	0	+ sl.	−
Aspergillus flavus	1	−	−	−	0	−	−
Penicillium sp.?	1	+ sl.	−	−	0	+ v. sl.	−
Actinomyces griseus	1	−	−	−	0	−	−

+, positive; −, negative.

test. The turbidity, varying hydrogen-ion concentrations and interfering substances probably account for most of the inaccuracies obtained by testing the untreated culture. None of the 18 aerobic spore-forming bacteria tested, liberated indol or skatol; similarly, 4 cocci, 2 yeasts and 2 molds liberated no indol from pepton under the conditions of the experiment. The principal indol liberating bacteria are those of the colon-aerogenes and proteus groups. Several organisms like *P. vulgaris, Bact. dysenteriae, Bact. aerogenes*, and *Bact. paratyphosum* B apparently vary as to their ability to form indol from pepton, some cultures and sub-species being positive and others negative. All cultures were tested for skatol by the dimethylanilin test. All were negative with the exception of *Cl. skatol* n. sp. which was strongly positive.

On the whole the vanillin (Steensma) test was least satisfactory for it gave many positive results which could not be confirmed by other tests. One reason for this is that the color produced is read directly without extraction by any solvent as in the Ehrlich test. It is also believed that interfering substances are often present in bacterial cultures which give orange or red colors with the reagent, thus making the test less accurate and reliable. The chief use of the Steensma vanillin test is in its confirmatory value. When used alone it is unreliable. The Salkowski test is suitable where large amounts of indol are present, but it is not sensitive enough for ordinary purposes. Furthermore the production of a color closely resembling the indol-red color results when acid alone or acid plus sodium nitrite are added to indol-acetic and indol-propionic acids. When the Salkowski test is performed upon the distillate, however, it is much more sensitive and reliable, though it hardly compares with the modified Ehrlich technic in these respects. It is recommended mainly as a confirmatory test.

SUMMARY

1. A critical review of the literature on the indol reaction as applied to bacterial cultures has been compiled.

2. The Ehrlich, vanillin, Salkowski, β-naphtho-quinone, dimethylanilin, Konto, nitroprusside, Gnezda, furfural, glyoxylic

acid, methyl alcohol, Baudisch and other tests for indol and skatol were carefully studied and compared for reliability, accuracy, simplicity and practicability.

3. The Ehrlich test was found to give the best results. A suitable technic for performing the test was worked out with excellent results when applied to bacterial cultures. It was found that not only the Ehrlich but most other indol and skatol tests when applied directly to the liquid medium did not yield consistent nor accurate results particularly when only small amounts of indol or skatol were present.

4. It was found necessary to distil the culture and make the tests upon the distillate. By the use of standards prepared from pure indol or skatol the technic may be made quantitative. Small quantities of indol may be detected by this method which are entirely overlooked when testing the culture direct. Indol acetic acid and other interfering substances which are often present in bacterial cultures do not seriously affect this test. Steam or direct distillation may be used. Satisfactory recoveries of indol are obtained by either method of distillation. Precautions in carrying out the technic were carefully worked out and must be closely followed if quantitative results are sought.

5. The test is accurate to 1 part to 25,000,000.

6. In the characterization of bacterial species it is recommended that the indol and skatol tests be applied quantitatively and following an approved technic. The present chaos surrounding the indol test in bacteriology is largely attributable to the diversity of methods, tests, mediums and many other variable factors.

7. The dimethylanilin test for skatol as described in this paper is recommended as the most satisfactory reaction for this substance. The test should be made upon the distillate otherwise it may give negative results. Of 53 species tested for skatol only one, *Cl. skatol* n. sp. gave positive results. The Ehrlich reaction also serves as a valuable indicator of skatol.

8. It is not safe to rely upon the production of a red color in the culture tube as a positive indol test regardless as to whether the test be the Ehrlich, vanillin or Salkowski, unless the color is

pronounced and soluble in chloroform. Although distillation by steam or directly requires a little time, yet it has been found that once the apparatus is set up, the tests may be run very expeditiously. It is believed that the quantitative and reliable nature of the results obtained are more than sufficient justification for the use of such a method.

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