LXXVI. INDOLE FORMATION BY *BACTERIUM COLI*.

I. THE BREAKDOWN OF TRYPTOPHAN BY WASHED SUSPENSIONS OF *BACTERIUM COLI*.

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KUHNE [1875] recognised indole as one of the products of the bacterial decomposition of proteins. The discovery of tryptophan by Hopkins and Cole [1901] was followed by the proof by these authors that tryptophan was the parent substance of bacterially formed indole [Hopkins and Cole, 1903]. They succeeded in isolating indole and indoleacetic acid from the products of the aerobic growth of bacteria (including *Bact. coli*) on a medium consisting of pure tryptophan and gelatin. Under anaerobic conditions they were able to isolate indolepropionic acid.

Hertzfeldt and Klinger [1915] studied, in roughly quantitative manner, the relationship between the disappearance of tryptophan and the production of indole with both indole-positive and indole-negative organisms. They used peptone medium and also a medium of pure tryptophan and salts, in which tryptophan was the sole source of nitrogen. In the latter case it is noteworthy that a form of bacterial suspension was used. Raistrick and Clarke [1921] investigated the bacterial decomposition of both the side chain and ring of tryptophan, but *Bact. coli* was not used. Fellers and Clough [1925] made a careful survey of the tests available for the detection of indole and skatole in bacterial cultures and applied them to a large number of bacterial species, but peptone and similar complex media were employed.

Bact. coli was chosen for the following work since it is a strongly indole-positive organism. An additional advantage is that *Bact. coli* does not decompose indole [Hertzfeldt and Klinger, 1915].

EXPERIMENTAL.

Most of the previous work on indole production has been done by means of growth experiments; it was decided here to employ washed bacterial suspensions for the following reasons. (a) The reaction occurs much more rapidly—about 36 hours instead of 3–4 days. (b) Pure tryptophan may be used as substrate and the reaction carried out in phosphate buffer, thus avoiding the complications incident to the use of bacterial media, such as the production of volatile phenolic substances. (c) Possible use of the tryptophan for bacterial growth is avoided. (d) Indole is the only volatile product.

In order to determine whether the indole produced could be recovered and estimated quantitatively from the bacterial suspension the following trial experiment was carried out. Distillation *in vacuo* of the cultures was tried in place of the steam-distillation, direct distillation or extraction with organic

solvents previously used in this class of work. 2 ml. of stock *Bact. coli* suspension were added to 10 ml. of indole solution (containing 0.01–0.1 mg. indole per ml.). 10 ml. of this mixture were adjusted to p_H 9.2 [Zoller, 1920], using phenolphthalein as internal indicator, and washed into a small Claisen flask with 10 ml. water. This was distilled *in vacuo* almost to dryness, the tip of the condenser dipping below 10 ml. of water in an ice-cooled receiver. Two further amounts of 10 ml. of water were run into the flask and treated similarly. The temperature of the bath was not allowed to exceed 40°. The distillate and washings were made up to 100 ml. and were kept at 0° till estimated.

The distillation method of indole recovery has been criticised [Berthelot, 1914] on the grounds that some indole derivatives, *e.g.* β -indolecarboxylic acid decompose to indole on boiling. A number of indole derivatives (β -indolecarboxylic acid, β -indoleacetic acid, β -indolepropionic acid, β -indolepyruvic acid, β -indoleacrylic acid) have been submitted to distillation *in vacuo*, but no indole was detected, the lower temperature of distillation preventing any possible decomposition.

From a number of colorimetric methods available for the quantitative estimation of indole, that of Herter and Foster [1905], modified by Bergeim [1917] was chosen after trial. It has the advantage that the coloured compound formed is stable, and that the reaction is specific, no colour being given with skatole. Ammonia gives a green colour, but blank experiments in which culture distillates were shaken with synthetic permutit before estimation proved that sufficient ammonia to interfere was never present.

50 ml. of the distillate and 50 ml. of standard indole solution were run into separating funnels and to each were added 0.5 ml. of freshly prepared 1 % sodium β -naphthoquinonemonosulphonate and 1 ml. of 10 % potassium hydroxide. After standing for 15 minutes the blue solution was extracted successively with 5 ml. and 3 ml. of chloroform. The orange-red chloroform solution was run into 10 ml. volumetric flasks and made up to volume. The unknown and standard solutions were compared in a colorimeter with micro-plungers and cups (1 ml.). With concentrations of indole above 5 ml. of stock standard in 100 ml. the solutions were diluted with their own volume of water before estimation.

Stock standard. About 10 mg. of indole were weighed accurately, dissolved in a minimum volume of ethyl alcohol and diluted to 100 ml. with water. The solution remained constant in strength for 4–5 days if kept at 0°. Standard solutions of required strength were prepared from this stock standard.

Table I gives the percentage recovery of added indole from *Bact. coli* suspensions using the above method.

Table I.

Indole added (mg.)	Indole recovered (mg.)	% re-covered	Indole added (mg.)	Indole recovered (mg.)	% re-covered
0.927	0.901	97.2	0.515	0.500	97.1
0.927	0.888	95.8	0.515	0.492	95.6
0.927	0.895	96.5	0.206	0.199	96.4
0.721	0.684	94.9	0.206	0.197	95.7
0.721	0.695	96.4	0.103	0.100	97.3
0.515	0.499	96.9	0.103	0.097	94.5
					Mean 96.2

Preparation of bacterial suspensions. *Bact. coli* was grown on Roux bottles containing tryptic caseinogen broth agar. After 18 hours' incubation the growth of six bottles was washed off in the usual way with sterile precautions, washed and centrifuged twice with sterile Ringer's solution and finally suspended

in 60 ml. of Ringer's solution and stored at 0°. The dry weight of 1 ml. suspension was 0.024 ± 0.003 g.

Tryptophan. The tryptophan used in this work was prepared from caseinogen according to Hopkins and Cole [1901], as modified by Dakin [1918]. It gave the following analytical figure:

Nitrogen % (micro-Dumas) 13.6 % Theoretical 13.7 %.

Indole formation from tryptophan.

In the following experiments small quantities of tryptophan were used since:

(a) the reaction is slow and if larger amounts are used a much longer time is needed for the completion of the reaction;

(b) it has been reported [Kilborn *et al.*, 1928] that 0.1 % of indole is toxic to *Bact. coli*. The concentration of indole formed in the experiments to be described did not exceed 0.01 %.

In view of the differing products obtained by Hopkins and Cole from the action of *Bact. coli* on tryptophan according to the degree of aeration, it was desirable to maintain good aerobic conditions. Air was bubbled through the suspensions at a steady rate. For each experiment the following quantities were used: 100 ml. tryptophan solution in phosphate buffer p_H 7.2 containing 20 mg. of tryptophan; 20 ml. stock *Bact. coli* suspension. The tryptophan solution was sterilised by filtration through a Seitz filter, and the apparatus was also sterilised. The solutions were run in with sterile precautions. The air current was turned on and the apparatus incubated at 37°. 10 ml. samples were removed aseptically at intervals and the indole formed was estimated by the method described. The p_H was checked with each sample and was found to remain constant.

In Fig. 1, curves *a*, *b* and *c* show some typical results obtained. Curve *d* shows the negligible autolytic production of indole when *Bact. coli* suspensions

Fig. 1.

are incubated with water instead of tryptophan solution. Between 85 and 90 % of the theoretical yield of indole was obtained, though the time taken to reach this maximum varied between 16 and 36 hours. The yield of indole began to fall after reaching the maximum owing to volatilisation and the continuous aeration. 100 ml. of a solution of indole (of strength chemically equivalent to the

tryptophan solutions) was aerated together with 20 ml. of *Bact. coli* suspension (Fig. 1e). This curve gives the rate of loss of indole due to aeration. An experiment in which indole was aerated with a killed suspension of *Bact. coli* (Fig. 1f) proves that this loss is due to aeration and not to breakdown of tryptophan by *Bact. coli*, for the rate of loss of indole is identical in the two cases.

In Fig. 2 the curves *b* and *c* of Fig. 1 have been corrected for the steady loss of indole shown above and further for the average 4 % loss occurring in the distillation process. In each case the indole formed is shown to be 100 % of that theoretically obtainable from the tryptophan added. Four other experiments gave similar results.

Fig. 2. — *l*-Tryptophan; - - - *dl*-tryptophan.

Fig. 3.

Hopkins and Cole [1903] isolated indoleacetic acid, as well as indole, from the products of the aerobic growth of *Bact. coli* on a medium of tryptophan, salts and gelatin; it will be seen that in the experiments described above there was no formation of this acid. This distinction is probably due to the different conditions under which the experiments were performed, for the present work using washed suspension differed from that of Hopkins and Cole in that: (a) the

cultures were aerated, (b) a small amount of tryptophan and a large quantity of *Bact. coli* were used and (c) there were no other substrates present. It is possible that the presence of alternative sources of carbon and nitrogen modifies the course of the reaction. The course of breakdown of tryptophan by *Bact. coli* has been shown to be modified considerably by the addition of glucose and other sugars [Hertzfeldt and Klinger, 1915].

Effect of aeration. The degree of aeration was found to have a marked effect on the course and completeness of the reaction. In Fig. 3, curve *a* represents the (uncorrected) production of indole when air was continuously passed through the apparatus; curve *b* is from a non-aerated experiment; and curve *c* is from an experiment in which the culture was contained in a 500 ml. Ehlenmeyer flask. Attempts to detect indoleacetic acid in the culture filtrates at the end of the experiments were unsuccessful.

Rate of breakdown of tryptophan.

It was hoped that by studying the disappearance of tryptophan concurrently with the formation of indole further light might be thrown on the course of the reaction. In the previous work of Hertzfeldt and Klinger [1915] these had been shown to be roughly in agreement.

Two methods of estimating tryptophan were used and estimations of indole were carried out at the same time in the usual way.

(1) A colorimetric method making use of the condensation reaction between tryptophan and *p*-dimethylaminobenzaldehyde in the presence of hydrochloric acid. (Details as to quantities are from a private communication from Dr R. S. Alcock.) An aeration experiment with tryptophan solution and *Bact. coli* suspension was performed as described above and 10 ml. samples were removed for the estimation of indole and 15 ml. samples for the tryptophan determinations. The latter were filtered through kieselguhr and 12 ml. of the filtrate precipitated with 2 ml. of 30 % trichloroacetic acid and filtered with kieselguhr to remove proteins. 10 ml. of the filtrate were extracted three times with ether to remove indole and freed from ether by aeration. 4 ml. or less (according to the concentration of tryptophan, which should be 0.2–0.5 mg. in not more than 4 ml.) were taken for estimation. The tryptophan standard was diluted to the same volume as the test solution. To each were added 4 drops of 4 % *p*-dimethylaminobenzaldehyde (in alcohol) and 15 ml. of concentrated hydrochloric acid. After standing 2–3 days in the dark to develop the maximum blue colour, the solutions were diluted to 25 ml. and compared colorimetrically with a standard prepared in a similar way. In the later stages when the concentration of tryptophan had become very low, 6 and 8 ml. of the indole-free solution were taken for estimation and fuming hydrochloric acid was used instead of concentrated.

A typical result is shown in Fig. 4 (*a* and *b*). The broken curve (*a*) represents the percentage of tryptophan oxidised, the full line (*b*) the percentage of indole formed (corrected). It will be seen that there is complete correspondence.

(2) As tryptophan was the only amino-acid present it was possible to estimate it by determinations of amino-nitrogen. The manometric method of Van Slyke [Peters and Van Slyke, 1932] was chosen.

15 ml. samples from a tryptophan-*Bact. coli* aeration experiment were de-proteinised by the addition of 4 ml. of 30 % trichloroacetic acid and filtered with kieselguhr. To 10 ml. of the filtrate were added 4–5 drops excess of Phillips's "milk of magnesia" and the solution was boiled down to half-volume in order to remove ammonia. After the addition of a slight excess of glacial acetic acid, the

clear solution was diluted to 10 ml. and 4 or 5 ml. samples were taken for estimation. A blank experiment was performed without tryptophan in order to determine the endogenous production of amino-N by *Bact. coli* from its own protein. The blank values were subtracted from the values obtained in the tryptophan experiment and the difference gives the amount of amino-N due to the tryptophan.

Fig. 4. ——— Indole formed, %; - - - - tryptophan broken down, %.

Table II gives the results of such an experiment.

Table II.

Time hrs.	Tryptophan exp. amino-N mg./100 ml.	Blank exp. amino-N mg./100 ml.	Amino-N due to tryptophan mg./100 ml.	% tryptophan remaining	% indole formed (corrected)
0	1.146	0.387	0.776	100	0
3.0	1.310	0.637	0.673	86.7	17.1
6.3	1.280	0.743	0.537	69.2	30.8
12.0	1.170	0.840	0.330	41.0	64.0
22.5	0.914	0.933	—	0	99.3

The results are given in graphical form in Fig. 4, curves *c* and *d*. It will be seen that identical results are obtained by the two methods; the rate of formation of indole is equal to the rate of disappearance of tryptophan and these experiments do not indicate any accumulation of intermediate products. The second method also measures the rate of deamination of tryptophan and the results lead to the conclusion that if more than one step is involved in the breakdown of tryptophan to indole, then the whole process is limited by the rate of deamination, since this is identical with the rate of formation of indole.

Oxygen uptake of the reaction.

The oxygen uptake of the oxidation of tryptophan to indole was measured in Barcroft manometers. 1 ml. *Bact. coli* suspension was placed in both cups; the left cup also contained 1 ml. buffer p_H 7.2 and 1 ml. water, and the right cup 1 ml. buffer and 1 ml. tryptophan solution (containing 1–5 mg. of tryptophan). The centre tubes of both cups contained 40 % KOH for the absorption of carbon

dioxide. The reaction proceeded very slowly, however, and after 12 hours' shaking only 40 % of the theoretical amount of indole had been formed. Attempts were made to accelerate the reaction by adjustment of the concentration of the reactants, and also by the addition of oxygen carriers such as methylene blue, but with no success.

It was decided to put up a series of experiments and to compare the oxygen uptake with the indole formed at different periods. The contents of the Barcroft cups were washed out and diluted to a suitable volume and the indole was estimated in the usual way (see Fig. 5). The straight line relationship was more

Fig. 5.

marked than was expected, for the high endogenous oxygen uptake of the thick *Bact. coli* suspensions necessary to bring about the reaction at a reasonable rate render small errors in the volume of suspension added very serious. The period of shaking (up to 12 hours) was also much longer than that normally used in accurate manometer work. The straight line relationship between oxygen uptake and indole formation renders justifiable a calculation of the oxygen uptake for the complete reaction. A series of eight experiments gave an average oxygen uptake of 472 μ l. of O₂ per mg. of indole formed (Table III).

Table III.

O ₂ uptake (μ l. of O ₂ at N.T.P.)	Indole formed mg.	μ l. O ₂ per mg. of indole	O ₂ uptake (μ l. of O ₂ at N.T.P.)	Indole formed mg.	μ l. O ₂ per mg. of indole
101	0.191	528	278	0.608	457
134	0.312	428	279	0.628	444
190	0.418	455	281	0.543	517
207	0.432	479	345	0.735	469
					Mean 472

Theoretically, if the oxidation of 1 molecule of tryptophan is brought about by:

- (a) 6 atoms of oxygen, then 570 μ l. of O₂ per mg. of indole are required;
- (b) 5 " " 475 " " "
- (c) 4 " " 380 " " "

The conclusion seems unavoidable that 5 atoms of oxygen are involved in the oxidation of each tryptophan molecule. This agrees with an equation postulating

the final complete oxidation of the tryptophan side chain to carbon dioxide and water, together with the elimination of nitrogen as ammonia:

Experiments with inhibitors.

The effect of enzymic inhibitors on the course of the oxidation of tryptophan was studied. Barcroft manometric experiments were used for this purpose, since any change or stoppage of the reaction would be reflected by a change in the oxygen uptake. The following treatments were used:

(1) *Toluene*. The bacterial suspension was shaken with toluene, incubated for 10 min. at 37° and freed from toluene by aeration. The normal oxygen uptake was completely inhibited.

(2) *Chloroform*. The treatment was the same as with toluene, and in Barcroft experiments there was again no oxygen uptake.

(3) *Acetone*. *Bact. coli* suspension was centrifuged down, rubbed up with acetone, washed twice by centrifuging and finally suspended in the original volume of Ringer's solution. There was no oxygen uptake after this treatment.

(4) *Cyclohexanol*. The bacterial suspension was made 2 % with respect to cyclohexanol, incubated at 37° for 8–9 hours and washed as in (3). Oxygen uptake was again totally inhibited.

These results show the reaction to be very sensitive to inhibitors, and no information was obtained from them as to the course of the breakdown of tryptophan.

Anaerobic experiments.

A series of 75 ml. Thunberg tubes each containing 20 ml. of tryptophan solution of the usual strength and 4 ml. of *Bact. coli* suspension was set up. The tubes were evacuated, filled with nitrogen and incubated at 37°. At intervals during 48 hours tubes were taken out and indole was estimated. A regular formation of a small amount of indole, amounting in 48 hours to only 5·8 % of that theoretically possible, was noted. This is probably due to the presence of endogenous hydrogen acceptors in the bacterial suspension.

Hopkins and Cole [1903] have isolated β -indolepropionic acid from the products of the anaerobic growth of the Rauschbrand bacillus on tryptophan-gelatin medium. It was found impossible to devise a satisfactory method for the quantitative estimation of indolepropionic acid, but its anaerobic formation by *Bact. coli* suspensions from tryptophan was confirmed in the following way. After 48 hours' incubation the culture was filtered through kieselguhr, proteins were precipitated by warming at p_{H} 3·8 and the liquid was again filtered through kieselguhr. The filtrate was adjusted to p_{H} 1 and extracted with ether. The aqueous layer gave negative tests for tryptophan with bromine water and with the formaldehyde test. The ether solution, after concentration, was extracted twice with 1 % sodium carbonate solution and the extracts were diluted to the original volume of the tryptophan solution. Tests for β -indolepropionic acid were made on this solution by colour reactions given in detail in Table I of the following paper [Woods, 1935, p. 651]. A solution of β -indolepropionic acid of strength equivalent to the original tryptophan solution gave identical reactions of approximately the same intensity. No trace of any other indole derivative could be detected.

These results show that tryptophan is completely broken down by suspensions of *Bact. coli* under anaerobic conditions, and that the production of indolepropionic acid is almost quantitative.

Experiments with racemic tryptophan.

A sample of racemic tryptophan was prepared from *l*-tryptophan by the method given by du Vigneaud and Sealock [1932]. The production of indole from racemic tryptophan was studied by aeration experiments with *Bact. coli* suspension, similar to those already described for *l*-tryptophan.

A maximum production of 45.2 % of the theoretical quantity of indole was found. Correction for indole losses (see p. 643) brought this figure up to 51.0 %. The result of an experiment is shown in the broken curve of Fig. 2. The rate of fall of indole after the maximum was slower than usual, suggesting that there is a very slight and slow breakdown of the *d*-tryptophan. These experiments show that if *Bact. coli* is able to bring about the formation of indole from *d*-tryptophan at all, then it does so at a rate incomparably slower than from *l*-tryptophan.

SUMMARY.

1. The formation of indole from *l*-tryptophan by thick washed suspensions of *Bact. coli* has been studied. Under conditions of continuous aeration there is quantitative production of indole.

2. The degree of aeration has a marked effect on the rate and completeness of the reaction.

3. The rate of disappearance of tryptophan is identical with the rate of formation of indole.

4. The oxidation of one molecule of tryptophan to indole by *Bact. coli* requires five atoms of oxygen.

5. *d*-Tryptophan is broken down, if at all, at a very slow rate.

6. Under anaerobic conditions β -indolepropionic acid is formed.

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