LXXVII. INDOLE FORMATION BY *BACTERIUM COLI*.

II. THE ACTION OF WASHED SUSPENSIONS OF *BACTERIUM COLI* ON INDOLE DERIVATIVES.

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A CONVENIENT technique for the quantitative investigation of indole formation having been described in the preceding paper [Woods, 1935], it was decided to study the action of thick washed suspensions of *Bact. coli* on various indole derivatives. Such derivatives, if intermediate stages in the breakdown of tryptophan by *Bact. coli*, should give rise to indole at least to the same extent and as rapidly as tryptophan itself under the same conditions.

Frieber [1922], in a series of growth experiments with *Bact. coli*, substituted a number of indole derivatives for tryptophan in his growth medium. He found no production of indole from the following compounds: α -indolecarboxylic acid, $\alpha\beta$ -indolecarboxylic acid, skatole, β -indolealdehyde, β -indoleacetic acid, β -indolepyruvic acid, β -indole-ethylamine. Saito [1933] found that, in growth experiments, *dl*- β -indolelactic acid (but not *l*-indolelactic acid) gave rise to indole when incubated in a suitable medium with *Bact. coli*. This formation of indole took place only in the presence of an alternative source of nitrogen—a mixture of ammonium lactate and asparagine was used. Unfortunately the tests for indole were qualitative only, and no data as to concentration of either indolelactic acid or indole are given, so that it is difficult to assess the importance of this result. Saito also finds that with β -indolepyruvic acid there is formation of indole if fresh cultures of *Bact. coli* are used. This was presumably in the presence of alternative sources of nitrogen, though this is not definitely stated; results were again qualitative. Saito's result with indolepyruvic acid does not agree with that of Frieber, whose medium also contained ammonium lactate, though not asparagine.

Preparation of the indole derivatives.

β -Indolepropionic acid was prepared from adipic acid by a series of reactions quoted by Berg *et al.* [1929]. The final colourless crystalline product melted at 133° (uncorrected). N (micro-Dumas), 7.35 %. Theoretical, 7.41 %. Equivalent (by titration in alcoholic solution), 188. Theoretical, 189.

β -Indolealdehyde was prepared as described by Majima and Kotaki [1922, 1], modified by Putochin [1926]. It was found more expedient to start the Grignard reaction in ether solution and to add the benzene solutions gradually. A considerable loss occurs during the steam-distillation of the crude product owing to resinification. This was avoided in the following way. The crude aldehyde formed on hydrolysis of the Grignard compound was centrifuged down, sucked dry on a Büchner funnel and washed on the funnel with a small amount of pure benzene. After drying at 37° it was ground up twice with light petroleum, filtered and dried. This treatment removes *N*-formylindole, indole and other unchanged

reactants. The product was finally recrystallised twice from boiling water. From 10 g. of indole 3 g. of faint gold-coloured crystals m.p. 194° were obtained. N (micro-Dumas), 9.60 %. Theoretical, 9.66 %.

β -Indoleacetic acid was made from indole according to Majima and Hoshino [1925]. The chloroacetonitrile used was prepared from chloroacetamide [Steinkopf, 1908]. The product decomposed at 160 – 165° to skatole and carbon dioxide. N (micro-Dumas), 8.05 %. Theoretical, 8.00 %. Equivalent (by titration in alcoholic solution), 173. Theoretical, 175.

β -Indolecarboxylic acid. The method of Majima and Kotake [1922, 2] was used. The final product was faintly pink and melted at 215° (uncorrected). N (micro-Dumas), 8.64 %. Theoretical, 8.70 %.

β -Indoleacrylic acid was synthesised from β -indolealdehyde by the procedure described by Bauguess and Berg [1934]. The yellow crystals obtained melted with decomposition at 194° (uncorrected). N (micro-Dumas), 7.56 %. Theoretical, 7.49 %. Equivalent (by titration in alcoholic solution), 186. Theoretical, 187.

β -Indolepyruvic acid was prepared according to Ellinger and Matsuoka [1920] from β -indolealdehyde. The product, after removal of acetic acid of crystallisation, was buff-coloured. It darkened at 200° , softened at 206° and melted at 211° (all uncorrected). Berg *et al.* [1929] give these figures as 198° , 204° and 212° . N (micro-Dumas), 9.56 %. Theoretical, 9.66 %.

Experiments with indole derivatives.

In the work to be described the conditions were identical with those described in the preceding paper [Woods, 1935] in studying the breakdown of tryptophan. The following quantities were used: 100 ml. of a solution of the indole derivative in phosphate buffer p_H 7.2 (containing the chemical equivalent of 20 mg. of tryptophan). 20 ml. of *Bact. coli* suspension (prepared as described in the last paper). It was necessary to dissolve the derivatives in a small amount of dilute alkali as they are all soluble with difficulty in cold water. The p_H was adjusted to 7.2, buffer was added and the solutions were diluted to the required volume. They were sterilised by filtration and all experiments were conducted at 37° .

The culture filtrates used for testing for the derivatives were prepared in the following way. The suspension was filtered through kieselguhr and proteins were precipitated by warming at p_H 3.8. After filtering through kieselguhr the clear solution was adjusted to p_H 1–2 and extracted well with ether. The ether solutions were concentrated, extracted twice with 2 % sodium carbonate solution and the extracts diluted to the original volume of the suspension.

The colour reactions used for the detection of the various indole derivatives are given in detail in Table I.

Anaerobic experiments were conducted in Thunberg tubes, which were evacuated and filled with nitrogen.

β -Indolealdehyde. An aeration experiment with *Bact. coli* suspension was performed as described in the last paper. In 48 hours there was only the normal 1–2 % autolytic production of indole from the suspension itself.

β -Indolecarboxylic acid. There was no formation of indole from this derivative when aerated together with *Bact. coli* suspension.

β -Indoleacetic acid. This is a particularly interesting compound, since Hopkins and Cole [1903] have isolated it from the products of the aerobic growth of *Bact. coli* on tryptophan, whilst the washed suspension technique has been shown [Woods, 1935] to give only indole (in theoretical yield) and no trace of β -indole-

Table I.

All tests are on solutions containing 10 mg. of indole (or their equivalent) in 100 ml.

Test (3 ml. solution)	β -Indoleacrylic acid	β -Indoleacetic acid	β -Indolepropionic acid	Skatole	Indole
1 ml. 50 % H_2SO_4 and 1-2 drops 2 % KNO_2	Yellow, then light pink colour, soluble in amyl alcohol to a pink solution	Cherry-red colour soluble in amyl alcohol	Golden yellow intensifying on warming. With higher concentrations crystalline needles on cooling. Soluble in amyl alcohol	Yellow in the cold, more orange on boiling. Soluble in amyl alcohol and in $CHCl_3$	Cherry-red colour, soluble in amyl alcohol, insoluble in $CHCl_3$
3 drops 2 % <i>p</i> -dimethylaminobenzaldehyde (in alcohol) and 1 ml. conc. HCl	Purplish red colour insoluble in $CHCl_3$, soluble in amyl alcohol, giving cherry-red solution with absorption band at 550 $m\mu$ (1) No colour even on heating	No colour is given	Light rose, deepening on adding 1 drop 2 % KNO_2 . Colour quickly goes orange and fades	Blue-violet colour on boiling, soluble in amyl alcohol and $CHCl_3$ to a pure blue solution. Addition of 1 drop 2 % KNO_2 gives intense blue colour, soluble in amyl alcohol and in $CHCl_3$	Red-violet colour soluble in amyl alcohol and $CHCl_3$ to a red-violet solution. Addition of 1 drop 2 % KNO_2 gives a more orange tint, soluble as before
3 drops 2 % <i>p</i> -dimethylaminobenzaldehyde (in alcohol) and 3 ml. fuming HCl	Orange on boiling. On adding 1 drop 2 % KNO_2 colour goes greenish yellow (soluble in amyl alcohol)	Orange on boiling. Similar to preceding test (above)	Red-violet colour on warming. Addition of 1 drop 2 % KNO_2 gives a deep blue-violet colour soluble in $CHCl_3$ to a deep blue-violet solution	Similar to preceding test (above)	Similar to preceding test (above)
1 drop conc. HCl and a few drops of very dilute $FeCl_3$	Orange-brown on boiling	Red-violet on boiling. Soluble in amyl alcohol	Orange solution on warming	Orange-red on boiling, intensified by 1 drop 2 % KNO_2	Very pale yellow on boiling. 1 drop 2 % KNO_2 gives a cherry-red colour
2 drops 1 % vanillin (in alcohol) and 3 ml. conc. HCl	Orange colour on boiling. Addition of 1 drop 2 % KNO_2 gives colour as in (1)	Pale pink on boiling, soluble in amyl alcohol. Colour intensifies on adding 1 drop 2 % KNO_2 , soluble in amyl alcohol to a pink-violet solution	Light purplish pink on boiling, insoluble in $CHCl_3$. Addition of 1 drop 2 % KNO_2 gives a deep violet colour soluble in $CHCl_3$	Pernanganate colour on boiling, soluble in amyl alcohol and $CHCl_3$ to a violet solution. 1 drop 2 % KNO_2 gives a deep violet colour soluble as before	Deep orange colour soluble in amyl alcohol, almost insoluble in $CHCl_3$. Addition of 1 drop 2 % KNO_2 causes colour to fade
Shaken with 1 drop dimethylaniline and 3 ml. conc. HCl added	Orange on boiling	Pink on boiling, insoluble in $CHCl_3$, soluble in amyl alcohol	Blue-violet colour on heating, soluble in $CHCl_3$ giving a purple-red extract	Violet colour on boiling, soluble in amyl alcohol and in $CHCl_3$	Pale orange pink on boiling, partly soluble in $CHCl_3$, soluble in amyl alcohol
50 ml. solution, 0.5 ml. 1 % Na β -naphthoquinonemonosulphonate and 1 ml. 10 % KOH				No colour is formed	Blue colour develops in 15 mins. (or precipitate with strong solutions), soluble in $CHCl_3$ to an orange-red solution

acetic acid. However, aeration experiments showed that, in 48 hours, this acid gave rise to no indole, beyond the normal 1–2 % blank, when incubated with *Bact. coli* suspensions. No trace of skatole was observed. A culture filtrate at the end of the experiment gave reactions identical with the original indole-acetic acid solution, and there was no trace of any other derivative.

Equal quantities of β -indoleacetic acid and tryptophan were mixed and an aeration experiment performed; a normal curve for the production of indole from tryptophan was obtained, showing that indoleacetic acid is neither toxic to *Bact. coli* nor inhibitory of the production of indole.

Anaerobic experiments were also done with β -indoleacetic acid and *Bact. coli* suspension in order to determine if skatole could be formed under these conditions. After 50 hours' incubation the culture distillates showed no trace of indole or skatole. Salkowski E. and H. [1885] have shown that the sodium salt of indoleacetic acid when sown with a mixed putrefactive culture gave no indole or skatole.

β -Indolepropionic acid. This is the end-product of the anaerobic action of *Bact. coli* on tryptophan. Aeration experiments showed that it was not broken down to indole under aerobic conditions, as in 48 hours only the usual small blank was obtained. When mixed with tryptophan it did not inhibit the normal production of indole from this amino-acid. A culture filtrate was prepared at the end of the experiment; it gave tests identical with a solution of pure indole-propionic acid and there was no trace of indoleacetic acid or of any other derivative. These experiments show that the product of the anaerobic breakdown of tryptophan is quite unchanged when incubated with *Bact. coli* aerobically.

β -Indolepyruvic acid. In view of the oxidative nature of the breakdown of tryptophan to indole, it was thought possible that an initial stage might be an oxidative deamination to β -indolepyruvic acid. Some results by other workers with this derivative have already been mentioned. Aeration experiments were performed using freshly made suspensions of *Bact. coli*, but only the normal blank of 2 % indole was obtained in duplicate experiments (Fig. 1, curve *a*). Tests of the culture filtrate indicated that the acid was unchanged, and in addition identical derivatives with 2:4-dinitrophenylhydrazine were obtained from the culture filtrate and from a solution of pure indolepyruvic acid.

The usual experiment to test the possibility of the derivative itself inhibiting the formation of indole led to a surprising result. When a solution containing both indolepyruvic acid and tryptophan was aerated in the presence of *Bact. coli* suspension, 10–15 % more indole was formed than could have been derived from the tryptophan present. This is shown in curve *b* of Fig. 1. Curve *c* shows the formation of indole from the same weight of tryptophan as was used for *b*. The excess formation of indole is outside the limits of experimental error (see previous communication, Table I). Bearing in mind that Saito's [1933] experiments—in which indole was obtained from β -indolepyruvic acid—were conducted in the presence of an alternative source of nitrogen, it seemed of interest to try the effect of replacing tryptophan by other nitrogen compounds. Ammonia was chosen as the simplest possible.

In one experiment 30 mg. of ammonium sulphate were added to 20 mg. of indolepyruvic acid in 100 ml. of solution; in another the indolepyruvic acid was dissolved in dilute aqueous ammonia and the p_H adjusted to 7.4 (the ammonia present was equivalent to 300 mg. of ammonium sulphate). The results of aeration experiments with these solutions are shown in Fig. 1, curves *d* and *e*. It is seen that indole was formed in appreciable quantity (8–10 %) in the first 8–10 hours and then remained steady for 20 hours, after which there was a slight fall.

Since indole is continually lost by aeration (see previous paper, p. 650), the constant period is really a period of slightly increasing indole concentration reaching 1–2 % above the maximum recorded. The experiments are good duplicates, showing that increasing the concentration of ammonia has no effect. Further, the addition of more ammonium sulphate during the steady period did not increase the yield of indole.

Fig. 1.

The effect of tryptophan in the experiment shown in curve *b* is probably due, therefore, to ammonia formed from the deamination of tryptophan during its breakdown to indole, for the amount of extra indole is approximately equal to the indole obtained in the presence of ammonia. The possible significance of these results will be discussed later.

The action of *Bact. coli* on β -indolepyruvic acid under anaerobic conditions was also tried. The usual proportions of substrate and suspension were incubated anaerobically for 48 hours and a protein-free filtrate prepared. On testing the filtrate there was no trace of β -indolepropionic acid and the indolepyruvic acid was unchanged.

β -Indoleacrylic acid. When an aeration experiment with *Bact. coli* was carried out there was no formation of indole beyond the normal 2 % blank. The possible inhibitory action of β -indoleacrylic acid on indole production was tested and a normal formation of indole from tryptophan was found. The addition of ammonium sulphate did not bring about any formation of indole, and in all cases tests on the culture filtrate showed the acid to be unchanged, no trace of any other indole compound being detectable.

Anaerobic experiments were also done:

- (a) With indoleacrylic acid and *Bact. coli* suspension alone.
- (b) With indoleacrylic acid and suspension in the presence of formate as a hydrogen donator (10 mg. sodium formate to 20 ml. of solution).
- (c) With indoleacrylic acid and suspension in the presence of both formate and ammonium sulphate (10 mg. of each to 20 ml. of solution).

The culture filtrate after 50 hours' incubation showed no formation of indolepropionic acid in any of the experiments, and the indoleacrylic acid was unchanged.

DISCUSSION.

The growth experiments of Frieber [1922] were repeated in a number of cases (β -indolealdehyde, β -indoleacetic acid and β -indolepyruvic acid) by the washed suspension technique and his results were confirmed. It seems probable, therefore, that his other experiments, of which β -indole-ethylamine is of most physiological importance, would also be confirmed. This duplication of results provides support for the validity of the use of washed suspensions. Work described in the previous paper shows that a theoretical yield of indole can be obtained from tryptophan by the action of washed suspensions of *Bact. coli*; there seems little reason to doubt that, if any of the indole compounds used had been intermediates in the breakdown of tryptophan to indole, then they would have given rise to indole in something like quantitative yield, especially as in all the cases tried the derivatives themselves were not inhibitory of the normal production of indole from tryptophan.

The only case in which some indole was obtained—that of β -indolepyruvic acid—is of peculiar interest. In growth experiments, such as those of Saito [1933], it is reasonable that an alternative source of nitrogen should be necessary, since nitrogen is essential for growth of the organism and *Bact. coli* is not able to split the indole ring to obtain this nitrogen. But that an alternative source of nitrogen should be necessary for the formation of indole from indolepyruvic acid in washed suspension experiments must cast doubt on any suggestion that β -indolepyruvic acid is an intermediate, quite apart from the fact that only 8 % of the indole theoretically possible is obtained, compared with 100 % from tryptophan. A possible explanation of this formation, and also of the formation of indole from *dl*- β -indolelactic acid noted by Saito [1933], is that there is some synthesis of tryptophan from indolepyruvic acid and ammonia, and that this tryptophan is then oxidised in the usual way to indole.

It has been suggested, Harden [1930], that the bacterial decomposition of tryptophan occurs by the following route: tryptophan $\rightarrow$ β -indolepropionic acid $\rightarrow$ β -indoleacetic acid $\rightarrow$ indole. The work here reported gives no support to this hypothesis.

In the breakdown of tryptophan to indole, decarboxylation may either precede or follow deamination. In the former case β -indole-ethylamine should result, but Frieber's [1922] work showing that this amine does not give rise to indole with *Bact. coli* is evidence that this does not occur. In the latter case an oxidative deamination to β -indolepyruvic acid seems probable in view of the aerobic nature of the reaction, but, as has been already pointed out, experiment does not support this theory.

Raistrick [quoted by Cole, 1933] has suggested that the primary reaction in both aerobic and anaerobic breakdown of amino-acids by bacteria is a deamination to the unsaturated acrylic acid. This suggestion is based on the isolation of unsaturated acids from the corresponding amino-acids (histidine, tyrosine and aspartic acid) after bacterial action. Indoleacrylic acid has not, so far as the author knows, been reported as having been obtained in this way. The experiments which have been described show that β -indoleacrylic acid does not give rise either to indole aerobically or to indolepropionic acid anaerobically. Raistrick further suggests that the breakdown may proceed as follows:

This avoids β -indolepyruvic acid, but postulates instead a β -keto-acid as an

intermediate product. This β -substituted compound would be very unstable and difficult to prepare in a free form, so it was not possible to test this experimentally. Since indole was produced from neither β -indoleacrylic acid nor β -indolecarboxylic acid, it seems unlikely that this series of reactions actually occurs. Indolecarboxylic acid has been detected by Berthelot [1914] in 15-day cultures of *B. proteus* on tryptophan-gelatin medium, but there is no justification for generalising from one bacterial species to another, and in any event the acid is probably a stable end-product formed under the given conditions.

It has been shown in the preceding paper that 5 atoms of oxygen are involved in the oxidation of the tryptophan molecule to indole, and that the side chain is completely oxidised to carbon dioxide, water and ammonia. It was also demonstrated that the rate of indole formation is limited by the rate of deamination of the tryptophan, but the results given in the present paper do not suggest any scheme for the course of the reaction. It is possible, as in all metabolic work, that the intermediate compounds are highly unstable substances of short lifetime which are passed on immediately from one enzyme to the next. The formation of indoleacetic acid as well as indole when the conditions are different [Hopkins and Cole, 1903] suggests that a series of reactions does occur, β -indoleacetic acid being the stable end-product of a branch from the main course of the action; it is improbable that indole is formed by a direct splitting off of the side chain of tryptophan from the indole nucleus.

SUMMARY.

1. The action of washed suspensions of *Bact. coli* on certain indole derivatives has been investigated.
2. There was no formation of indole from the following compounds: β -indole-aldehyde, β -indolecarboxylic acid, β -indoleacetic acid, β -indolepropionic acid and β -indoleacrylic acid.
3. β -Indolepyruvic acid, but only in the presence of ammonia, gives rise to a 10 % production of indole.

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