ON THE SYNTHESIS OF TRYPTOPHANE BY CERTAIN BACTERIA AND ON THE NATURE OF INDOLE FORMATION.¹

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PART I.

IT is a recognised characteristic of many micro-organisms that they form indole in peptone broth or peptone water. This property, indeed, has been used as a means of differentiating strains and establishing the identity of organisms. Such indole, however, is not so much *formed* as *liberated from the peptone*. All that we mean when we speak of indole being "formed" is that indole appears free in the culture and can be detected by certain reagents. *A priori*, of course, one would expect that all bacteria should possess the power of making indole.

It is a characteristic of plants as distinguished from animals that they can and do synthesise their ring from open chain compounds. Indeed, it might even be made a test of the vegetable nature of bacteria that they should be able to produce their own indole and synthesise their own tryptophane. On the other hand, bacteria have led a saprophytic, or in some cases a parasitic, life for such an infinitude of generations that it is quite conceivable that some of them have lost this power and become entirely dependent on their environment for the indole radical in their proteins.

It appears, therefore, of interest to determine whether bacteria do form indole and its compounds synthetically. The most simple test, of course, is to see whether they can be grown indefinitely in media which contain no indole compounds. Two such media have been used by the author, namely, that described by Uchinsky and another simpler medium devised by Voges and Fränkel.

Uchinsky's medium has the following composition:—

Water, 1000 c.c.; glycerin, 30–40 c.c.; NaCl, 5 to 7 grms; CaCl₂, 0.1 grms.; MgSO₄, 2.4 grms.; K₂HPO₄, 0.2 to 0.25 grms.; ammonium lactate, 6 to 7 grms.; sodium asparaginate, 3 to 4 grms.

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Voges and Fränkel's medium is somewhat simpler. Its composition is as follows: Sodium chloride, 5 grms.; neutral sodium phosphate, 2 grms.; ammonium lactate, 6 grms.; asparagin, 4 grms.

The following method was adopted in the case of *B. coli*:—

A series of tubes of the medium was prepared and one was inoculated with *B. coli*. From this after twenty-four hours' incubation at 37° C., a second tube was inoculated, and from this after a similar period of incubation a third tube. This was continued till ten tubes had been inoculated. From the tenth tube a flask containing 100 c.c. of the medium was inoculated. This was incubated three days and a good turbidity was obtained. It was then treated with an equal volume of alcohol and centrifuged at a high velocity. The deposit of bacilli was then tested for the presence of tryptophane by Hopkins and Coles' modification of the Adamkiewicz reaction; that is to say, a solution of oxalic acid was reduced by means of sodium amalgam, and this was added to concentrated sulphuric acid in the proportion of 1 part of solution to 2 parts of sulphuric acid. The sediment was then dissolved in a little of the mixture, and a violet colour was obtained. A portion was dissolved in sulphuric acid diluted with water, to compare the colour obtained by charring, but even without this precaution the violet colour of the tryptophane reaction was unmistakable.

To obviate the criticism that this tryptophane may have been derived from the original culture from which the series was inoculated, a second series was inoculated and tested, incubation being omitted. In this case two loopfuls of each dilution were transferred to a test-tube and tested with the reagent. The two loopfuls from the first tube represented, of course, the amount used to inoculate the second, and so on. It was found that tryptophane was not detectable even in the second dilution. The tryptophane in the sediment must therefore have been derived by a synthetic action of the bacteria from the open chain compounds in the medium; that is to say, from ammonium lactate and asparagin (or sodium asparaginate).

In performing this experiment with Voges and Fränkel's medium, it was found that the strain of *B. coli* used would not at first grow in the medium. As the latter was found to be somewhat acid, it was made faintly alkaline with ammonium hydrate, and the organism was trained by growing it in very weak peptone solution containing the ingredients of the proteid-free medium. After this it grew vigorously, but unfortunately no experiments were done at the time to see whether one of these measures would by itself have sufficed.

The relationship of indole and tryptophane is, of course, quite a simple one. Indole is a double-ring compound of which one constituent is the benzene, and the other the pyrrol ring. Tryptophane is indole with an alanine chain attached to the pyrrol ring.

The two organisms which have, with the author, grown best in proteid-free media are the strain of *B. coli*, mentioned above, which was isolated by Dr. Cruickshank at Crichton Asylum, and a strain of Friedländer's bacillus obtained from Dr. C. H. Browning of the Bland-Sutton Institute, Middlesex Hospital.

Other organisms have grown rather feebly and died out quickly, but the following Gram-negative bacilli have grown in a second culture:—

<i>B. friedländer</i>	.	.	.	.	.	.	.	.	.	+	+	+
<i>B. coli</i>	.	.	.	.	.	.	.	.	.	+	+	
<i>B. dysenteriae</i> (Flexner)	.	.	.	.	.	.	.	.	.		+	
<i>B. dysenteriae</i> (Y)	.	.	.	.	.	.	.	.	.		+	
<i>B. dysenteriae</i> (Shiga)	.	.	.	.	.	.	.	.	.	Fair		
<i>B. typhosus</i>	.	.	.	.	.	.	.	.	.		+	
<i>B. paratyphosus</i> (A)	.	.	.	.	.	.	.	.	.	+	+	
<i>B. paratyphosus</i> (B)	.	.	.	.	.	.	.	.	.		+	
<i>B. enteritidis</i> (Gaertner)	.	.	.	.	.	.	.	.	.	+	+	
<i>B. Schweineseuche</i>	.	.	.	.	.	.	.	.	.	Fair		
<i>B. fluorescens</i> (?)	.	.	.	.	.	.	.	.	.			„

In the case of some of these organisms growth did not always take place. The author was unable to determine the reason for this; tubes of the same make or medium giving sometimes fair growth and sometimes no growth.

The cultures were tested by making subcultures on agar and in 2 per cent. peptone water, and growth was obtained in each case where a positive result has been recorded.

Attempts to detect indole in the supernatant fluid, after centrifuging down the bacteria, always failed even when concentration methods were used (extraction with ether). The indole synthesised appears, therefore, to have been just sufficient for the needs of the organism. It is possible, of course, that laborious extraction of large amounts of culture might yield traces, but the author has not extracted more than 300 c.c. of any culture. Indole is slightly volatile in ether vapour, and this difficulty has not been overcome. Indole left for long periods seems even to volatilise. At all events a small crystal, left in a warm room in a current of warm air, gradually diminished and vanished.

It may, therefore, be concluded that indole, at least in the form of tryptophane, can be synthesised by these organisms (*i.e.*, the Gram-negative bacilli mentioned above), but that free indole is not by ordinary means detectable in the proteid-free medium.

The question now arises whether free indole, such as can be detected, for example, in a peptone-water culture of *B. coli*, is split off from the tryptophane of the medium, or is formed in excess by the organism owing to the abundance of food materials; and, secondly, the question whether free indole can be absorbed by the organism and utilised in tryptophane formation.

In this connection the following experiment by the author seems of great importance.

Three hundred c.c. of 3 per cent. peptone water were inoculated with *B. coli* and allowed to stand at 37° C. till the indole reaction was well marked but had not yet attained a maximum. The culture was then sterilised at 60° C. and divided into three portions in three 100 c.c. flasks. One of these was left sterile. Both the others were again inoculated, and to one of them enough

glucose was added to make a 1 per cent. solution. The experiment is most neatly done by adding the glucose as sterile 10 per cent. solution in 0.5 per cent. salt solution and adding an equal amount of 0.5 per cent. salt solution to each of the other flasks. The concentration of indole was tested colorimetrically by the following method. A series of dilutions was prepared from each in small wide tubes, and each tested with the same amount of Ehrlich's paradimethylamidobenzaldehyde reagent and saturated persulphate of potash solution. One part of each reagent was added to 5 parts of each dilution. The series corresponded exactly. All these flasks were now placed in the same incubator at 37° C., and on the third day after the second inoculation 5 c.c. were withdrawn from each flask and tested. It was found that the flask which had not been inoculated gave practically the same colour as before. That which had been inoculated and contained glucose showed a marked diminution in the reaction, while that which was inoculated but contained no glucose showed marked increase. These changes were gross, and the exact differences were not estimated.

From this it appears that the effect of the glucose is to cause a more rapid absorption or more complete use of indole, probably owing to the increased rapidity of growth of the organisms, while in the absence of glucose the organism finds it convenient to discard indole and set it free into the medium.

The increased rapidity of growth of *B. coli* in peptone solution containing glucose is easily demonstrated by preparing sets of tubes containing from 0.5 per cent. to 2.5 per cent. of peptone, and comparing the growth in these with that in similar tubes containing 1 per cent. glucose. The increased growth in the glucose tubes is visible on naked-eye comparison of the tubes and in the counting chamber of the Thoma-Zeiss hæmocytometer as well as on plate cultures from suitable dilutions of corresponding cultures. In the case of the higher concentrations of peptone the growth in the tubes without glucose ultimately surpasses that in the tubes containing glucose, though in the case at least of the peptone used by the author in this set of experiments, there was no corresponding increase in the indole reaction, the concentration of tryptophane having presumably fallen too low.

It is often stated, and various experiments tend to prove, that the presence of the acid prevents the appearance of indole. This has been given as a cause of the non-appearance of indole in the presence of glucose. In this connection it may be pointed out that where bacteria are concerned acid means organic acid. Free mineral acids, even when present only in minute traces, are highly bactericidal. This means that in practice the acidity is due to a substance which is either itself a potential "food" of the open chain type or is an indication of the presence of such a food as one or more of the constituents of a fermentable substance. With regard to the first suggestion it is true that the gradual disappearance of the acid reaction, say, in a culture of *B. paratyphosus* (B) in litmus milk, may be accounted for by the production of ammonia from the peptone. One can indeed detect ammonia in these alkaline cultures by boiling the culture and testing the vapour with hydrochloric acid vapour when a white cloud is formed, or even by

letting the vapour impinge on moist red litmus paper which becomes blue gradually. That the blue colour is not due to atmospheric ammonia is shown by boiling a tube of water and letting the vapour impinge on another test paper. The vapour from the culture blues the litmus very distinctly quicker.

But at the same time it should be remembered that these ammonia compounds of organic acids may serve as food substances for the bacteria like the ammonium lactate in Uschinsky's medium.

That acidity does not of itself prevent indole formation is proved by the fact that in cultures containing enough glucose to inhibit indole formation, partially but not completely, indole only appears after fermentation has taken place and acidity has developed. Wyeth (1919³) has noted the same fact in the case of lactose, maltose, mannite, and saccharose; cf. also Fischer (1915¹) and Zunz and György (1917⁴).

In connection with her extremely interesting work on tryptophane, Homer has suggested (1916²) that the inhibition of indole formation by glucose is due to the formation of a condensation product of glucose and tryptophane. It is true that, as she says, media containing glucose darken more than media without glucose during sterilisation by heat, and that this darkening may be due to the formation of condensation products such as she suggests; but this cannot explain the disappearance of indole when glucose is added to a *B. coli* culture in which indole has already been formed; more especially since the disappearance only takes place in the presence of living *B. coli*.

It seems more probable that the splitting of tryptophane into indole and alanine is a reversible process brought about by an enzyme of the bacillus. The typhoid bacillus does not produce free indole in peptone water, and although it ferments glucose it does not, when grown with glucose and indole, cause disappearance of the indole. Presumably, therefore, *B. typhosus* does not possess this enzyme and can neither set indole free from tryptophane nor make use of free indole.

One may therefore discard all the theories that have been put forward in favour of the simple statement, that if the food supply is such that *B. coli* needs tryptophane it does not form free indole and may even absorb indole already in solution in the medium; but if tryptophane is abundant, and the alanine chain is more useful, then indole is split off and thrown out into the medium.

SUMMARY AND CONCLUSIONS.

1. Certain Gram-negative bacilli have been grown in protein-free media and have been shown to synthesise tryptophane. The strains which gave the best results were one of *B. coli* and one of *B. friedländer*, both of which appeared capable of indefinitely growing in the medium.

2. This synthesis of a ring compound supports the view of the

vegetable nature of bacteria in spite of their motility (shared by certain reproductive cells of plants) and the alleged discovery of chitin in their envelopes.

3. It has been shown that the addition of glucose to a living culture of *B. coli* causes the rapid disappearance of indole already formed. This seems to indicate that the effect of glucose is to cause increased demand for indole on the part of the organism, and not merely, by the development of acidity, to prevent the splitting off of indole from the peptone.

4. On this hypothesis indole "formation" is a consequence rather of diminished use of indole as tryptophane than of a special formation by the organism.

5. The splitting off of indole appears to be a reversible chemical change dependent upon the chemical balance between indole, tryptophane, and other substances.

6. The difference between *B. coli* and *B. typhosus*, with respect to indole formation and reabsorption, is most probably due to the fact that *B. coli* possesses an enzyme which enables it to split off and utilise the open chain (alanine) portion of the tryptophane molecule.

7. In view of the fact that, when the amount of glucose added to the medium is insufficient to prevent the formation of free indole, the indole appears late, and after the glucose is fermented, acid formation cannot be the cause of the inhibition of indole formation by glucose.

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