CCXXXVII. THE *COLI*-TRYPTOPHAN-INDOLE REACTION.

I. ENZYME PREPARATIONS AND THEIR ACTION ON TRYPTOPHAN AND SOME INDOLE DERIVATIVES.

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WOODS [1935, 1, 2], in two papers on indole formation by *B. coli*, adequately reviews the position up to the present. In his own experiments, using twice washed and presumably viable suspensions of the organism, he demonstrates the complete oxidation of tryptophan to indole and concludes that five atoms of oxygen are required for each molecule of tryptophan used. He presents a strong presumptive case for the complete and quantitative breakdown to β -indolepropionic acid under anaerobic conditions. In the second of his two papers he examines the action of his washed suspensions upon a number of possible intermediates and he is forced to reject both the hypothesis of Harden [1930] and that ascribed to Raistrick by Cole [1933]. We ourselves, using dead suspensions of *B. coli*, have been unconsciously covering so much of the same ground that it seems advisable to publish those of our findings which bear upon the investigations of Woods.

Our first object was to obtain if possible an enzyme preparation from the organisms which would convert tryptophan into indole and this we have achieved by methods one of which requires the use of toluene and chloroform, which according to Woods prevent the reaction; it seems unlikely that these substances can be classed as simple enzyme poisons.

With regard to the actual mechanism of the breakdown of tryptophan to indole by *B. coli*, we began with a belief that the breakdown would occur *via* the unsaturated β -indoleacrylic acid in a manner analogous to the breakdown of histidine to urocanic acid [Raistrick, 1917] passing *via* the keto-acid to indolecarboxylic acid and thence to indole. This would account for the production of indolepropionic acid under anaerobic conditions and of indole under aerobic conditions. Unfortunately neither β -indoleacrylic acid nor indolecarboxylic acid gives rise to the production of indole when aerated with the indole-producing enzyme. In this direction our results are at the moment entirely negative, but it is possible that with enzyme preparations available this problem may be brought nearer to solution.

Indole in our experiments was determined by the method of Happold and Hoyle [1934]. Since the error in these estimations was $\pm 5\%$ we have felt content with the method. Woods uses a vacuum distillation method since steam-distillation may cause decarboxylation of indolecarboxylic acid; this method seems unnecessarily time-consuming to us, and in many bacteriological media, especially media containing bile salts, in which we have had to estimate indole, the method would be impracticable on account of excessive frothing.

During the course of the investigation we have used many strains of *B. coli* isolated from both faecal and urinary sources and have also used old stock

cultures. Most freshly isolated strains are slow indole formers; in peptone water for example practically no indole is produced in the first 15 hours, whereas with old laboratory strains indole production begins almost at once and reaches a maximum within 12 hours. The addition of sodium deoxycholate in concentrations of 1/1000 or 1/2000, whilst it does not affect the rate of indole production of old strains, accelerates considerably the indole production of freshly isolated organisms. Higher concentrations of deoxycholate inhibit growth of the organism and consequently indole production.

Our work on enzyme preparation has been done with old laboratory strains which were powerful indole formers.

Preliminary attempts to obtain an enzyme preparation.

Our first experiments were done with living washed suspensions of *B. coli*. Such suspensions, continuously aerated at room temperature under toluene, were found to produce indole from tryptophan. This result led us to a series of attempts to produce a killed preparation which would show a similar activity.

Destruction of the bacteria by irradiation with ultraviolet light resulted in complete inactivation, as also did lysis with 1:50 sodium deoxycholate.

Shaking the suspension with toluene was found to be an uncertain method of killing the bacteria and resulted in considerable loss of potency of the preparation, but successful results were obtained by shaking with chloroform, and chloroform-killed suspensions have been used in most of our work. These suspensions however were found to deteriorate on keeping, and for this reason attempts were made to produce a desiccated preparation. This was achieved by precipitating the bacterial suspension with alcohol, washing with alcohol and with alcohol-ether, and desiccating *in vacuo*.

An attempt was made to obtain a cell-free preparation by suspending the bacteria in phosphate solution ($M/5$, p_H 8.5) and allowing to autolyse for 5 days. The suspension was then shaken with chloroform and centrifuged. The supernatant fluid was quite inactive, the chloroform layer was inactive, but the layer of intact bacteria retained some potency, though its activity was much reduced.

The enzymes appear to be strictly intracellular as the supernatant fluid obtained by centrifuging *B. coli* cultures or suspensions, after removal of indole by extraction with light petroleum and shaking with chloroform, is quite inactive.

The two successful methods which we have elaborated are described below.

Methods of preparation of the indole-producing enzymes.

(1) *Type of medium employed.* The medium used for the cultivation of the organism has a pronounced effect upon the potency of enzyme preparations. The most powerful preparations were obtained when the organism was grown on 1:10,000 tryptophan solidified with agar, with no other source of nitrogen and carbon (p_H 7.8). This type of medium is however unsatisfactory for general use since, owing to the poor growth obtainable on it, the bulk of enzyme from a given number of Roux bottles is small. The ratio of potency to protein content of enzyme is however higher with this medium than any other.

Excellent results are obtained when the bacteria are grown on Fränkel's synthetic medium [Stephenson, 1930] + 1:10,000 tryptophan, a very potent enzyme resulting.

Media containing pure tryptophan are somewhat expensive, and for most purposes satisfactory results can be obtained by the use of ordinary nutrient agar or Fairchild's peptone agar.

The following results were amongst those obtained when chloroform-killed bacterial suspensions of approximately equivalent cell content were allowed to act on 2 mg. of tryptophan at room temperature at p_H 8.5 for 40 hours. The number of Roux bottles required is given in brackets.

(1) 7.5 ml. of suspension from 1/10,000 tryptophan solidified with agar (18 Roux bottles). Indole formed: 0.65 mg.

(2) 7.5 ml. of suspension from Fränkel's medium *plus* 1/10,000 tryptophan (4 Roux bottles). Indole formed: 0.40 mg.

(3) 7.5 ml. of suspension from ordinary agar ($3\frac{1}{2}$ Roux bottles). Indole formed: 0.11 mg.

In the latest experiments, since it was desirable to obtain enzymes of greater potency, the bacteria have been grown on tryptic digest of caseinogen solidified with agar. A chloroform-killed suspension from 6 Roux bottles of culture producing 0.86 mg. of indole from 2 mg. of tryptophan is a typical finding. This represents almost 80% conversion. Such preparations are certainly much stronger in their ratio potency/protein content than those obtained on Fränkel's medium containing tryptophan 1/10,000; they are less powerful in this sense than those grown on tryptophan solidified with agar.

Experiments were done to determine if the presence of tryptophan in the medium is essential for the elaboration of the indole-producing mechanism. In one experiment a living suspension of *B. coli* from Fränkel's synthetic medium aerated under toluene with tryptophan for 36 hours completely failed to produce indole whilst a parallel suspension from ordinary agar did so.

In two later experiments, however, done with chloroform-killed suspensions from Fränkel's medium, some activity was obtained, in one case the potency being 1/10 and in the other 2/5 of the potency of parallel suspensions from Fränkel's medium + 1:10,000 tryptophan.

Preparation of chloroform-killed bacterial suspensions.

Bacteria are grown overnight on large Roux bottles of suitable medium. The bacteria are then washed off with saline, centrifuged, the supernatant liquid discarded and the deposit resuspended in saline, 10 ml. of saline being used for every 10 Roux bottles (average surface area 250 cm.²). The suspension is then extracted successively with light petroleum (B.P. 40–60°) until completely free from indole (about 4 or 5 extractions are usually necessary).

Chloroform is added to the indole-free suspension in the proportion of 1 vol. of chloroform to 2 vols. of suspension. The mixture is then shaken vigorously in a mechanical shaker (200 r.p.m.) for 4 hours. This has always been in our experience sufficient to sterilise. The emulsion is subcultured to verify its sterility, and used at once, no attempt being made to separate out the chloroform. Preparations made in this manner are very active, but should be used immediately since they show some deterioration on keeping.

The following is a typical result obtained with an enzyme preparation made in this way. (A similar method has been used by Gordon and Cooper [1932] in the preparation of bacterial phosphatase.)

Action of chloroform-killed suspensions of B. coli on tryptophan.

Flasks were set up as below, and continuously aerated at room temperature under toluene for 40 hours.

Flask (1): 7.5 ml. bacterial suspension, 4 ml. 1:2000 tryptophan solution
10 ml. phosphate buffer p_H 8.4.

Flask (2): 7.5 ml. bacterial suspension, 4 ml. water, 10 ml. phosphate buffer p_H 8.4.

Flask (3): 7.5 ml. bacterial suspension (heated at 100° for 5 mins.), 4 ml. 1:2000 tryptophan, 10 ml. buffer p_H 8.4.

Note. 7.5 ml. of suspension were equivalent to the growth of 8 Roux bottles.

Result. Subculture of bacterial suspension: no growth.

Subculture of flasks (1), (2), (3) after aeration: no growth.

Indole content after 40 hours' aeration: (1) 0.20 mg. (2) Nil. (3) Nil.

A note is necessary regarding the accurate estimation of indole in this type of experiment. In estimating by the extraction technique, owing to the large amount of protein material present separation of the liquids may be incomplete, with the result that the final rosindole solutions contain precipitated protein. This precipitated protein should be removed, either by centrifuging or by diluting with 4 vols. of alcohol and filtering, before attempting the colorimetric estimation.

Preparation of dried enzyme.

Bacteria are grown on Roux bottles as above, washed off, centrifuged and resuspended in saline. The suspension is then treated with 66% alcohol (at 0°), filtered through a Büchner filter and the precipitated bacteria are washed with ice-cold absolute alcohol and with ice-cold anhydrous alcohol-ether (1:2) and desiccated *in vacuo*. The dry preparation thus obtained is indole-free and usually sterile and has the advantage that it can be stored without apparent loss of activity. The following experiment illustrates its action on tryptophan.

Action of desiccated B. coli on tryptophan.

Flasks were set up as below and continuously aerated under toluene for 42 hours.

Flask (1): dried bacteria from 7 Roux bottles (0.224 g.), 4 ml. of 1:2000 tryptophan. 20 ml. phosphate buffer p_H 8.4.

Flask (2): dried bacteria from 7 Roux bottles (0.224 g.), 4 ml. water. 20 ml. phosphate buffer p_H 8.4.

Result. Subcultures before and after aeration: no growth.

Indole content: flask (1) 0.110 mg. (2) Nil.

Factors modifying the action of the enzyme preparations.

(1) *Aeration.* Indole formation by living cultures of *B. coli* is considerably affected by the degree of aeration, inadequate aeration resulting in a considerable reduction of the indole formed, and this was found to apply also to the action of killed suspensions.

The following results were obtained when 3.5 ml. of chloroform-killed suspension were allowed to act for 40 hours on 2 mg. of tryptophan at room temperature under toluene, one flask being continuously aerated while a parallel control was placed in a narrow tube plugged with cotton wool and not aerated.

Aerated flask: indole content 0.325 mg.

Unaerated flask: indole content 0.031 mg.

(2) *Reaction.* A number of experiments have been done to determine the effect of p_H on the activity of the enzyme preparation, the preparations being allowed to act on tryptophan in buffered solutions (p_H 2–5 citrate, 5–8.4 phosphate, 8.4–11 borate). The optimum reaction was found to be p_H 8.5. The activity dropped rapidly between p_H 7.5 and 6.5, was almost negligible below

6.0 and finally terminated at 5.0. The alkaline limit was more variable, almost all preparations were active up to p_H 9.5, the majority were inactive above p_H 10.0, but occasional preparations were feebly active up to p_H 11.0. It is probable that the alkaline limit of action is conditioned by the resistance of the cells to lysis by alkali, which resistance may vary with different preparations. The accompanying curve shows a typical result (Fig. 1).

Fig. 1. Effect of p_H on action of 3 ml. chloroform-killed *B. coli* suspension on 2 mg. of tryptophan; flasks aerated at room temperature under toluene for 40 hours.

(3) *Heat resistance.* All our preparations have been completely inactivated by heating to 100° for 5 mins.

(4) *Sodium deoxycholate.* It has been stated above that the addition of 1:1000 sodium deoxycholate to the medium accelerates the production of indole by certain freshly isolated strains of *B. coli*. This is not due to acceleration of action of the tryptophan-splitting enzymes, since the addition of this concentration of deoxycholate to enzyme preparations has a pronounced inhibiting effect on indole production. In one experiment parallel chloroform-killed suspensions were allowed to act on tryptophan in the presence and absence of 1:1000 sodium deoxycholate. In the absence of deoxycholate 0.4 mg. of indole was produced in 48 hours whilst in the presence of deoxycholate only a faint trace of indole was produced.

The effect of deoxycholate on indole production by the living organism must therefore be due to some other factor.

(5) *Effects of chloroform and toluene.* In a recent personal communication Woods suggested that some of the differences between his results and our own were due to differences in potency between his viable suspensions and our enzyme preparations and that chloroform and toluene exerted considerable inhibitory effects although they did not completely inactivate as was suggested in his paper. He pointed out that the yield of indole in his experiments was much higher than in ours. It must be noted however that whereas his experiments were done at 37° ours were done at room temperature. It was decided to extend our study under parallel conditions of the relative potencies of viable suspensions prepared by Woods's technique and enzyme preparations made by our methods, and also to study the effects of chloroform and toluene on these preparations.

(A) *Relative potency of viable suspensions and desiccated enzyme.* Bacteria were grown on Roux bottles of ordinary agar, washed off and divided into equal parts. One part was used to prepare viable suspensions by Woods's technique

and the other to prepare desiccated enzyme. Each preparation was then allowed to act upon 2 mg. tryptophan at room temperature for 48 hours and the indole produced estimated. The average results of 3 experiments were as follows:

Viable suspension from 6 Roux bottles yielded 0.26 mg. indole.

Desiccated enzyme from 6 Roux bottles yielded 0.21 mg. indole.

It appears that the desiccated enzyme is not appreciably less potent than the viable suspensions, the slight difference observed being probably attributable to slow growth of the viable suspensions.

(B) *Effect of chloroform.* Chloroform exerts a definite inhibitory effect upon the desiccated enzyme, the potency of preparations being decreased by 75–80 % when aerated in the presence of a layer of chloroform. Chloroform-killed suspensions show a reduced potency when compared with parallel viable suspensions, but it was not found possible to obtain an accurate comparison of the relative potencies since counts indicated that the viable suspensions were slowly growing and the reduction of potency is variable.

(C) *Effect of toluene.* It has been previously stated that shaking a bacterial suspension with toluene has much more inhibitory effect than shaking with chloroform. It was also found that the desiccated enzyme was very sensitive to toluene, a considerable reduction in its potency being obtained when aerated under a layer of toluene. In some of our original experiments we thought that the desiccated enzyme was inferior in potency to chloroform-killed suspensions and very variable results were obtained when desiccated preparations were used. It has later become evident that this apparent inferiority was really due to the great sensitivity of the desiccated preparation to toluene, and that the irregular results were due to variation in the degree of emulsification of the toluene layer which occurred during aeration. A somewhat unexpected result was obtained when the sensitivity of viable suspensions and chloroform-killed suspensions to toluene was tested. The activity of parallel suspensions with and without a layer of toluene was tested with the result that in both cases the indole production was more than doubled in the presence of toluene. It is suggested that this is due to removal of indole from the aqueous layer by the toluene, with a consequent acceleration of the reaction.

It is to be noted that when washed suspensions and chloroform-killed suspensions are aerated under toluene the toluene layer does not tend to become emulsified, whilst with desiccated preparations considerable emulsification of the toluene layer occurs and washed suspensions shaken with toluene tend to form stable emulsions. It is possible that the inhibitory effect observed with these latter preparations is largely physical.

*Action of the enzymes on some possible intermediates of the
tryptophan-indole reaction.*

Indole-3-aldehyde was prepared according to the original method of Ellinger [1906] and by the later and greatly improved method of Boyd and Robson [1935]. The details of the latter method were kindly supplied to us some time ago by Dr Robson to whom we would express our thanks. Both preparations were indole-free and had m.p. 194° (uncorrected). The enzyme preparation from 24 Roux bottles was made up to 40 ml. with saline and 20 ml. of the suspension were inactivated by boiling. Four aeration flasks were set up as follows and aeration continued for 2 days.

(1) 10 ml. enzyme + 10 ml. buffer p_H 8.4 + 4 ml. 1/2000 tryptophan + 10 ml. toluene.

(2) 10 ml. inactivated enzyme + 10 ml. buffer p_H 8.4 + 4 ml. 1/2000 tryptophan + 10 ml. toluene.

(3) 10 ml. enzyme + 20 ml. saturated solution of indole-3-aldehyde in buffer p_H 8.4 + 10 ml. toluene.

(4) 10 ml. inactivated enzyme + 20 ml. saturated solution of indole-3-aldehyde in buffer p_H 8.4 + 10 ml. toluene.

From the tryptophan present in flask (1) about 30% of the theoretical yield of indole was obtained. No indole was detected in the others.

The concentration of indole-3-aldehyde has been varied considerably but in no single instance has indole been detected.

Growth experiments with indole-3-aldehyde also yielded negative results.

Indolecarboxylic acid was prepared both according to the method of Ciamician and Zotta [1888] from skatole and by that of Ellinger [1906] from the 3-aldehyde. The yields by both methods were extremely poor, the compounds appeared to be identical and had m.p. 214° (uncorrected). The four flasks set up with the aldehyde were duplicated with the carboxylic acid, 10 ml. of a saturated solution of the acid in phosphate buffer p_H 8.4 were used. Indole was not detected.

β -*Indoleacrylic acid* was prepared according to the method of Bauguess and Berg [1934]; the yield was again poor, m.p. 195° (uncorrected). The acid was tested with the enzyme preparation as above. The amount used was 10 ml. of a saturated solution of the acid in phosphate buffer. No indole production was detected.

Through the courtesy of Mr Woods who sent us specimens of indolepyruvic indolepropionic and indoleacetic acids we were enabled to examine the action of our enzyme upon these substances. No indole was produced from quantities of 2 mg.

DISCUSSION.

The name "tryptophanase" is suggested for the complete system of enzymes which induce and catalyse the production of indole from tryptophan and which are present in killed suspensions of *B. coli*. In our experiments we have restricted the concentration of tryptophan so as to obtain yields of indole which could be extracted and estimated without too much labour. An increase in the concentration of tryptophan in the system considerably increases the total amount of indole produced, though the percentage of tryptophan converted into indole is decreased. The greatest indole production has been obtained using a chloroform-killed suspension of *B. coli* which was grown on trypsinised caseinogen solidified with agar. The growth from 6 Roux bottles converted 80% of 2 mg. tryptophan into indole when aerated at laboratory temperature under toluene. The same suspension converted 42% of 5 mg. tryptophan into indole. It will have been noted that although *B. coli* grown on synthetic media free from tryptophan is not entirely deficient in tryptophanase the activity is considerably reduced, and that the most active preparations are those which have been grown on media with a high concentration of free tryptophan. It seems that the development of tryptophanase by the organism is controlled in part by the concentration of free tryptophan in the media. It is hoped to discuss this in a subsequent communication since we have reason to believe that we are dealing with an adaptive enzyme system.

With cultures grown on ordinary nutrient agar indole production has been greatest with washed viable suspensions of *B. coli* aerated with tryptophan under a layer of toluene. There is no doubt that under these conditions, whilst the suspensions may remain viable for a period greater than the duration of the

experiment, the death rate so exceeds the growth rate that viable counts at the end of the experiment are in the region of thousands per ml. as against thousands of millions in the absence of toluene. In many experiments the suspensions under toluene appear to be sterile. The maximum indole production from 2 mg. tryptophan using suspensions from 6 Roux bottles of ordinary agar under toluene was 0.58 mg. or a 50 % conversion. In the absence of toluene the yield was approximately 23 % of the theoretical. Indole is extracted from the aqueous phase by the toluene and it may be this removal of indole from the reacting system which accounts for the enhanced production under toluene.

It remains to explain the apparent inactivation of suspensions shaken with toluene at high speeds. Such suspensions form a very stable toluene-water emulsion and a coating of this emulsion probably interferes in a physical sense with the action of the enzymes. It has been noted that the dried preparation acting without toluene is approximately as effective as the chloroform-killed suspensions under toluene but that its activity is reduced in the presence of toluene. This has been discussed; the chloroform-killed suspensions remain separated in the aqueous phase whereas the dried product forms an apparent foam in a toluene-water emulsion. Woods obtained complete inactivation of his suspensions by shaking with chloroform and toluene, but it seems evident from our results that these substances cannot be regarded as enzyme poisons, although the potency of suspensions is reduced by shaking with chloroform and more markedly by shaking with toluene.

Working with viable suspensions, Woods showed that indole was not produced from indole-lactic, -propionic, -acrylic, -pyruvic, -acetic and -carboxylic acids, or from indole-3-aldehyde, and that whilst small amounts of indole were produced from the pyruvic acid in the presence of ammonia or other source of nitrogen this was probably due to synthesis of tryptophan which was then broken down. The preparations of tryptophanase which we have elaborated have similarly failed to produce indole from these derivatives (with the exception of indolelactic acid which has not been tested), so that in our present state of knowledge it is not possible to suggest a mechanism for the breakdown of the tryptophan side chain, and in particular the promising hypothesis of breakdown *via* the acrylic acid is not supported by experimental evidence.

SUMMARY.

1. Killed preparations of *B. coli* which convert tryptophan into indole have been obtained. The name tryptophanase is suggested for the enzyme system found in these preparations.

2. The tryptophanase is active between p_H 5.0 and 10 with an optimum at p_H 8.5.

3. The tryptophanase does not produce indole from indolepropionic indole-acetic, indoleacrylic, indolepyruvic or indolecarboxylic acid or from indole-3-aldehyde.

4. Tryptophanase is present in *B. coli* grown on synthetic media free from tryptophan, but the potency of preparations is much increased when the bacteria are grown on media with a high tryptophan content; the maximum potency is obtained when the bacteria are cultivated on a medium in which tryptophan forms the sole source of nitrogen.

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