

CCVII. THE PRODUCTION OF INDOLE BY SUSPENSIONS OF *BACT. COLI*

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WOODS [1935, 1] has studied the conversion of tryptophan into indole by washed suspensions of *Bact. coli*. He found that the process was an oxidation requiring 5 atoms O per mol. tryptophan. The conversion of tryptophan to indole was complete and the rate of disappearance of tryptophan was the same as the rate of formation of indole. The coli suspensions used by Woods were prepared from growths on trypsin-digested casein solidified with agar, and thus had always been produced in the presence of tryptophan. Happold & Hoyle [1935] also studied the oxidation of tryptophan and found on two out of three occasions that slight activity was present in suspensions grown in the supposed absence of tryptophan on Fraenkel's synthetic medium. Since this medium, however, contained natural asparagine [Dr Happold, private communication] it is possible that tryptophan might have been present as an impurity in biologically significant amounts. It was therefore decided to test the production of the enzyme under conditions in which tryptophan was certainly absent.

Technique

(1) *Medium used for growth of the suspensions.*

KH_2PO_4	4.5 g.
$(\text{NH}_4)_2\text{SO}_4$	0.5 g.
NH_4Cl	0.5 g.
$M/2$ Na lactate	50 ml.
N NaOH	26 ml.
Water to	950 ml.

This mixture was adjusted to pH 7.6, distributed in 9.5 ml. volumes in tubes and autoclaved.

The following solutions were prepared separately: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4% (autoclaved); ferrous ammonium sulphate $M/500$ in $N/50$ HCl (filtered); Na dithiodiacetate $M/200$ (filtered).

These were mixed in the proportions Mg 1.0, Fe 2.5, dithioacetate 2.0, and 0.5 ml. added per tube. The dithiodiacetic acid was prepared as stated by Fildes & Richardson [1937]. Na lactate was prepared from three times recrystallized Zn lactate.

The mixtures, having been prepared in the test tubes and inoculated were poured into 50 ml. Erlenmeyer flasks and incubated at 37° in air + 5% CO_2 .

All apparatus was treated in the usual way for excluding the presence of unknown substances.

(2) *The strains used.* Two laboratory cultures maintained on agar and four recent isolations were used. These gave the fermentation reactions of *Bact. coli*, *Bact. coli communis* and *Bact. acidi lactici*. All grew copiously after little hesitation on the medium described and in serial subculture from one drop of the previous culture in 10 ml. All produced indole rapidly when tryptophan

was added to the culture medium. Though some of the observations to be described were checked on more than one strain, a single strain was used as the standard. This was obtained from the cultures of the Bland-Sutton Institute and was described as No. 86 of the National Collection of Type Cultures. After 20–24 hr. growth the cultures were centrifuged and the supernatant liquid discarded. The bacteria were washed once with distilled water and suspended to a standard opacity in *M*/18 phosphate buffer (*pH* 7.6). The dry weight of bacteria from 1 ml. of this suspension was 1.5 mg.

(3) *Estimation of activity of the suspensions.* The bacterial suspension (7 ml.) was mixed in a 100 ml. Erlenmeyer flask with *M*/18 phosphate at *pH* 7.6 (12.5 ml.) and 0.5 ml. 0.175% tryptophan, and incubated at 37°. The quantity of tryptophan used represented a possible maximum yield of indole of 25 µg./ml. Woods used four times this concentration.

The indole produced in the mixture was estimated by a simple method which appeared to be sufficiently accurate and gave results comparable with those of Woods.

A standard solution of indole was prepared containing 20 µg./ml. and from this a series of 10 dilutions with 25% differences. One ml. of each dilution was mixed with 1 ml. of Ehrlich's indole reagent, prepared freshly from a sample of *p*-dimethylaminobenzaldehyde as colourless as possible. This reference series was remade after 4 hr.

The suspensions to be tested were centrifuged and the supernatant liquid diluted with water. One ml. of each dilution was mixed with 1 ml. Ehrlich reagent and after a few minutes tested against the reference series. The average of the estimates obtained from three or four dilutions was taken as the correct reading.

Various indole derivative tests by this technique at concentrations equivalent to 20 µg. indole/ml. gave negative results, namely indoleacrylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid. Indolecarboxylic acid gave a mere trace of colour which was due to indole which the preparation contained.

The activity of suspensions of bacteria grown without tryptophan

Each of the six strains grown under the conditions stated actively converted tryptophan into indole. Fig. 1 shows examples of the action of different suspensions of one strain (No. 86, N.C.T.C.) grown on different occasions. No. 86 was equally active after 40 subcultures since its last contact with tryptophan.

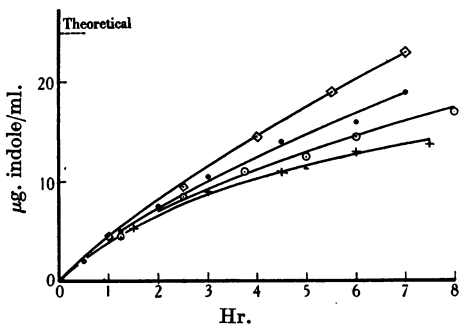


Fig. 1.

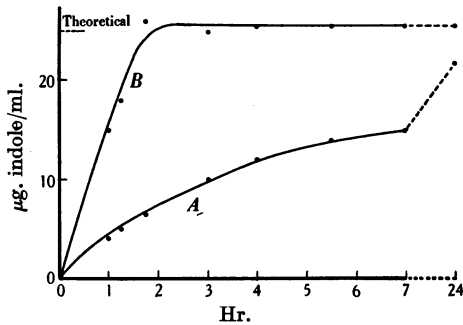


Fig. 2.

Fig. 1. Rate of production of indole by suspensions grown without tryptophan.

Fig. 2. Rate of production of indole by suspension *A* grown without tryptophan, compared with *B* grown with tryptophan.

The effect of growth in the presence of tryptophan

Parallel cultures were put up, one containing 0.5 ml. 0.175% tryptophan per 10 ml. The suspensions were gathered after 22 hr. and gave the results shown in Fig. 2. The presence of the tryptophan did not increase the yield of the culture. Fig. 2 shows that growth in the presence of tryptophan much increased the activity of the suspension.

That the effect of growth in tryptophan is quantitative rather than qualitative is shown in Fig. 3. The suspension from a tryptophan culture was diluted serially with buffer until the initial activity was equal to that of the suspension grown without tryptophan. The course of the curves was exactly the same. In this experiment it was found that the activity of two suspensions could not be estimated from the initial courses of the curves. According to these it was assumed that the tryptophan culture was about five times more potent whereas it had to be diluted twenty-five times before its activity was the same as that of the culture grown without tryptophan.

These results obtained when tryptophan was present in the culture were in general confirmation of those of Woods and indicated that the less elaborate method used for estimating indole did not lead to any notable error.

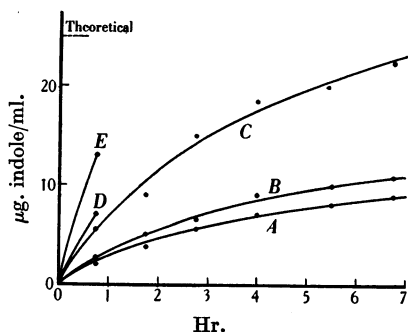


Fig. 3.

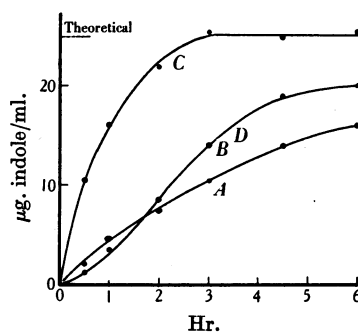


Fig. 4.

Fig. 3. Comparative activity of suspensions grown without tryptophan (A) and with tryptophan. B=tryptophan suspension diluted 1/25; C=1/6; D=1/4; E=undiluted.

Fig. 4. Effect of growth in glucose. A grown in lactate; B in glucose; C in lactate + tryptophan; D in glucose + tryptophan.

The effect of growth in glucose on the activity of the suspensions

It is a well-known phenomenon that the presence of glucose in a tryptophan-containing medium inhibits the production of indole by colon bacilli growing therein. Happold & Hoyle [1936] have concluded that this is due to a non-production of the "tryptophanase" enzyme in bacteria growing in the presence of glucose.

To test this possibility, flasks of the standard medium were prepared without, however, lactate; other ingredients were added as follows:

A, Medium + $M/2$ lactate 0.5 ml.

B, Medium + $M/2$ glucose 0.5 ml.

C, Medium + $M/2$ lactate 0.5 ml. + 0.175% tryptophan 0.5 ml.

D, Medium + $M/2$ glucose 0.5 ml. + 0.175% tryptophan 0.5 ml.

The cultures were grown for 20 hr. Those containing glucose were found to have given a smaller yield than those with lactate and thus the four suspensions

to be tested were made 1/3 the usual concentration. The centrifuged liquid of flasks *B* and *D* still contained glucose. Flask *C* contained quantities of indole equiv. 100% conversion of tryptophan. Flask *D* contained traces of indole, < 1 $\mu\text{g./ml.}$ and approximately 100% of unconverted tryptophan. The presence of glucose in the culture had therefore almost entirely inhibited the production of indole. Fig. 4 shows the action of the washed suspensions on tryptophan. It will be noticed that the suspension grown in glucose, after a preliminary delay which appears from other experiments to be constant, showed at least as much activity as that grown in lactate and that the presence of glucose had entirely inhibited the excess activity due to the tryptophan.

Thus it is possible to distinguish two "fractions" of activity in suspensions grown in the absence and presence of tryptophan.

(a) One fraction which is always present in *Bact. coli*, which does not depend for its production upon the presence of tryptophan in the external medium and which is unaffected by glucose therein. This fraction may be looked upon as "constitutive" in the sense of Karström [1938].

(b) Another fraction which is produced as a result of tryptophan in the external medium, which action is inhibited by the presence of glucose therein. This fraction may be called "adaptive".

It may readily be shown colorimetrically that the washed bacteria contain tryptophan which they have synthesized from NH_3 and thus it is possible that both fractions of activity result from an action of tryptophan.

The effect of indole upon the activity of suspensions

Three suspensions were made from cultures grown without added tryptophan. To each was added tryptophan to make a final conc. equivalent to 25 $\mu\text{g. indole/ml.}$ To one was also added $\frac{1}{2}$ equiv. of indole and to another 1 equiv. Fig. 5 shows that the activity of the suspension was entirely inhibited by 1 equiv. indole and largely inhibited by $\frac{1}{2}$ equiv.

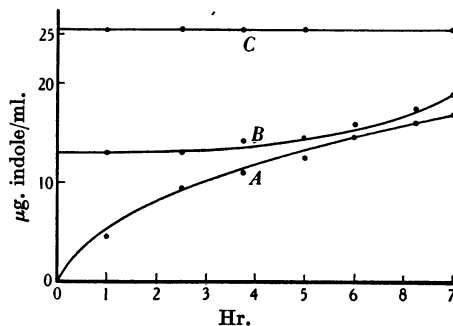


Fig. 5. Inhibition by indole. *A* = action of suspension on tryptophan; *B* on tryptophan + $\frac{1}{2}$ equivalent of indole; *C* on tryptophan + 1 equivalent of indole.

The same result was obtained when suspensions were used from cultures grown in tryptophan, but only when they were diluted to an activity similar to that of a suspension grown without tryptophan. When these suspensions were less diluted, more indole was required to produce a total inhibition. Thus the inhibitory action of indole depends upon the extent of enzyme surfaces present rather than upon the concentration of the substrate. The suspensions which had been proved to be inactive in the presence of tryptophan + indole

were shown to be potentially active by washing and resuspension in tryptophan. None of the indole derivatives already mentioned had any effect on the activity of suspensions under these conditions.

The effect of growth in the presence of indole and derivatives

In view of the effect of tryptophan in increasing the activity of suspensions of bacteria grown in a medium containing this substance, the actions of indole itself and of derivatives were tested.

As has been found by others the presence of indole in a culture is inhibitory to growth. In the present series when indole was added in equivalent concentration to the tryptophan, growth was delayed until the 2nd day and was not normally heavy until the 3rd. With indoleacrylic acid very little growth took place during the first 3 or 4 days but a heavy growth was found on the 4th or 5th. No other indole derivative was found to inhibit. By means of serial subculture in the inhibitory solutions, it was possible to banish the inhibition by indole and to reduce that by indoleacrylic acid so that full growth took place on the 2nd day. In the following experiment therefore the activities of suspensions were tested after 24 hr. growth except in the case of indoleacrylic acid, in which the bacteria were grown for 48 hr. The serial subcultures did not affect the general result of other similar experiments, but allowed a more exact comparison of activity to be made.

All cultures were prepared as before. Indole and its derivatives were dissolved in water or when necessary in *M*/20 NaOH in conc. equiv. 0.175% tryptophan and 0.5 ml. portions of these solutions were added to 10 ml. of medium, after sterilization by Seitz filtration.

The following derivatives were used: skatole, indolecarboxylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid, and indoleacrylic acid.

The suspensions were washed as usual and put up with tryptophan equiv. 25 μg . indole/ml.

Fig. 6 shows that an equal stimulation of the activity of the suspensions had resulted from growth in the presence of indole and indoleacrylic acid though the other compounds had no stimulatory effect.

The production of indole from indoleacrylic acid

It has been shown by Woods [1935, 2], Happold & Hoyle [1935] and others that suspensions of *Bact. coli* are unable to produce indole from indoleacrylic acid or any of the other derivatives mentioned. These results have been confirmed in the present work.

On the other hand in experiments in which the bacteria were grown in the presence of these substances, the result was different. Indole was formed from indoleacrylic acid added to the culture but not from the other derivatives even after 5 days' incubation.

The production of indole from indoleacrylic acid appears to have little relation to the amount of growth which has taken place. For instance in two experiments in which the growth was moderate in 24 hr. as a result of continuous subculture in indoleacrylic acid, the production of indole was only 1 μg ./ml.

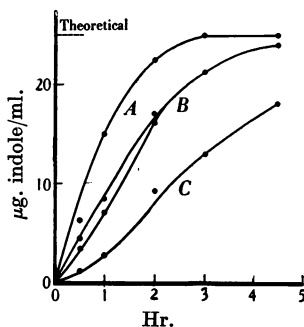


Fig. 6. Activity of suspensions grown in (A) tryptophan; (B) indole or indoleacrylic acid; (C) indole-pyruvic, -propionic, -carboxylic or -acetic acids, or in skatole, or with no addition.

and $< 1 \mu\text{g./ml.}$ (theoretical $50 \mu\text{g./ml.}$). On the 2nd day when growth was full, 2.5 and $4 \mu\text{g./ml.}$ had been produced. In another experiment using a strain unadapted to indoleacrylic acid the relation of growth to indole was as follows:

Days	Growth	Indole $\mu\text{g./ml.}$
1st	0	0.0
2nd	Trace	8.5
3rd	Trace	23.5
4th	+++	33.0

Under similar conditions the production of indole by suspensions grown in tryptophan would have been complete ($50 \mu\text{g./ml.}$) in less than 24 hr.

The mechanism by which the indole is produced is not clear but as a suspension of *Bact. coli* is unable to oxidize this substance directly, it would appear probable that under the conditions of growth *Bact. coli* is able slowly to synthesize tryptophan from indoleacrylic acid and that this is then oxidized to indole.

These results allow the conclusion that, whatever significance may be attached to the stimulation of activity by growth in tryptophan or indole, a similar significance need not be associated with the action of indoleacrylic acid, since this could be ascribed to the indole produced.

All workers are agreed that there is no experimental evidence to suggest that the oxidation of tryptophan to indole involves a passage through any of the theoretically possible intermediates tested. The present results with indoleacrylic acid are of interest in connexion with Raistrick's suggestion [quoted Cole, 1933] that this substance produced by a deamination of tryptophan may be the first step in the production of indole. If, as seems probable, *Bact. coli* can synthesize tryptophan from indoleacrylic acid the reverse process should also be possible, in agreement with Raistrick's idea. In practice it is not possible to demonstrate this action but this may merely be due to the much greater rapidity with which tryptophan is oxidized by another route. In any case the evidence still remains that indoleacrylic acid cannot be oxidized further and therefore this potential deamination of tryptophan can hardly be a step in the production of indole.

It appears to be generally held that the formation of indole from tryptophan probably takes place in steps catalysed by a series of enzymes. On the other hand if the detachment of the indole ring took place in one step under the influence of one enzyme it would be more easy to account for the phenomena noted here. The fact that growth in tryptophan or in indole leads to an increase in the enzyme activity is more in accordance with a view that these are substrate and end-product of one enzyme, rather than substrate and end-product of two different enzymes. Further the fact that indole completely inhibits the action on tryptophan is also reminiscent of the well-known inhibition of enzymes by their own end-products.

SUMMARY

1. Suspensions of *Bact. coli* grown in the absence of tryptophan are always capable of oxidizing tryptophan to indole.

2. When grown in the presence of tryptophan, the activity is some twenty-five times greater.

3. The activity of the former suspensions is unaffected by the presence of glucose in the medium; the excess activity of the latter is entirely inhibited by glucose.

4. The activity of suspensions is inhibited by indole.

5. The activity of suspensions is increased by growth in the presence of indole or indoleacrylic acid. Other indole derivatives are inactive in this respect.

6. Indole is produced during growth in the presence of indoleacrylic acid, but not in the presence of other derivatives.

7. It is suggested that a growing culture can synthesize tryptophan from indoleacrylic acid.

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