34. Indole Formation in *Bacterium coli commune**

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Hopkins & Cole [1903] showed 39 years ago that tryptophan is the parent substance of the indole formed by bacteria. Since then many attempts have been made to elucidate the intermediary mechanism of indole formation [see Frieber, 1922; Saito, 1933; Woods, 1935; Happold & Hoyle, 1935; Baker & Happold, 1940]; all these attempts have been unsuccessful: no intermediates have so far been isolated, nor has a substance related to tryptophan been shown to yield indole. Woods [1935] and Happold and his co-workers [1935; 1940] have tested the indole formation from 17 substances, among them 3-indolepyruvic acid, 3-indolelactic acid, 3-indolepropionic acid, 3-indoleacrylic acid, 3-indoleethylamine, 3-indoleglycine, 3-indolealdehyde, 3-indolecarboxylic acid. All these compounds may be excluded as intermediates.

The list of substances already tested comprises most of the compounds which might be expected to arise from tryptophan assuming that the decomposition starts in the side chain. The negative results obtained with these substances may be taken to suggest that the breakdown of tryptophan does not start in the side chain. This conclusion led us to examine a hypothesis of indole formation not involving a primary breakdown of the side chain. In forming such a hypothesis we were guided by the two following facts:

(1) In certain organisms (rabbit, *B. subtilis*) tryptophan is converted into kynurenine, a reaction which Kotake [1931] and Kotake & Otami [1933] formulate thus:

In this case the decomposition of tryptophan starts in the ring, with the oxidation of the carbon atom 2.

(2) Certain aniline derivatives, e.g. *o*-aminophenylacetaldehyde, or *o*-amino- β -phenylethyl alcohol readily form indole or indole derivatives *in vitro*:

* A brief account of this paper appeared in *Chemistry and Industry*, 1941, 61, 723.

On the basis of these observations we assume that the conversion of tryptophan into indole may start with an oxidation of the ring similar to reaction (1) leading, via oxindole-alanine, to an *o*-aniline derivative similar to or identical with kynurenine; that the side chain of this compound is consequently broken down to *o*-aminophenylacetaldehyde which yields indole according to (2). If this hypothesis, which has already been discussed by Stephenson [1939], is correct, kynurenine and some other *o*-aniline derivatives should yield indole in *B. coli*. We have tested several compounds of this type and among them we found one substance, *o*-amino- β -phenylethanol, to react in the expected manner.

EXPERIMENTAL

Bacteria. The organism used in this work was a strain of *Bacterium coli commune*. It was grown at 37° on tryptic casein digest [Gladstone & Fildes, 1940] containing 2 % or 3 % agar. The period of incubation varied between 18 hr. and 5 days. Cells from young cultures show a high O₂ uptake when no substrate is added; in old cultures the 'endogenous' metabolism is smaller, and this is an advantage in some experiments.

When the period of incubation was completed the cells were washed twice on the centrifuge with 0.9 % NaCl and then suspended in a small volume of 0.9 % NaCl for storage in the refrigerator. For experiments the stock suspension was diluted with a saline medium containing 0.05 *M* phosphate buffer (*pH* 7.4) and 0.45 % NaCl. Unless otherwise stated 3 ml. suspension containing about 25 mg. cells (dry weight) were measured into a conical Warburg cup and shaken at 40° under varying conditions.

Indole determination. The method of Herter & Foster [1905] as modified by Bergeim [1917] and Woods [1935] was used in most experiments. The experiment was interrupted by the addition of H₂SO₄, and an aliquot of the suspension containing about 0.1 to 0.2 mg. indole was analysed. After the addition of Na- β -naphthaquinonesulphonate and alkali to the distillate the mixture was allowed to stand 40 min. before the dye was extracted with CHCl₃.

The method of Fellers & Clough [1925] served to distinguish between indole and dihydroindole (indoline). Indole gives a pink colour, dihydroindole a yellow colour with *p*-dimethylaminobenzaldehyde. The reagent of Herter & Foster (Na- β -naphthaquinone sulphonate) shows no marked differences towards indole and indoline, a pink colour being formed with both compounds.

Substrates. The specimen of *o*-amino- β -phenylethanol used in this work was prepared by Bennett & Hafez [1941]. Dihydroindole was made according to the directions of the same authors. *o*-Aminocinnamic acid was obtained by reduction with FeSO₄ in ammoniacal solution of the respective nitro-compound. *o*-Aminoethylbenzene was prepared from *o*-nitroethylbenzene by treatment with iron filings and HCl.

Indole fermentation from o-amino- β -phenylethanol

It is shown in Table 1 that indole is readily formed from *o*-amino- β -phenylethanol. The rate of this reaction depends on the concentration of the substrate: the highest rate is observed when the concentration is about 6 mg./ml. Very high concentrations inhibit the metabolism. At the optimum concentration the rate of indole formation from *o*-amino- β -phenylethanol is more than three times higher than that from tryptophan. The O₂ uptake is increased by the addition of *o*-amino- β -phenylethanol. In the experi-

Table 1. *Indole formation from o-amino-β-phenylethanol and from tryptophan*

(22.5 mg. *B. coli* in 3 ml. medium; shaken in air for 6 hr. at 40°.)

Substrate added	0	2.5	5.0	10.0	20.0	40.0	0.5	1.0	10.0
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
		o-Amino-β-phenylethanol				Tryptophan			
mg. indole formed	0	0.104	0.389	0.636	1.25	1.17	0.155	0.306	0.416
μl. O ₂ absorbed	615	714	770	915	956	790	746	931	1230

ment quoted in Table 1 the increase is considerably greater than would be expected for the reaction $\text{NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{CH}_2 \cdot \text{CH}_2\text{OH} + \frac{1}{2}\text{O}_2 = \text{indole} + 2\text{H}_2\text{O}$, as will be seen from the last two lines of Table 1. We cannot offer a satisfactory explanation for this discrepancy. Generally the discrepancy appears to be large when the 'blank' respiration is large. In cells from old cultures where the 'blank' is small, the agreement between indole formation and extra O₂-uptake is fairly satisfactory, as seen in Table 2.

Table 2. *Indole formation from o-amino-β-phenylethanol in B. coli under varying conditions*

Conditions of culture (37°)	mg. cells used (in 3 ml. phosphate buffer)	Period of incubation at 40° hr.	μl. O ₂ used by 3 ml. bacterial suspension		mg. indole	
			With 10 mg. amino-phenyl-ethanol		Found	Calc. from extra O ₂ absorbed
			Blank			
1. Casein digest; 19 hr.	25	3	806.5	1074	1.5	2.9
2. Casein digest; 19 hr.	23.8	2	590	758	0.705	1.76
3. Casein digest; 2% glycerol; 44 hr.	26.9	3	538	770	0.295	2.43
4. Casein digest; 2% glycerol; 5 days	27.1	4	232.5	318	0.554	0.895
		6	297.5	417	1.107	1.242
5. Broth; 2% glycerol; 5 days	26.3	3	251	282	0.443	0.472
		6	420	450.5	1.005	0.320
6. Casein digest; 5 days	27.8	2	33	82.5	0.635	0.518
		4	61	169	1.253	1.13

Table 3. *Course of O₂ uptake and of indole formation in the presence of o-amino-β-phenylethanol*

(13 mg. *B. coli* in 3 ml. medium; 40°.)

Time hr.	No substrate μl. O ₂ absorbed	4 mg. o-amino-β-phenylethanol	
		μl. O ₂ absorbed	mg. indole formed
1	90.5	135	0.085
2	157	224	0.159
4	258	361	0.333
22	700	772	1.34
30	794	820	1.48
48	927	881	1.74

The time curve of indole formation and O₂ consumption is shown in Table 3. It will be noted that the rate of O₂ uptake is increased in the earlier stages of the experiment, but it falls below that of the control in the later stages. This inhibition may be due to the indole formed; we find that a concentration of 0.5 mg. indole per ml. inhibits the 'blank' O₂ uptake of *B. coli* by 25%.

The formation of indole from *o*-amino-β-phenylethanol was confirmed by Ehrlich's test, by the nitroso-indole test [see Zoller, 1920] and by the characteristic odour.

No indole was formed when the conditions were anaerobic. The tests for dihydroindole were negative aerobically and anaerobically. Addition of dihydroindole did not cause any indole formation or O_2 consumption. It may be concluded from these observations that reaction (3) does not occur in *B. coli* and that the oxidation is the primary, the ring closure the secondary reaction when *o*-amino- β -phenylethanol is converted into indole. The intermediary compound is expected to be *o*-aminophenylacetaldehyde. This compound has not been described in the literature. Under conditions in which it was expected to arise, indole was found instead [Stephen, 1925]. The aldehyde is unstable and reacts at once to form indole (reaction 2).

We have tested two other ortho-derivatives of aniline, *o*-aminocinnamic acid and *o*-aminoethylbenzene. These did not form indole in *B. coli*.

DISCUSSION

It may be argued that the formation of indole from *o*-amino- β -phenylethanol is not necessarily connected with the formation of indole from tryptophan. *B. coli* oxidizes ethyl alcohol and several of its homologues to the corresponding aldehydes. As the aldehyde arising from *o*-amino- β -phenylethanol is known to yield indole spontaneously, this alcohol might be expected to form indole on oxidation; the indole formation would in this case be 'accidental' and different from the mechanism of indole formation from tryptophan.

We prefer to assume that there is a link between the two indole-yielding reactions. We believe that indole is formed when, or because, a substrate yields *o*-amino- β -phenylacetaldehyde in the course of its decomposition. *o*-Amino- β -phenylethanol is not necessarily an intermediate in indole formation from tryptophan; in fact, its rather slow rate of reaction at low concentrations is against this. But the corresponding aldehyde is, in our view, an intermediate. This hypothesis has the advantage of explaining the various types of tryptophan breakdown (formation of kynurenine in the rabbit and *B. subtilis* [Kotake, 1931], of kynurenic acid in the dog, of anthranilic acid in *B. subtilis* [Kotake & Otami, 1933], of indole in *B. coli*) as modifications of one major scheme. In every case the primary step is the formation of an oxindole derivative followed by ring opening; the further breakdown of the side chain then differs from case to case. Removal of 2H atoms leads to kynurenine; a further oxidation, as suggested by Kotake [1931], leads to $NH_2.C_6H_4.C(OH):CH.CO.CO_2H$ and thence by ring closure to kynurenic acid; it is not difficult to draw up a series of reactions leading to *o*-amino- β -phenylacetaldehyde, though at the present this scheme would have to be entirely hypothetical.

The hypothesis also explains the failure of indole derivatives other than tryptophan to yield indole and is in accordance with the conclusion of Baker & Happold [1940] that the breakdown of tryptophan to indole requires an intact side chain.

SUMMARY

It is shown that *o*-amino- β -phenylethanol yields indole in *B. coli*, if O_2 is available. The primary step is probably the formation of *o*-aminophenylacetaldehyde. This reacts spontaneously to form indole.

The following hypothesis accounts for the experimental observations so far available. The biological breakdown of tryptophan begins with an oxidation of carbon atom 2' of the indole ring leading to an oxindole derivative; this is followed by a ring opening yielding an *o*-aniline derivative; in the case of indole-forming organisms the side chain of the *o*-aniline compound is broken down to *o*-aminophenylacetaldehyde, whence indole arises spontaneously.

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