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The Synthesis of Indole by Washed Cell Suspensions of *Escherichia coli*

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The biosynthesis of the aromatic amino acids by micro-organisms has been mainly investigated by studies on the nutrition of normal and mutant cells and the accumulation of intermediate compounds in culture media. Recently several of the reactions concerned in aromatic synthesis in *Escherichia* and *Aerobacter* have been examined in detail with cell-free extracts (Kalan & Srinivasan, 1955; Mitsuhashi & Davis, 1954; Yaniv & Gilvarg, 1955; Yanofsky, 1955).

During an investigation of the formation of indole by bacteria it was found that washed-cell suspensions of certain mutants of *Aero. aerogenes* and *Esch. coli* would form aromatic compounds from glucose and ammonium salts. The formation of indole, anthranilic acid and an unidentified indole-like compound has been briefly reported by Gibson, Jones & Teltscher, 1955. This paper concerns the formation of indole.

MATERIALS AND METHODS

Strains of Esch. coli used. These (Table 1) were mutant strains derived originally from *Esch. coli* 518. They were obtained after ultraviolet irradiation and penicillin selection by the technique described by Davis (1948). Strains were maintained by serial subculture on heart-infusion agar.

Glassware and chemicals. Glassware was cleaned with a chromic acid cleaning mixture. The chemicals were the purest obtainable commercially; no attempt was made to purify them further.

Media. Two media were used for the routine preparation of cell suspensions.

(a) *Heart-infusion agar.* To 1 l. of tap water were added 500 g. of minced defatted ox heart, 10 g. of Evans peptone and 5 g. of NaCl. After steaming for 1 hr. the broth was filtered, the pH adjusted to 8.4, and the liquid autoclaved (15 lb./in.² for 20 min.) and re-filtered. Agar (1.5%) was added, and after autoclaving at 15 lb./in.² for 20 min. to dissolve the agar the pH was adjusted to 7.2–7.4. The medium was then autoclaved at 15 lb./in.² for 20 min.

(b) *Synthetic medium.* The medium used was that described by Davis & Mingioli (1950). Glucose was added with the other ingredients before autoclaving. Double-strength liquid medium dispensed in 7.5 ml. volumes was sterilized at 10 lb./in.² for 10 min. and added aseptically to an equal volume of sterile molten 3% agar.

Preparation and use of cell suspensions. Agar plates (4 in. diam.) were inoculated by spreading over them 0.2 ml. of a light (approx. 4×10^6 cells/ml.) suspension of organisms in distilled water. The inoculum was taken from a heart-

Table 1. *Strains of Esch. coli*

Mutant no.	Strain	Growth requirement*	Parent strain
—	518	None	—
1	7-4	Tryptophan	518
2	T2-15	Tryptophan (10^{-4} M, DL) plus serine (10^{-3} M, DL) or plus glycine (10^{-3} M)	7-4

* Supplement needed for growth in minimal medium (Davis & Mingioli, 1950), and concentration used during present experiments.

infusion agar slope incubated for 24 hr. at 37°. The inoculated plates were incubated for 16 hr. at 37° and the cells washed off with 0.9% NaCl solution. For cells grown on heart-infusion agar 10 ml. of saline was used per plate, and for synthetic medium agar, 5 ml. of saline. The cells were washed twice with saline and resuspended in 0.2M phosphate buffer, pH 7.7 ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$). The density of the suspension was measured turbidimetrically and adjusted to approx. 1.5 mg. dry wt./ml. Freshly harvested cells were used for each experiment.

Tests for indole synthesis were carried out by adding a suspension of organisms prepared as described to an equal volume of the mixture of substrate solutions to give a final volume of 5 ml. in 6 in. $\times$ $\frac{3}{4}$ in. tubes. The tubes were then incubated in a water bath at 37°.

Generally no increase in turbidity was observed in the course of an experiment, indicating that no growth was taking place. In a few experiments some increase in turbidity was observed which, however, did not exceed 50% of the original turbidity. For example, in experiments with mutant 1, when tryptophan and MgSO_4 were included in the synthesis system the turbidity increased by about 50%.

Assay of indole. Indole was estimated by a modification of the method of Wood, Gunsalus & Umbreit (1947). To 6 ml. of toluene was added 0.5 ml. of sample. After shaking for 2 min., 3 ml. of the toluene layer was transferred to a fresh tube and 1 ml. of a 5% solution of *p*-dimethylaminobenzaldehyde in ethanol added, followed by 1 ml. of acid alcohol (500 ml. of ethanol plus 40 ml. of conc. H_2SO_4). The colour was allowed to develop for 12–15 min. and the absorption compared with a reagent blank by means of an EEL portable colorimeter with $\frac{1}{2}$ in. tubes and a 626 m μ . filter. A standard curve was prepared with known concentrations of indole. Results are expressed in terms of μm -moles of indole formed/mg. dry weight of cells.

Confirmation of identity of indole. The formation of indole was confirmed by growth tests with mutants responding to indole, and by comparison of the ultraviolet absorption spectra of experimental samples with those of solutions of indole (Gibson *et al.* 1955).

RESULTS

Mutant 1 required tryptophan for growth (Table 1). Since tryptophan cannot be replaced for this organism by indole or anthranilic acid the pathway of tryptophan synthesis is presumably blocked between indole and tryptophan. This has been confirmed by experiments with washed-cell suspensions of mutant 1, which failed to form tryptophan from indole plus serine, although the same suspensions did form indole from tryptophan.

'Cross-feeding' experiments with the technique described by Gibson & Jones (1954) indicated that mutant 1 accumulated indole in culture, supporting the growth of strains requiring indole.

Washed-cell suspensions of mutant 1 formed indole from glucose and ammonium chloride. Examination of a number of other strains in which tryptophan synthesis was apparently blocked at the same stage as that of mutant 1 showed that there was a constant correlation between the ability of

a strain to 'cross-feed' indole-requiring strains in culture and the ability of washed-cell suspensions to form indole.

The amount of indole formed by suspensions of mutant 1 varied greatly when the cells were harvested from different media. The experiments to be described have therefore been grouped according to the media from which the test cells were harvested.

Indole synthesis by cells grown on heart-infusion agar

When suspensions of mutant 1, harvested from heart-infusion agar, were incubated with glucose and ammonium chloride, indole was formed in considerable amounts (Table 2). Ammonium chloride could be replaced by other ammonium salts such as ammonium nitrate or ammonium sulphate. The optimum concentration of glucose was 0.1M, and that of ammonium chloride 0.005M. Preliminary experiments showed that with 0.1M phosphate buffers the optimum initial pH for indole synthesis lies in the range 7.5–8.0. There was a considerable drop in pH during the course of the experiments, for example from pH 7.7 to 6.5–7.0.

Many amino acids when added to the glucose–ammonium chloride medium had no significant effect upon indole synthesis by mutant 1, but several inhibited the synthesis and one, serine, stimulated it (Tables 2 and 3).

Serine therefore might be essential for some reaction on the pathway of indole synthesis, and, if so, mutant 1 must synthesize some serine from glucose and ammonium chloride. It appeared that by using a strain of *Esch. coli* which could not form serine from these substrates it would be possible to control the amount of serine available to the cells. Irradiation of mutant 1, followed by penicillin selection, gave mutant 2, which required for growth, in addition to tryptophan, either serine or glycine

Table 2. *Effect of serine on indole synthesis by mutant 1*

Suspensions were incubated for 4 hr. at 37° with substrates in 0.1M phosphate buffer, pH 7.7. In this and all further experiments shown, the amount of cells present was within the range 0.64–0.80 mg. dry wt./ml. MgSO_4 (10^{-4}M) was added to substrates for cells harvested from synthetic medium. +, Compound was added to suspension; –, compound was not added.

Substrate	Cells harvested from					
	Heart-infusion agar			Synthetic medium		
Glucose (0.1M)	+	+	+	+	+	+
NH_4Cl (0.005M)	–	–	+	–	+	+
DL-Serine (0.01M)	–	–	+	–	–	+
Indole formed (μm -moles/mg. dry wt.)	39	188	286	<10	238	325

(Table 1). Mutant 2 presumably could not form serine except from glycine.

In contrast with mutant 1, mutant 2 did not form any more indole from glucose and ammonium

Table 3. *Effect of amino acids on indole formation by Esch. coli 7-4*

Amino acids added to give 0.01M final concentration to glucose (0.1M) and NH_4Cl (0.005M) in 0.1M phosphate buffer, pH 7.7. Summary of several experiments. Incubation time, 3-5 hr. at 37°.

Amino acid	Effect
DL-Serine	Variable stimulation (15-100%)
DL-Alanine, L-arginine, DL-aspartic acid, DL-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-methionine, DL-proline, DL-tyrosine, DL-valine	No stimulation or inhibition less than 10%
L-Cysteine, DL-glutamic acid, glycine, DL-homoserine, DL-lysine, DL-phenylalanine, DL-threonine, DL-tryptophan	Inhibition, greater than 10%

Fig. 1. Effect of serine, glycine, threonine and homoserine on indole synthesis by cells of mutant 2 grown on heart-infusion agar. Cells were incubated with substrates as shown, in 0.1M phosphate buffer, pH 7.7, for 5 hr. at 37°.

chloride than from glucose alone (Fig. 1). When serine was added indole was formed in considerable amount. DL-Serine was used in the experiments described and D-serine was found to be inactive.

No other amino acids would completely replace serine for indole synthesis by mutant 2, and the only others which were at all effective were glycine, threonine and homoserine, in that order. The amounts of indole formed in the presence of serine and the three amino acids mentioned above are shown in Fig. 1. Under the conditions shown in Fig. 1 the omission of ammonium chloride did not affect indole formation when DL-serine was present. Serine was rapidly deaminated and could therefore act as a source of ammonium ions. For optimum synthesis with glycine as substrate, ammonium ions were essential and they were therefore included in all experiments. The effects of the four amino acids have been examined in greater detail with cells harvested from synthetic medium and are described below.

In Fig. 2 the amount of indole formed by mutant 1 with serine present is plotted against time. The curve shows an initial lag phase followed by a period in which indole is formed at a constant rate. The length of the lag period and the constant rate of formation varied somewhat in different experiments, but were of the order of 2 hr. and 100 μm-moles of indole formed/mg. dry weight of cells/hr., respectively.

Fig. 2. Rate of indole synthesis by mutant 1. Curve 1: cells from heart-infusion agar incubated at 37° in 0.1M phosphate buffer, pH 7.7, with glucose (0.1M), NH_4Cl (0.005M) and DL-serine (0.01M). Final vol., 17 ml. Curve 2: cells from synthetic agar incubated as above with the further addition of MgSO_4 (10^{-4}M). Final vol., 10 ml.

Indole synthesis by cells grown on synthetic medium

Cell suspensions of mutant 1 harvested from synthetic medium containing tryptophan (10^{-4} M) formed less indole in a given time than did suspensions harvested from heart-infusion agar. Indole synthesis was stimulated by the addition of cell extracts, obtained as the supernatants after treating heavy cell suspensions of mutant or wild-type *Esch. coli* with boiling water and centrifuging. Various mixtures of vitamins or amino acids failed to replace the cell extracts, which could, however, be replaced by magnesium salts. A number of salts were tested to see if they would also stimulate indole formation. From the results shown in Table 4 it can be seen that, at the concentrations tested, magnesium and manganous ions both stimulated indole synthesis, and zinc and cobalt caused inhibition. At a concentration of 10^{-5} M, magnesium stimulated synthesis much more effectively than any other metal. In some experiments Fe^{2+} ions stimulated indole formation. The concentrations of Mg^{2+} ions needed to stimulate synthesis are shown in Fig. 3.

As with cells grown on heart-infusion agar, indole formation by mutant 1 was stimulated by serine (Table 2) and there was a lag period before a constant rate of synthesis was reached (Fig. 2).

For the growth of mutant 2 either serine or glycine, in addition to tryptophan, had to be added to the synthetic medium (Table 1). Cells harvested from either a glycine or a serine medium failed to form indole from glucose and ammonium chloride, but would do so if serine was added. Other amino acids were tested to see if they would replace serine, and again only glycine, threonine or homoserine would promote indole formation.

Table 4. Effect of metals on indole synthesis by mutant 1 harvested from synthetic medium

Salts as shown were added to indole synthesis system consisting of cells, glucose (0.1M), NH_4Cl (0.005M) and DL-serine (0.01M) in 0.1M phosphate buffer, pH 7.7. Incubation time, 4 hr. at 37° . In the control no salt was added and the indole formed was 133 $\mu\text{m-moles/mg.}$

Salt added	Indole formed ($\mu\text{m-moles/mg.}$) in the presence of salt added to final concn. of	
	10^{-5} M	10^{-4} M
CaSO_4	117	121
$\text{AlK}(\text{SO}_4)_2$	117	111
Na_2SO_4	117	122
ZnSO_4	122	10
CoSO_4	42	9
$\text{Fe}_2(\text{SO}_4)_3$	138	132
FeSO_4	142	144
MnSO_4	136	248
MgSO_4	310	316

The amounts of indole formed with different amino acids as substrates were markedly influenced by the medium on which the cells were grown. As can be seen from Fig. 4, with cells harvested from a glycine medium, the effectiveness of serine, glycine, threonine and homoserine as substrates for indole synthesis diminished in that order. With cells grown on a serine medium, serine was most effective in promoting indole formation. Glycine, threonine and homoserine gave rise to only a small amount of indole (Fig. 4).

Fig. 3. Stimulation of indole synthesis by Mg^{2+} ions. Cells from synthetic medium agar incubated at 37° with glucose (0.1M), DL-serine (0.01M), NH_4Cl (0.005M) and MgSO_4 as shown, for 4 hr. in 0.1M phosphate buffer, pH 7.7.

Fig. 4. Serine, glycine, threonine and homoserine as substrates for indole synthesis: mutant 2 grown on synthetic media containing (a) serine or (b) glycine. Basal synthesis system; glucose (0.1M), NH_4Cl (0.005M) and MgSO_4 (10^{-4} M). Cells incubated at 37° in 0.1M phosphate buffer, pH 7.7. Incubation time (a) 5 hr., (b) 4 hr. Concentration of amino acid, 0.01M.

Table 5. *Effect of growth medium and magnesium on indole formation*

Cells were incubated in 0.1M phosphate buffer, pH 7.7, for 4 hr. at 37° with substrates shown.

Agar growth medium	Indole formed in	
	Basal system* (μ m-moles/ mg. dry wt.)	Basal system* +MgSO ₄ (10 ⁻⁴ M) (μ m-moles/ mg. dry wt.)
Heart-infusion	286	336
Heart-infusion + 10 ⁻³ M DL-tryptophan	63	237
Difco heart-infusion	63	246
Difco proteose no. 3	29	382
Difco tryptose	23	248
Difco brain-heart-infusion	152	242
Difco yeast-beef	24	133

* Basal system: glucose, 0.1M; NH₄Cl, 0.005M; DL-serine, 0.01M.

*Indole synthesis by cells grown in
various other complex media*

Early attempts to replace the stock laboratory heart-infusion agar with Difco heart-infusion agar or other Difco media yielded suspensions of cells which formed relatively little indole. This is shown in the results of the experiments summarized in Table 5 (middle column).

It was thought, for reasons which are given below, that the concentration of tryptophan in the growth medium might influence indole formation. Cells harvested from heart-infusion agar to which had been added 10⁻³M DL-tryptophan did form much less indole than cells grown on heart-infusion agar alone, as can be seen from the results shown in Table 5 (middle column).

After the importance of magnesium in indole formation had been established the problem of relatively 'inactive' cells was reinvestigated. It was found (Table 5) that synthesis by 'inactive' cell suspensions, whether they were obtained by growth on Difco media or by the addition of tryptophan or indole to heart-infusion agar, was greatly stimulated by magnesium.

DISCUSSION

The results presented show that indole is formed from glucose and ammonium salts by washed-cell suspensions of a mutant strain of *Esch. coli* (mutant 1) unable to convert indole into tryptophan. The fact that cell suspensions will form indole from these simple precursors indicates that their use may be of value in the study of aromatic synthesis.

Serine, or a product of serine metabolism other than glycine, is probably concerned with some stage

in indole synthesis. Several experimental findings support this suggestion. First, serine alone of the amino acids tested stimulated indole formation by mutant 1. Secondly, with another mutant (2) isolated from mutant 1 it was possible to limit the amount of serine available to the indole-synthesis system. Suspensions of mutant 2 would not form indole from glucose plus ammonium chloride but required the addition of serine or a related amino acid. If the cells were grown on a synthetic medium containing serine, only serine was effective in promoting active indole formation. Cells harvested from a glycine medium would use in decreasing order of effectiveness serine, glycine, threonine and homoserine as substrates for indole synthesis. As suspensions of cells of *Esch. coli* requiring serine or glycine for growth readily form glycine from homoserine or threonine (Gibson & Woods, unpublished observations) the most likely explanation of the activity of the latter two amino acids is that they are converted through glycine into serine, which is the intermediate most closely concerned with indole formation.

The stage at which serine acts as an intermediate in indole formation is unknown, although preliminary experiments on anthranilic acid formation by washed-cell suspensions (Gibson *et al.* 1955) indicate that it is probably before the anthranilic acid stage.

Although no attempts were made during the experiments to prepare cells deficient in metal, the addition of some metals, notably magnesium, was found to stimulate indole synthesis under certain conditions. Knowledge of the stage or stages in indole synthesis at which these metals function must await further work on the individual reactions concerned in the metabolic pathway.

Since cells grown on a glycine synthetic medium were more efficient at using glycine and related amino acids as substrates for indole synthesis than cells grown on a serine medium, it is probable that there is some adaptive process concerned in the conversion of glycine into serine. Similar results have been found with regard to the utilization of serine and glycine as substrates for the methylation of homocysteine (Gibson & Woods, unpublished observations). In addition, the initial increasing rate of indole formation suggests an adaptive process.

One of the reasons for the differing activities of the cell suspensions of mutant 1 harvested from various complex media may be the tryptophan concentration in the medium. Thus the addition of tryptophan to the stock heart-infusion agar gave cells of low activity. It was thought originally that mutant 1 may have produced indole from the tryptophan in the medium, which in turn depressed indole synthesis in a manner similar to that described for

methionine and the synthesis of methionine by cell suspensions of *Esch. coli* (Wijesundera & Woods, 1953; Cohn, Cohen & Monod, 1953). However, the addition of indole to the growth medium stimulated rather than depressed indole synthesis, therefore the effect of tryptophan remains unexplained.

SUMMARY

1. Indole is formed from glucose and ammonium salts by washed-cell suspensions of a mutant strain of *Escherichia coli* (strain 7-4) unable to convert indole into tryptophan.

2. The medium on which the cells are grown influences the amount of indole formed by the harvested cells.

3. Magnesium ions greatly stimulate indole formation by cells grown on either a synthetic medium or on various complex media. Manganous and ferrous ions stimulate to a lesser extent.

4. Serine appears to be concerned in indole formation. This is shown by experiments with cell suspensions of both *Esch. coli* 7-4 and a further mutant (*Esch. coli* T2-15) which requires tryptophan plus serine or glycine for growth.

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Synthesis and Metabolism of some Substances Related to δ -Aminolaevulinic Acid

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In a preliminary communication (Neuberger & Scott, 1953) reasons were given which had led us to believe that δ -succinamidolaevulinic acid might be an intermediate in the biosynthesis of porphyrins. But this succinamido compound, unlike δ -aminolaevulinic acid (ALA) itself, was not converted into porphyrins by the intact cells of *Rhodospseudomonas spheroides*, as observed by Dr J. Lascelles, nor did it show any activity, according to the experiments of Drs E. Dresel and J. E. Falk, in their system of lysed fowl red cells. ALA itself is the immediate precursor of porphobilinogen (PBG), which in turn gives rise to porphyrins (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953). In the present paper further observations with rats are presented which indicate that succinamidolaevulinic

acid is not converted into ALA to a significant extent. Experiments are also reported which indicate that *N*-succinylglycine and *N*- β -carboxypropionylglycine, unlike *N*-acetyl glycine or *N*-propionylglycine, are not converted into glycine in the rat. This suggested that the negative results obtained with succinamidolaevulinic acid might be due to a general inability of the organism to split the succinamido bond. δ -Acetamidolaevulinic acid was therefore synthesized, but biological tests indicate that this compound is not hydrolysed in the body to a significant extent.

It is now assumed that the condensation of glycine and 'active' succinate gives rise in the first place to α -amino- β -oxoadipate, which loses one of its carboxyl groups to yield ALA. Free β -keto acids containing an α -amino group have so far not been prepared, and nothing is known of the stability of compounds of this type. As a preliminary to a possible synthesis of the α -amino- β -keto acid itself, several esters of the acid have been synthesized, and

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