The competition between formation and breakdown of polyacetylene is shown by the general form of the curves. As the medium becomes depleted of glucose, polyacetylene formation becomes slower and is eventually outstripped by the increasing rate of breakdown. Comparison of the steepness of the descending slopes in Fig. 5 suggests that breakdown is more rapid at higher polyacetylene concentrations, and this is confirmed by the results in Fig. 4. Reduction of temperature has several effects, of which the most marked appears to be a lower rate of breakdown, so that a higher maximum concentration of polyacetylenes is eventually attained (Fig. 3).

SUMMARY

1. The polyacetylenic compounds produced by the Basidiomycete B. 841 during growth and in various replacement cultures are formed from glucose; they are not metabolic end-products but can be broken down by the fungus at rates comparable with their rates of synthesis. They are formed in highest yield in replacement cultures supplied with 4% aqueous glucose only, and the extent of their synthesis is reduced when cornsteep liquor (a source of essential growth factors for the fungus) is added.

2. It is suggested that the synthesis of polyacetylenes by the fungus B. 841 represents an alternative metabolic pathway for some intermediate in glucose breakdown, the other metabolic routes of which show a partial requirement for factors present in cornsteep liquor.

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The Synthesis of Indole from Anthranilic Acid by *Escherichia coli*

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It is widely accepted that in *Neurospora* anthranilic acid serves as a precursor of indole in the biosynthetic pathway leading to tryptophan (see reviews by Bonner, 1951; Bonner & Yanovsky, 1951; also Partridge, Bonner & Yanovsky, 1952). Evidence is accumulating that tryptophan synthesis in bacteria occurs in a similar fashion to that in *Neurospora*. Thus anthranilate or indole will replace tryptophan for the growth of *Lactobacilli* (Snell, 1943; Green & Black, 1943; Wright & Skeggs, 1945; Schweigert, Sauberlich, Baumann & Elvehjem, 1946; Schweigert, 1947; Rhuland & Bard, 1952) and for some mutant strains of *Escherichia coli* (Davis, 1951). Further, anthranilate has been found to accumulate in the growth medium of a strain of *Bacterium typhosum* blocked, presumably, between anthranilate and indole (Rydon, 1948). The last worker showed also that the inhibition of growth of *Bact. typhosum* by 4- and 5-methylanthranilates was reversed by anthranilate, indole or tryptophan.

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On the other hand, Rhuland & Bard (1952) failed to show indole or tryptophan synthesis by washed cells of *Lactobacillus arabinosus* which metabolized anthranilate. They suggested that the utilization of anthranilate for growth in this organism may be different from that in *Neurospora*.

In the present paper are described some growth characteristics of mutant strains of *Esch. coli* blocked at various points in the biosynthetic chain to tryptophan, and conditions for obtaining indole synthesis from anthranilate in washed suspensions and extracts.

MATERIALS AND METHODS

Strains of Escherichia coli. Strain M 121–35 was kindly supplied by Dr B. Davis, and the other strains were supplied by Dr J. Monod. The bacteria were maintained on heart maceration–glucose agar. For experimental work they were grown for 16–18 hr. in a glucose–mineral broth (Davis & Mingioli, 1950), supplemented with tryptophan unless otherwise stated. Generally the cells were harvested by centrifuging and washed twice with 1% (w/v) KCl.

Enzymic experiments. Experiments with whole cells were carried out in inverted T-tubes with mechanical shaking (Monod, Cohen-Bazire & Cohn, 1951). Reactions with cell-free extracts were performed in stationary tubes under air. All experiments were carried out at 38°.

Preparation of cell-free extracts. Cell-free extracts used in this study were prepared in the Mickle vibrator. In general 11 ml. of bacterial suspension (about 20 mg. dry wt./ml.) was shaken for 20 min. in a cold room at −10° with 5 g. of no. 12 Ballotini glass beads. Under these conditions the temperature of the suspension remained at 0±2° during disintegration. The cells were suspended in a medium containing 1·5% (w/v) KCl, 0·005M potassium phosphate and 0·005M aminotrishydroxymethylmethane (tris) at pH 8·0. Omission of any one of these components resulted in extracts of considerably reduced activity. Addition of pyruvate, thioglycollate or glutathione to the suspending medium did not increase the activity of the extracts.

After disintegration, the mixture was centrifuged for 30 min. at approximately 3000*g* at 0°. Under the conditions used the supernatant contained 1·4–1·6 mg. of N/ml. and possessed all the enzymic activity of the disintegrate. The supernatant was used either as such or after dialysis against 1·5% (w/v) KCl + 0·005M phosphate. Undialysed extracts lost about 50% of their activity overnight at 2°, but dialysed extracts retained almost full activity for 24 hr. at 2°.

The activity of extracts depended markedly on the method by which the cells were grown. The most active preparations were made from bacteria grown with limiting tryptophan and incubated until the amino acid was exhausted. Cells harvested before tryptophan had been completely utilized gave extracts of very low or no activity.

Special chemicals. Anthranilic acid was recrystallized twice from water and gave a single diazotizable spot on paper chromatograms. Calcium pyridoxal phosphate, purity 70%, was obtained from the California Foundation for Biochemical Research. It was used as such after it had been established that the small amount of calcium did not affect the reactions under study. Two samples of adenosine triphosphate (ATP) were used: (*a*) disodium ATP, 98% pure, from Pabst Laboratories, and (*b*) barium ATP, about 75% pure, prepared according to LePage (1949) and converted into the sodium salt by precipitating the barium from an acid solution with sulphate.

Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN), both 98–100% pure, were from Pabst Laboratories.

Analytical methods

Indole and anthranilate. After precipitation of protein with trichloroacetic acid and centrifuging, the supernatant was made alkaline and extracted with two volumes of toluene. Indole was determined in a sample of the toluene layer by the method of Umbreit, Wood & Gunsalus (1946), and diazotizable matter in a sample of the water layer (Bratton & Marshall, 1939). Colour intensities were determined with the Klett–Summerson photoelectric colorimeter.

Total nitrogen. The method of Johnson (1941) was used.

Bacterial nitrogen. This was determined from the turbidity in the Muenier photoelectric colorimeter, which had been calibrated against known concentrations of bacteria.

Absorption spectra. These were determined with the Beckman model DU spectrophotometer.

Chromatography. Ascending chromatography was carried out on Whatman no. 1 paper, with butanol or *iso*pentanol, both saturated with water, as solvents. Spots were revealed by spraying with Ehrlich reagent (Tabone & Robert, 1948) or by exposing the papers to nitrous fumes and spraying with a mixture of equal parts of 0·5% ammonium sulphamate and 0·5% *NN*-diethyl-*N'*-α-naphthylpropylenediamine.

RESULTS

Growth characteristics of mutant strains of Esch. coli.

The growth requirements of the *Esch. coli* mutants used in this study are summarized in Table 1. *N*-Acetylanthranilate, *N*-methylanthranilate, phenylglycine and *o*-aminoacetophenone were examined also as possible intermediates in tryptophan biosynthesis, but they did not support the growth of any mutants.

A bacterial mutant may, during growth, liberate intermediates in the synthesis of the compound which it requires for growth, further metabolism of these intermediates being prevented by the enzymic deficiency of the mutant. The culture supernatants were therefore examined for the presence of materials reacting in the Ehrlich or diazotization tests. The accumulation experiments were carried out in media containing limiting concentrations of tryptophan. The compounds described below accumulated in significant amounts only towards the end of growth and the best yields were obtained when cultures were incubated for 3–4 hr. after exhaustion of tryptophan. The results are summarized in Table 1.

Table 1. *Growth requirements and accumulations by* Esch. coli *wild types and mutants*

Strain	Growth requirements	Accumulations*
W	None	Trace of indole
ML	None	Trace of indole
M 121–35	Anthranilate, indole or tryptophan	None
M 19–2	Indole or tryptophan	Anthranilate
B 37	Indole or tryptophan	Anthranilate
ML 304g	Tryptophan	BDC† and trace of indole
ML 328e	Tryptophan	BDC† and indole

* Diazotizable or Ehrlich-positive material after 16 hr. at 37° in Davis medium supplemented with $1·5 \times 10^{-5}$M L-tryptophan.

† See text.

Strains B 37 and M 19–2, which grew on either indole or tryptophan, produced large amounts of a diazotizable compound in their culture supernatants. This compound was extracted by ether from culture media at pH 6 but not at pH 9, supported the growth of M 121–35, and had the

same spectrum in the ultraviolet as anthranilate. The colour produced in the Bratton–Marshall test had the same spectrum as that produced by anthranilate. The compound moved as anthranilate on paper chromatograms and was inseparable from anthranilate when mixtures were chromatographed.

Mutant ML 328e, which grew only on tryptophan, produced large amounts of a compound with the properties of indole, and a substance which reacted in the Bratton–Marshall test to give a blue compound with an absorption maximum at 620 mμ. compared with 545 mμ. for anthranilate. Compounds with this property will be referred to as BDC. Strain ML 304g, with similar growth requirements, produced BDC, but only traces of indole, which may have arisen from a slight breakdown of tryptophan.

Table 2. *Growth substance in ML 304g growth supernatant*

The growth supernatant from ML 304g was extracted once with an equal volume of toluene and twice with ether to remove indole. Samples were then: *A*, sterilized by filtration; *B*, heated with 0·05 N-HCl at 100° for 10 min., neutralized and sterilized by filtration; *C*, sterilized by autoclaving. Each fraction was tested against mutants of *Esch. coli* in the manner described by Hirsch & Cohen (1953).

Fraction 1·5 ml. added to 5 ml. of test medium	Growth after 16 hr. (Muenier units)		
	B 37	M 19–2	M 121–35
A	0	0	0
B	0	29	28
C	0	121	116

Attempts to isolate BDC have so far been unsuccessful. It was extremely labile in acid solution, being destroyed within 10 min. at room temperature and pH 5. Partial destruction occurred at neutral or alkaline pH at 100–120°: it was stable for several weeks at 2° at neutral pH. The substance was not extracted by ether, toluene, *iso*-pentanol or butanol at neutral pH and was destroyed by shaking with chloroform. It was not precipitated by magnesium, barium, cuprous or mercuric ions.

Autoclaved growth-supernatants of ML 304g and ML 328e, from which indole had been removed by extraction with toluene and ether, supported the growth of M 121–35 and M 19–2 (Table 2). The growth factor responsible was largely destroyed by acid under conditions that also destroyed BDC. The growth factor was apparently accumulated in a labile, non-utilizable form, since supernatants sterilized by filtration were inactive unless boiled for about 10 min. No attempt has been made to isolate the growth factor or establish its identity or non-identity with BDC. No anthranilate was detected in supernatants of ML 328e or ML 304g.

Indole synthesis by washed suspensions

Washed suspensions of M 121–35, ML 304g, ML 328e and wild types W and ML metabolized anthranilate aerobically in the presence of glucose. B 37 and M 19–2 did not metabolized anthranilate. Only with ML 328e was indole formation demonstrated. With M 121–35 and wild types indole accumulation was prevented by a secondary conversion of indole into unknown products (Trudinger & Cohen, 1956), which was about ten times as

Table 3. *Metabolism of anthranilate by washed cells of ML 304g and ML 328e*

Basic system contained 0·02 M tris, pH 7·8, and 100 μmoles of glucose in a volume of 5 ml. Incubated 1 hr. with aeration.

Expt.	Strain (mg. of N)	Changes in conditions	Changes in Anthranilate (mμmoles/ 5 ml.)	Changes in Indole (mμmoles/ 5 ml.)	Changes in BDC (Klett units)*
1	ML 304g (3·5)	None	—	0	+100
		+1 μmole of anthranilate	−1000	0	+770
		+0·5 μmole of anthranilate	− 500	0	+310
		+0·5 μmole of anthranilate; no glucose	− 55	0	+ 10
2	ML 328e (1·5)	None	—	+ 13	—
		No glucose	—	+ 10	—
		+1 μmole of anthranilate	− 600	+176	—
		+1 μmole of anthranilate; no glucose	− 60	+ 40	—
3	ML 328e (0·9)	None	—	+ 12	—
		+50 μmoles of phosphate	—	+ 19	—
		+1 μmole of anthranilate	− 510	+190	—
		+1 μmole of anthranilate +50 μmoles of phosphate	− 670	+100	—
4	ML 328e (3·0)	None	—	+ 39	0
		+1 μmole of anthranilate	−1000	+350	+410
		+1 μmole of anthranilate *in vacuo*	− 100	0	0

* Klett units after diazotization, with the 540 mμ. filter.

rapid as anthranilate removal. B 37 and M 19–2 also metabolized indole but the reaction could not be demonstrated with either ML 328e or ML 304g.

Washed suspensions of ML 304g produced no indole from anthranilate, but instead a BDC, similar to that found in growth media of this strain, accumulated (Table 3). No growth factor for M 121–35 or M 19–2 was formed. Washed suspensions of ML 328e also produced BDC as well as indole. Anthranilate could be estimated in the presence of BDC after destruction of the latter with acid. However, the optical density at 545 mμ. of diazotized mixtures of anthranilate and BDC was up to 20% less than the sum obtained when each was diazotized alone, and varied with the ratio of anthranilate to BDC. Thus a quantitative study of BDC formation could be made only when little or no anthranilate remained in the reaction mixture.

Formation of indole and BDC from anthranilic acid by ML 328e and ML 304g is shown in Table 3. Other experiments showed that the optimum concentration of glucose was $1–2 \times 10^{-2}$M. Phosphate increased slightly the rate of anthranilate removal but decreased indole production. Other experiments indicated that phosphate increased the yield of BDC at the expense of indole. The small amounts of indole and BDC formed in the absence of anthranilate were probably due to synthesis of anthranilate by the washed suspensions (see Trudinger & Cohen, 1956). BDC was not formed from indole by washed suspensions of either ML 304g or ML 328e.

The formation of indole by ML 328e exhibited a slight lag period compared with anthranilate removal. BDC formation paralleled indole formation. In tris buffer, pH 7·8, about 50% of the anthranilate removed was converted into indole. Although indole production lagged behind anthranilate production no diazotizable materials other than anthranilate were detected chromatographically (cf. extracts below). Microbiological tests for intermediates with M 121–35, M 19–2 and B 37 were also negative.

Indole synthesis by cell-free extracts

Effect of ATP. ATP was essential for the conversion of anthranilate into indole by freshly prepared extracts. The concentration of ATP for maximum activity was about $2·5 \times 10^{-3}$M. At higher concentrations of ATP the rate of indole production fell markedly (Table 4). This was apparently not due to impurities, since two samples of ATP from different sources gave the same concentration pattern. ATP combines strongly with a number of metal ions (Raaflaub, 1954, 1955; Cohn, 1954). However, the inhibition of indole synthesis by ATP could not be reversed by the addition of either $MgCl_2$ or $MnCl_2$ up to 10^{-2}M.

Inorganic phosphate. Although the enzyme preparation contained phosphate, the activity was increased by the further addition of inorganic phosphate (Table 5). After dialysis for 2 hr. against 1·5% (w/v) KCl the system was completely dependent on phosphate. The optimum concentration was $0·7–1·0 \times 10^{-2}$M. As with ATP, relatively small increases in phosphate above the optimum reduced markedly the rate of indole synthesis.

Table 4. *Effect of ATP on indole synthesis*

Reaction mixture contained 0·025M tris and 0·01M phosphate both at pH 7·95, 0·75% (w/v) KCl, 0·25 μmole of anthranilate, 25 μmoles of $MgCl_2$, 25 μmoles of α-oxoglutarate, 0·25 μmole of pyridoxal phosphate and an undialysed extract of ML 328e (2 mg. N) in a volume of 5 ml. Incubated 2 hr.

ATP concn. (mM)	Indole formed (mμmoles)
0	15
1·25	65
1·75	200
2·50	225
3·75	190
5·00	65

Table 5. *Effect of phosphate on indole synthesis*

Reaction mixture contained 12·5 μmoles of ATP and variable amounts of inorganic phosphate. Other conditions as for Table 4.

Phosphate added (mM)	Indole formed (mμmoles)	
	Undialysed enzyme	Dialysed enzyme*
0	—	0
2·5	60	20
5·0	120	58
7·5	135	98
10·0	125	104
12·5	60	—
15·0	—	73
17·5	30	—
20·0	—	40

* 2 hr. against 1·5% (w/v) KCl.

Metals. The freshly prepared enzyme had an absolute requirement for magnesium or manganous ions (Table 6). The optimum requirements were about $1·5 \times 10^{-3}$M and $2·5 \times 10^{-4}$M respectively. At concentrations four times as great the activity was reduced. In the experiment cited two different enzyme preparations were used. Other experiments have shown that at their optimum concentrations both manganese and magnesium produced the same activity. Cobalt also stimulated and, at its optimum concentration, was about one-half as active as either Mn^{2+} or Mg^{2+}.

Table 6. *Effect of* Mg^{2+} *and* Mn^{2+} *on indole synthesis*

Conditions as for Tables 4 and 5 except that $MgCl_2$ was replaced by $MnCl_2$ or its concentration varied. The enzyme was not dialysed.

$MgCl_2$ (mM)	Indole formed (mμmoles)	$MnCl_2$ (10^{-4} M)	Indole formed (mμmoles)
0	0	0	0
0·5	60	0·5	8
1·0	100	1·5	55
1·5	120	2·5	95
2·5	120	5·0	95
5·0	120	7·5	83
10·0	98	10·0	65

Carbon compounds. Experiments with anthranilate labelled with ^{14}C in the carboxyl group have demonstrated that the carboxyl group of anthranilate is lost during its conversion into indole (Nyc, Mitchell, Heifer & Langham, 1949). Two carbon atoms are therefore required in the transformation. Freshly prepared extracts were active in the absence of added carbon donors, but were stimulated by the addition of either pyruvate or members of the tricarboxylic acid cycle. After 2 hr. dialysis the activity was markedly reduced, but was increased by a number of compounds, of which α-oxoglutarate, pyruvate and oxaloacetate were the most effective. Malate, fumarate, succinate,

Table 7. *Effect of pyridoxal phosphate and anthranilate on indole synthesis*

Conditions as for Tables 4 and 5 except that the concentrations of pyridoxal phosphate and anthranilate were varied; enzyme was dialysed 2 hr. against KCl–phosphate. Results expressed as mμmoles of indole formed.

Expt.	Pyridoxal phosphate (molarity)	Anthranilate (molarity) 0	5×10^{-5}	10^{-4}	$1{\cdot}5 \times 10^{-4}$	2×10^{-4}
1	4×10^{-5}	0	160	135	90	55
2	2×10^{-5}	—	105	—	45	—
	4×10^{-5}	—	145	—	70	—
	8×10^{-5}	—	148	—	90	—
	$1{\cdot}6 \times 10^{-4}$	—	125	—	105	—
	$2{\cdot}4 \times 10^{-4}$	—	95	—	107	—

Pyridoxal phosphate and substrate. The activity of freshly prepared extracts was stimulated three- to four-fold by the addition of pyridoxal phosphate, but after 2 hr. dialysis against KCl–phosphate an absolute requirement was observed. Maximum activity was obtained when the concentrations of substrate and pyridoxal phosphate were of the same order (Table 7, Expt. 2). At 4×10^{-5} M pyridoxal phosphate the rate of indole synthesis was reduced when the anthranilate concentration was increased above 5×10^{-5} M (Table 7, Expt. 1). This fact, with the relatively high concentration of pyridoxal phosphate required for optimum activity, suggested an interaction between the co-enzyme and substrate similar to that described by Jakoby & Bonner (1953) for a number of other amino compounds. That this was so is indicated in Expt. 2 of Table 7. With $1{\cdot}5 \times 10^{-4}$ M anthranilate about four times the concentration of pyridoxal phosphate was required for optimum activity as with 5×10^{-5} M anthranilate. Further, at the lower concentration of anthranilate, high concentrations of pyridoxal phosphate depressed the rate of indole synthesis. It should be noted, however, that at higher anthranilate concentrations the rate of indole synthesis in the presence of an optimum concentration of pyridoxal phosphate was still significantly less than that at low substrate levels.

Table 8. *Effect of carbon compounds on indole synthesis*

Conditions as for Tables 4 and 5 except that α-oxoglutarate was replaced by other carbon compounds. Expts. 1 and 2: enzyme dialysed 2 hr. against KCl–phosphate. Expt. 3: enzyme dialysed 24 hr.

Expt.	Additions	Indole formed (mμmoles) No DPN	5×10^{-4} M DPN added
1	None	10	—
	α-Oxoglutarate	130	—
	Pyruvate	120	—
	L-Malate	70	—
	Fumarate	72	—
	Oxaloacetate	120	—
2	None	8	—
	α-Oxoglutarate	186	—
	DL-Lactate	40	—
	Succinate	80	—
	Citrate	56	—
3	None	25	20
	α-Oxoglutarate	196	48
	Pyruvate	200	50
	L-Malate	30	120
	Oxaloacetate	195	—
	Citrate	25	22
	DL-Lactate	22	20
	Succinate	25	—

citrate and lactate were less active, and ethanol, formate and acetate were inactive (Table 8). After dialysis for 24 hr. only the three keto acids were active by themselves, the activity being almost the same as with fresh extracts in their presence. Malate stimulated after the addition of DPN. In only a few cases has it been possible to reduce to zero the activity in the absence of added carbon donors, indicating the presence in most extracts of a poorly diffusible or bound carbon donor. The optimum concentration for α-oxoglutarate was about 5×10^{-3} M (Table 9), and that for pyruvate was about the same. In some preparations pyruvate was less effective than α-oxoglutarate; the latter was therefore generally used in these experiments.

Table 9. *Effect of α-oxoglutarate concentration on indole synthesis*

Conditions as for Tables 4 and 5 except that the concentration of α-oxoglutarate was varied; enzyme dialysed 24 hr. against KCl–phosphate.

α-Oxoglutarate (mM)	Indole formed (mμmoles)
0	2
0·5	20
1·0	80
2·0	105
3·0	113
4·0	118
5·0	120
10·0	120

Table 10. *Effect of cocarboxylase on anthranilate metabolism*

Conditions as for Tables 4 and 5 except in Expt. 2, where anthranilate concentration was 6×10^{-4} M; enzyme undialysed.

Additions	Expt. 1 mμmoles of indole/5 ml.	Expt. 2 mμmoles of 'anthranilate'/5 ml.	
		No acid	Acid-treated*
None	200	2630	2945
Pyruvate (25 μmoles)	205	2620	3000
Cocarboxylase (10 μg./ml.)	203	2650	3040
Pyruvate + cocarboxylase	110	1300	3000

* Heated for 10 min. at 100° after addition of HCl and before addition of nitrite in the Bratton–Marshall test.

Effect of DPN and cocarboxylase. With pyruvate or α-oxoglutarate as carbon donor indole formation was inhibited by the addition of 5×10^{-4} M DPN (Table 8). DPN also reduced the rate of removal of diazotizable material and did not promote removal of indole by the extracts. The rate of indole production in the presence of either α-oxoglutarate or pyruvate and DPN was less than that in the presence of malate and DPN (Table 8); malate also partly reversed the inhibition of indole synthesis in the presence of α-oxoglutarate or pyruvate. The inhibition could not be reversed by increasing the concentration of carbon donors, and did not appear to involve removal of carbon donors by side reactions. The mechanism of inhibition has not been further investigated.

Indole production in the presence of pyruvate but not of α-oxoglutarate was inhibited by the addition of cocarboxylase. In the presence of pyruvate and cocarboxylase anthranilate was very rapidly converted into non-diazotizable material, which after heating with acid regenerated a compound that diazotized and reacted on the chromatogram as anthranilate (Table 10). This suggests that a decarboxylated product of pyruvate reacted with the amino group of anthranilate to render it less available for indole synthesis.

Effect of other cofactors. Extracts dialysed 24 hr. against KCl–phosphate were fully active in the presence of the cofactors already described. No further increase in activity was obtained on the addition of coenzyme A, α-lipoic acid, TPN, vitamin B_{12}, biotin or an extract of boiled *Esch. coli* cells.

Effect of pH. The rate of indole synthesis was optimum between pH 7·9 and 8·2 in tris buffer (Table 11).

Table 11. *Effect of* pH *on indole synthesis*

Conditions as for Tables 4 and 5 except that pH was varied.

pH	Indole formed (mμmoles)
6·50	35
6·82	60
7·25	90
7·75	128
7·95	140
8·22	140
8·70	100
9·00	70

Course of reaction. The course of indole synthesis and removal of diazotizable material with time is shown in Fig. 1. The removal of diazotizable material with time indicated the formation of acid-labile, non-diazotizable compounds. If protein was precipitated at 0° and centrifuging and diazotization were carried out rapidly, low colour values were observed after the reaction had proceeded for 10–15 min. These values were very irregular. After standing 5–10 min. in dilute acid at room temperature the diazotizable material in the reaction samples increased sharply until a stable point was

reached. Values obtained in this manner are plotted in Fig. 1. The removal of diazotizable material proceeded in an irregular fashion, probably owing to the formation of intermediates that reacted in the anthranilate test. Chromatography of samples, taken when indole synthesis was about 30% complete, showed the presence of a material with a lower R_F than anthranilate. This compound has not been identified.

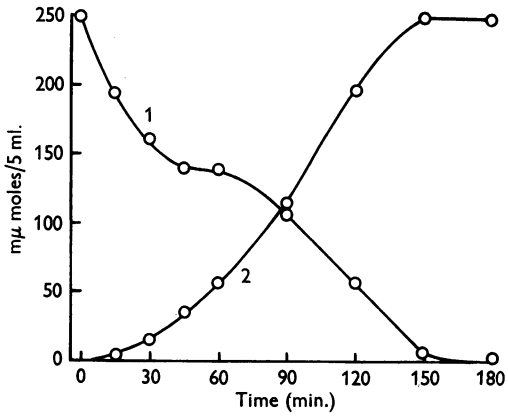


Fig. 1. Course of indole synthesis with time. Conditions as for Tables 4 and 5; undialysed enzyme (2·2 mg. of N). Curve 1: diazotizable material after acid treatment (see text) expressed as anthranilate. Curve 2: indole.

The product of anthranilate metabolism. Insufficient material was available to enable isolation of the product of anthranilate metabolism, but the following properties suggest strongly that the compound was, in effect, indole. The compound was steam-volatile and extractable by toluene, ether, benzene and chloroform under alkaline conditions. The product from a number of tubes was steam-distilled and concentrated from the distillate by extraction with ether. All Ehrlich-positive material was removed by this means, and the following properties were determined on this solution: (1) The compound supported the growth of M121–35, M19–2 and B37, but not of ML328e nor ML304g. The relationship between the colour intensity in the Ehrlich test and growth of B37 was the same as for authentic indole. (2) In the presence of serine and tryptophan desmolase (an aged, dialysed extract of B37) the compound was converted into a substance reacting as tryptophan (i.e. supported the growth of ML328e and ML304g); the tryptophan formed, estimated microbiologically, was equivalent to the Ehrlich-positive material originally present, expressed as indole. (3) The compound had a similar absorption spectrum in the ultraviolet to indole. (4) The absorption spectra of the Ehrlich colours from the compound and indole were identical over the range 400–700 mμ.

Indole formation in other strains of Esch. coli. Anthranilate was converted quantitatively into indole by extracts of ML304g. No formation of BDC was detected. The reaction occurred also in dialysed extracts of M121–35 and wild types W and ML. In undialysed extracts tryptophan was formed instead owing, no doubt, to the presence of serine or serine precursors in the extracts. No activity was found in extracts of M19–2 or B37. The growth studies with ML304g supernatant suggested that M19–2 may be blocked at a different point from that of the other mutant requiring indole or tryptophan. The effect of mixing extracts from the two strains was tried. No indole synthesis or anthranilate removal was obtained in these experiments.

Effect of extracts on BDC. Extracts of ML328e did not synthesize indole when incubated with either fresh or autoclaved solutions of BDC, produced by the action of washed suspensions of ML328e on anthranilate.

Tryptophan desmolase in Esch. coli *mutants.* The results of the growth studies lead to the conclusion that ML328e and ML304g lack tryptophan desmolase, the enzyme condensing indole and serine to tryptophan. This was confirmed by a survey of the enzyme in Mickle extracts of the various strains used. Under conditions where extracts of wild types or other mutants converted 2–3 μmoles of indole into tryptophan in 30 min. neither ML304g nor ML328e allowed significant conversion in 3 hr. These extracts were prepared from cells grown in limiting tryptophan and had little or no tryptophanase activity.

DISCUSSION

The results reported in this paper demonstrate that *Esch. coli* possesses the enzymes necessary for the conversion of anthranilate into tryptophan, with the intermediate formation of indole. Further, the accumulation of anthranilate by growing B37 and M19–2 and its formation by washed cells of B37 (Trudinger & Cohen, 1956) indicate that this compound, or a derivative of it, is an intermediate in the synthesis of indole by this organism. The rapid conversion of anthranilate into acid-labile, non-diazotizable compounds by extracts may mean that free anthranilate is not the actual intermediate in indole formation.

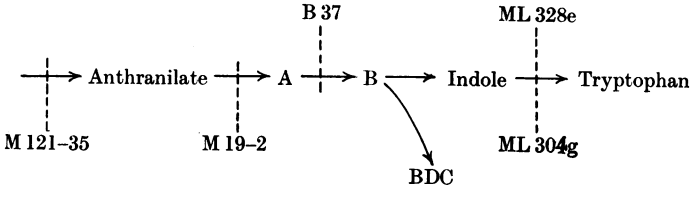
The failure of Rhuland & Bard (1952) to demonstrate indole synthesis from anthranilate in washed suspensions of *Lb. arabinosus* may have been due to further metabolism of indole, which they showed occurred readily in their system. Their incubation times also were relatively short (usually

30 min.) and corresponded to the 'lag' period in indole formation observed for *Esch. coli* in the present work.

Since tryptophan was not formed from indole and serine by washed suspensions, Rhuland & Bard concluded that the condensation reaction is not operative in *Lb. arabinosus*. However, this conclusion, based on results with whole cells, is not justified. Although tryptophan could not be detected when whole cells were incubated with indole and serine (see Trudinger & Cohen, 1956) the condensation occurred readily in extracts of *Esch. coli*.

The position of BDC in the biosynthetic chain is not clear. The following facts indicate that it is a side product of the reaction: (*a*) no growth factor was formed concurrently with its production by resting cells of ML 304g; (*b*) extracts of ML 328e did not synthesize indole from BDC or its autoclaved product; (*c*) no BDC was detected during indole synthesis by extracts. Since BDC is not formed from indole or by B 37, it would appear that BDC is most probably a derivative of a compound formed before indole and after the point at which B 37 is blocked.

Although enzymic studies did not confirm the point, the growth-stimulating properties of ML 304g growth supernatants suggest that M 19–2 is blocked at a different point from B 37. Thus on present evidence the biosynthetic chain may be illustrated by the following scheme (the interrupted lines indicating the metabolic blocks in the mutants):



The results with cell-free extracts suggest that the carbon donor required for the conversion of anthranilate into indole may be a keto acid or a derivative of a keto acid. Although in well-dialysed extracts no metabolism of keto acids could be detected, it should be emphasized that to obtain significant activation of indole synthesis, the keto acids were required at about 20 times the concentration of anthranilate. Thus a sufficient amount of a derivative of the keto acid could be produced without causing a detectable decrease in the keto acid concentration. It is unlikely that α-oxoglutarate acts after prior conversion into pyruvate, since (*a*) preparations were obtained in which α-oxoglutarate was superior to pyruvate, and (*b*) cocarboxylase inhibits the reaction in the presence of pyruvate but not in the presence of α-oxoglutarate.

The inactivity of other members of the tricarboxylic acid cycle in well dialysed extracts and in the absence of other cofactors suggests that if conversion of α-oxoglutarate and pyruvate into a common donor takes place it does not do so via the tricarboxylic acid cycle. Alternatively, the prime reaction may involve an interaction of either keto acid with anthranilate (or a derivative). The removal of excess of carbon atoms could then occur after the addition, and would obviate the necessity for prior metabolism of the keto acids. Since this work was completed Yanovsky (1955*a*, *b*) has reported that extracts of *Esch. coli* synthesize indole from anthranilate, utilizing as a carbon donor 5-phosphoribosylpyrophosphate or a derivative of this compound. That a two-carbon unit may be involved in the transformation has been suggested by Harley-Mason (1955) by analogy with the chemical combination of *N*-methylanthranilate with glycollic aldehyde to form *N*-methylindole.

The low production of anthranilate, indole and BDC during the early stages of growth of *Esch. coli* mutants was apparently due to an inhibition of the biosynthetic chain by tryptophan. Growth-factor levels of the amino acid (10^{-5} M) almost completely inhibit the synthesis of anthranilate by washed suspensions of *Esch. coli* (Trudinger, unpublished results). Wright & Skeggs (1945) have also obtained evidence that the rate of tryptophan synthesis in growing *Lb. arabinosus* is reduced in the presence of an external supply of the amino acid. These findings lend support to the proposal of Novick & Szilard (1954) that the rate of amino acid biosynthesis in the bacterial cell is conditioned by the internal concentration of amino acids other than by simple mass-action effects.

SUMMARY

1. Anthranilate, indole and a compound (BDC) that gives a blue colour in the test for anthranilate have been detected in the growth media of some *Esch. coli* mutants blocked at various stages in the biosynthesis of tryptophan.

2. Washed cells of two mutants blocked between indole and tryptophan synthesized indole or BDC or both when incubated with glucose and anthranilate.

3. An enzyme system converting anthranilate into indole has been extracted from *Esch. coli*. The reaction required ATP, inorganic phosphate, pyridoxal phosphate, Mg^{2+} or Mn^{2+} and a carbon donor. Pyruvate and α-oxoglutarate were the most effective of the donors studied.

4. The implication of these results on the problem of tryptophan biosynthesis is discussed.

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The Effect of 4-Methyltryptophan on Growth and Enzyme Systems of *Escherichia coli*

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During the study of the mechanism of tryptophan synthesis by *Escherichia coli*, the possibility of using 4-methyltryptophan (4-MT) as a blocking agent was examined. This compound has been reported to inhibit competitively the utilization of tryptophan for protein synthesis in *Bacterium typhosum* (Fildes & Rydon, 1947). However, certain discrepancies between the results of these workers and our own led to a more detailed analysis of the inhibition and, in particular, the effect of 4-MT on enzyme systems.

The results of this work are reported in this paper.

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MATERIALS AND METHODS

The strains of *Esch. coli*, general growth conditions and the methods for indole and anthranilic acid estimation have been reported in the preceding paper (Trudinger, 1956).

Cell-free tryptophan desmolase was prepared from *Esch. coli* strain ML, grown 16 hr. in aerated mineral medium (Davis & Mingioli, 1950). The bacteria (about 200 mg. dry wt.) were suspended in 10 ml. of 3% (w/v) KCl and shaken for 30 min. at 0° with 5g. of no. 12 Ballotini glass beads in the Mickle vibrator. The supernatant, after centrifuging for 15 min. at approx. 3000 *g*, was used in undialysed form. It contained no tryptophanase activity.

Cell-free tryptophanase was prepared in a similar manner, except that the bacteria were grown in a broth consisting of 1% peptone, 0·5% yeast extract (Difco) and 10^{-3} M L-tryptophan in distilled water.