Studies on the Inhibition of Induced Tryptophanase Synthesis in *Escherichia coli* by the Simultaneous Presence of Fermentable Carbohydrate and Aromatic Amino Acids during Growth

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The failure to produce indole by cultures of *Escherichia coli* in the presence of fermentable carbohydrate (glucose) is due to the non-production of the tryptophanase system (Happold & Hoyle, 1936). In simple salt media, glucose only partly inhibits the production of indole from tryptophan (up to about 80%), but complete inhibition is obtained by the addition of either phenylalanine or tyrosine in certain stoichiometrical relationships to the tryptophan present (Evans, Handley & Happold, 1941). No other amino acid acts in this way, and phenylalanine or tyrosine is ineffective in the absence of fermentable carbohydrate. The problem can therefore be resolved into two questions: (a) the mechanism of glucose inhibition and (b) the part played by the aromatic amino acids.

Three approaches were chosen to this problem: (1) The Michaelis constant of tryptophanase was measured in the presence and in the absence of aromatic amino acids plus glucose, by using *E. coli* cells with an already developed tryptophanase and cell-free extracts containing tryptophanase. (2) The disappearance of tryptophan from resting suspensions of *E. coli* was measured in the presence and in the absence of the aromatic amino acids with glucose also present. (3) The disappearance of L-tryptophan from growing cultures of *E. coli* was measured in the presence and in the absence of aromatic amino acids with glucose as the only other carbon source and ammonium sulphate as the nitrogen source.

MATERIALS AND METHODS

Organism; *Escherichia coli* NCTC 5928. This organism is no longer kept in the National Collection, but is still maintained in the Leeds Department of Biochemistry. It was subcultured on nutrient-agar slopes: 0.3% of Difco yeast extract, 1.0% of Bactopeptone, 0.5% of NaCl, 1.5% of agar adjusted to about pH 7.0 with N-NaOH.

Culture media. The simple salt medium used was: $(\text{NH}_4)_2\text{SO}_4$, 2 g./l.; KH_2PO_4 , 9 g./l.; glucose, 1.0 g./l.; sufficient 5N-NaOH to bring the pH to 7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g./l. The $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was added after pH adjustment and sterilization. Sterilization was effected by boiling for 15 min., leaving for 24 hr. at room temperature and boiling for a further 15 min.

Cells with preformed tryptophanase activity. The following medium was used: KH_2PO_4 , 9 g./l.; pancreatic digest of casein, 4 g./l.; sufficient 5N-NaOH to bring the pH to 7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g./l.) was added after sterilization. After growth overnight at 35° with aeration, the cells were centrifuged and washed four times with 100 ml. of phosphate buffer (9 g./l.; pH 7) to remove all residual indole and tryptophan.

Michaelis constant. A suspension of cells with tryptophanase activity was diluted so that 1 ml. produced 30–40 μg . of indole from 10 μmoles of L-tryptophan/3 min. at pH 7.8 and 35°; 1 ml. of this suspension was added to 9 ml. of reaction mixture and incubated at 35° for 3 min., and the reaction stopped by the addition of 5 ml. of 10% trichloroacetic acid. Indole was estimated by the method of Happold & Hoyle (1934), modified by Scott (1961). All the reaction mixtures contained KH_2PO_4 at a concentration of 9 g./l., sufficient NaOH to maintain a pH of 7.8, 0.4% of glucose and L-tryptophan (0.1–1.0 mm). L-Tyrosine, when present, was at a concentration of 1.0 mm.

Uptake of tryptophan by resting suspension of *Escherichia coli*. Cells were grown on the simple salt medium with aeration, centrifuged, washed and resuspended in the same volume of fresh simple salt medium containing glucose (0.1%), but no $(\text{NH}_4)_2\text{SO}_4$. Radioactive DL-tryptophan ($[3\text{-}^{14}\text{C}]\text{indolylalanine}$) was added, samples of the mixture were taken at intervals and centrifuged, and the radioactivity of the supernatant was measured at infinite thinness (Ormerod, 1956). This was repeated with L-tyrosine (1 mm) and with DL-phenylalanine (1 mm) in the medium. The concentration of DL-tryptophan was 0.05 mm (specific activity 22.9 $\mu\text{Ci}/\text{mg}$.) and the cell concentration 1100×10^6 cells/ml.

L-Tryptophan and indole patterns in growing cultures. The simple salt medium with various additions of tryptophan and aromatic amino acids was used. As an inoculum, 200 ml. of such a fully grown culture was centrifuged, and the cells were resuspended in 20 ml. of buffer (9 g. of $\text{KH}_2\text{PO}_4/\text{l}$, pH 7 with 5N-NaOH); 10 ml. of this suspension was added to 1990 ml. of simple salt growth medium, to give a cell concentration of 80×10^6 cells/ml. Incubation was at 35° with aeration (15 l. of sterile air/hr./2 l. of medium). Additions of L-tyrosine were made before sterilization of the main culture medium. L-Tryptophan was added aseptically just before the addition of the inoculum.

Samples of culture were collected by siphon at 15 min. intervals and centrifuged at 10000g for 5 min., and the supernatants stored at 15° and analysed next day for indole and tryptophan by the method of Scott (1961). Growth was followed turbidimetrically (Hilger Spekker, neutral and blue filters). There was a lag of about 15 min., followed by logarithmic growth (mean generation time

54 min.) to a stationary population of 1200×10^6 cells/ml. The various additions of amino acids had no detectable influence on the growth curve.

Tryptophanase activity. Acetone-dried cells (prepared according to Dawes, Dawson & Happold, 1947) were extracted with water in the cold for 1 hr. (0.2 g. of cells/10 ml. of water) and centrifuged to give a clear, straw-coloured extract. The extract was diluted about 20 times to give an approximate concentration of 0.3 mg. of protein/ml. (determined on the original extract by the biuret reaction, by using crystalline bovine serum albumin as the standard). Reaction mixtures consisted of 1 ml. of enzyme extract, 1 ml. of aqueous pyridoxal phosphate (200 μ g./ml.) and 5 ml. of phosphate buffer (9 g. of KH_2PO_4 /l., to pH 7 with 5N-NaOH) containing 100 μ g. of L-tryptophan. Enzyme and pyridoxal phosphate were added as one solution (2 ml.), which was prepared by incubating together equal volumes of aqueous pyridoxal phosphate and enzyme extract for 10 min. at 35°. Reaction mixtures were incubated at 35° for 5 min., and the reaction was stopped by the addition of 5 ml. of 10% trichloroacetic acid and 1 ml. of 1% dimedone (in ethanol). Indole was determined by the method of Scott (1961). Activities are expressed as μ g. of indole/min./mg. of protein.

Table 1. *Michaelis constant of tryptophanase*

Reaction mixtures (10 ml.) contained 90 mg. of KH_2PO_4 and 40 mg. of glucose, and the pH was adjusted to 7.8 with NaOH. L-Tyrosine, when present, was 1.0 mM. L-Tryptophan concentrations were as shown. Whole cells with tryptophanase activity or a cell-free extract of such cells previously incubated with pyridoxal phosphate (33.3 μ g./mg. of protein) were used. Incubation was for 3 min. at 35°. The reaction was stopped by the addition of 5 ml. of 10% trichloroacetic acid, and the indole determined according to Scott (1961).

Conditions	Concn. of L-tryptophan (mM)	Indole (μ g./3 min.)	Michaelis constant (mM.)
Whole cells, no tyrosine	0.1	11.25	0.256
	0.2	17.5	
	0.3	21.5	
	0.4	24.4	
	0.5	26.25	
	1.0	31.25	
Whole cells, with tyrosine	0.1	15.2	0.263
	0.2	24.4	
	0.3	29.4	
	0.4	33.3	
	0.5	28.5	
	1.0	42.6	
Whole cells, with tyrosine	0.1	9.0	0.25
	0.2	13.7	
	0.3	17.4	
	0.4	18.9	
	0.5	21.3	
	1.0	25.0	
Cell-free extract, no tyrosine	0.1	12.7	0.256
	0.2	19.6	
	0.3	23.8	
	0.4	27.7	
	0.5	30.0	
	1.0	35.7	

Tryptophan-synthetase activity. The method of Yanofsky (1955) was used, but the final indole measurements were made according to Scott (1961). Activities are expressed as μ g. of indole disappearing/15 min./mg. of protein.

RESULTS

Michaelis constant. With whole cells and no tyrosine, the Michaelis constant was 0.256 mM. Two determinations with whole cells and tyrosine gave values of 0.263 and 0.25 mM. A cell-free extract (no tyrosine) gave a value of 0.256 mM. The experimental findings are given in Table 1.

Uptake of tryptophan by resting suspensions. The uptake of tryptophan by suspensions of *E. coli* lacking tryptophanase was rapid during the first 10 min. in the presence or absence of tyrosine or phenylalanine (Fig. 1). From 10 to 45 min. the rate was slower and constant. At 1 hr., tryptophan

Fig. 1. Uptake of tryptophan by resting suspensions of *Escherichia coli*. *E. coli* cells with no tryptophanase activity were suspended, without aeration, in a simple salt medium containing radioactive DL-tryptophan ($[3\text{-}^{14}\text{C}]\text{indolyl-alanine}$) (0.05 mM) and glucose (0.1%), but no nitrogen source, at 35°. The total volume was 30 ml. Samples of the mixture were taken at intervals and centrifuged, and the radioactivity of the supernatant was measured at infinite thinness (O). This was repeated with 1.0 mM-L-tyrosine (●) and with 1.0 mM-DL-phenylalanine (Δ) present.

uptake ceased, when approximately three-quarters of the initial radioactivity had disappeared from the medium. There was no significant difference in the rate and amount of tryptophan disappearing in the presence or absence of other aromatic amino acids.

Utilization of L-tryptophan by growing cultures of Escherichia coli

Tryptophan utilization was measured in cultures growing aerobically in the salts-glucose medium with tryptophan but no other aromatic amino acid (Fig. 2). The starting concentrations of L-tryptophan were 0.025, 0.05, 0.075, 0.10 and 0.15 mM. At high starting concentrations of L-tryptophan (0.075 mM and above) there was a loss of L-tryptophan into the medium during the lag phase, followed by an uptake of tryptophan by the growing cells. The rate of uptake decreases sharply at the end of growth and the L-tryptophan level became steady at 9 hr. At initial L-tryptophan concentrations lower than 0.075 mM there was no initial loss of tryptophan during the lag phase, but a small loss with a maximum at 3½ hr. (not observed with higher initial tryptophan concentrations) occurred. The extent of this loss increased

with increasing initial L-tryptophan concentration. After 15 min., the initial L-tryptophan concentration of 0.15 mM rose to 0.162 mM, 0.10 to 0.104 mM and 0.075 to 0.0775 mM.

Fig. 2 also gives the corresponding curve for indole production. There was a small peak of indole production during the lag phase. Indole in the medium reached a maximum during the logarithmic phase and then decreased again until the end of growth, when a rapid and large indole production began and was still incomplete at 9 hr. The general pattern of indole production was the same for all starting concentrations of L-tryptophan. With a starting L-tryptophan concentration of 0.15 mM, the indole concentration was 0.0069 mM at 15 min., 0.0137 mM at 2½ hr. and 0.0463 mM at 9 hr. For 0.025 mM-L-tryptophan, there was no detectable indole production at 15 min., 0.0032 mM at 2½ hr. and 0.0044 mM at 9 hr. These experiments were repeated, with an initial concentration of tryptophan of 0.075 mM and with the addition of tyrosine (0.5 mM), or tyrosine (0.5 mM) plus DL-serine (1.0 mM). The corresponding patterns of tryptophan uptake and indole production are given in Figs. 3 and 4 respectively. These patterns were

Fig. 2. Disappearance of L-tryptophan and appearance of indole in growing cultures of *Escherichia coli*. The starting cell concentration was 80×10^6 cells/ml. in a simple salt medium containing glucose (0.1%) and the additions as shown. Incubation was at 35° with aeration. Samples were taken at intervals and centrifuged, and the supernatants analysed for indole and L-tryptophan. $\bigcirc-\bigcirc$, Indole production, and $\bullet-\bullet$, L-tryptophan uptake, with 0.075 mM-L-tryptophan added; $\bigcirc---\bigcirc$, indole production, and $\bullet---\bullet$, L-tryptophan uptake with 0.075 mM-L-tryptophan and 1.0 mM-DL-serine added; $\blacktriangle$, growth of the organism.

Fig. 3. Influence of L-tyrosine on the concentrations of L-tryptophan and indole in growing cultures of *Escherichia coli*. *E. coli* was grown (starting cell concentration, 80×10^6 cells/ml.) on a simple salt medium containing glucose (0.1%), L-tyrosine (0.5 mM) and L-tryptophan (0.075 mM). Growth was at 35° with aeration. Samples were taken at intervals and centrifuged, and the supernatant was analysed for L-tryptophan and indole. $\bullet$, Indole; $\bigcirc$, L-tryptophan; $\blacktriangle$, growth of the organism.

Fig. 4. Influence of L-tyrosine and DL-serine on the concentrations of L-tryptophan and indole in growing cultures of *Escherichia coli*. *E. coli* was grown (starting cell concentration, 80×10^6 cells/ml.) on a simple salt medium containing glucose (0.1 %), L-tyrosine (0.5 mM), DL-serine (1.0 mM) and L-tryptophan (0.075 mM). Growth was at 35° with aeration. Samples were taken at intervals and centrifuged, and the supernatant was analysed for L-tryptophan and indole. ●, Indole; ○, L-tryptophan; ▲, growth of the organism.

Table 2. *Tryptophanase and tryptophan-synthetase activities of Escherichia coli*

E. coli was grown on a simple salt-glucose medium with different concentrations of L-tryptophan and other additions as shown. Cells were acetone-dried after growth for 6 hr. and assayed for tryptophanase and tryptophan-synthetase activities.

Initial concn. of L-tryptophan (mM)	Tryptophanase activity (μ g. of indole/min./mg. of protein)	Tryptophan-synthetase activity (μ g. of indole used/15 min./mg. of protein)
0	0.0	16.7
0.025	2.28	12.1
0.05	6.70	10.0
0.075	13.10	8.23
0.1	17.56	9.75
0.15	26.30	5.84
0.075 + 1.0 mM-DL-serine	12.03	11.12
0.075 + 0.5 mM-L-tyrosine	3.4	—
0.075 + 0.5 mM-L-tyrosine + 1.0 mM-DL-serine	9.21	—

more complicated; several peaks of indole and tryptophan secretion and uptake were found. With tyrosine alone, these peaks coincided; with tyrosine and serine they alternated and the tryptophan peaks were increased, the indole peaks decreased. All the above experiments have been duplicated. The overall patterns of duplicate experiments can be superimposed. Agreement between individual duplicate points is good, except in those regions where the fluctuations are rapid. This is understandable, since the samples were centrifuged for 5 min. and sampling could not be controlled within less than ± 30 sec. For example, between $2\frac{1}{2}$ and $2\frac{3}{4}$ hr. in Fig. 3 the tryptophan concentration rises at the rate of about 0.01 mM/min., and there is a measurable change in the tryptophan concentration in 10 sec.

Similar cultures were grown again and harvested after 6 hr. The cells were acetone-dried, and tryptophanase and tryptophan-synthetase activities determined as described. The results are given in Table 2.

As the initial L-tryptophan concentration in the medium was increased, there was a corresponding increase in the tryptophanase activity of the cells after 6 hr. growth, and a decrease in the tryptophan-synthetase activity. Initial tryptophan concentration versus tryptophanase activity gave a practically linear plot, whereas tryptophan synthetase plotted against initial tryptophan concentration showed a more erratic distribution of points.

In agreement with previous work by Evans *et al.* (1941), addition of tyrosine to the growth medium caused a decrease in tryptophanase production. Serine partially abolished the inhibition of tryptophanase production by tyrosine. Serine alone, however, had no significant effect on tryptophanase production, but caused a small increase in the level of tryptophan synthetase.

DISCUSSION

Cell-free extracts and whole cells of *E. coli* with tryptophanase activity give the same Michaelis constant for tryptophanase in the presence or absence of phenylalanine or tyrosine. This indicates that L-tryptophan has free passage to the active site of the enzyme in whole cells, indole has a free passage out of the cell, and these two processes are not influenced by the presence of other aromatic amino acids in the reaction mixture. To produce cells with tryptophanase activity, *E. coli* must be grown in a tryptophan medium. Since adaptive permeability is possible (Clarke & Meadow, 1959) this first experiment does not necessarily show that tryptophan diffuses freely, unimpeded by other aromatic amino acids, across the wall of a cell containing no tryptophanase.

Aromatic amino acids do not, however, decrease the rate of uptake of radioactive tryptophan by resting cells with no tryptophanase (see Fig. 1). Presumably all the L-tryptophan was utilized by the cells and the remaining radioactivity in the supernatant was due to D-tryptophan.

In a growing culture, the disappearance of L-tryptophan from the medium was very slow for the first 2 hr. (Fig. 2). At higher initial tryptophan concentrations, there was even a secretion of L-tryptophan into the medium during the lag phase. The cells in the inoculum had been grown in a tryptophan-free medium and must have had a fully developed system of enzymes for synthesizing sufficient L-tryptophan for growth. The L-tryptophan of the new medium was therefore unnecessary for growth. Further, since the exogenous L-tryptophan could enter the cell, the concentration of free intracellular L-tryptophan would be unnecessarily high. There is no tryptophanase to deal with this excess of L-tryptophan, which is lost from the cell. It is difficult to understand why this resulted in an increase in the total free L-tryptophan of the system or why its magnitude depended on the initial concentration of L-tryptophan in the medium. An over-compensation by the cell is a possible but only partial explanation.

The secretion of L-tryptophan was accompanied by a very small production of indole (Fig. 2). The L-tryptophan in the medium could have influenced the equilibrium, $\text{indole} + \text{L-serine} \rightleftharpoons \text{L-tryptophan}$ (Yanofsky, 1952), and caused an accumulation of indole. This interpretation is supported by the finding that serine in the medium suppressed this indole production and simultaneously increased the amount of L-tryptophan secreted (Fig. 2).

With initial L-tryptophan concentrations of 0.075 mM and below, an additional peak occurred in the L-tryptophan uptake curve (Fig. 2) just at the end of the logarithmic phase. At this point, indole production, which increased during the logarithmic phase, now showed a small decrease. Serine increased this L-tryptophan peak and prevented indole production completely until the end of growth. This suggests that the equilibrium, $\text{indole} + \text{L-serine} \rightleftharpoons \text{L-tryptophan}$, is again involved and the increase of tryptophan and decrease of indole could be due to the continued synthesis of serine at the end of growth.

Inclusion of L-tyrosine in the simple salt-glucose-tryptophan medium exaggerated the results obtained without L-tyrosine. The initial increases in the indole and L-tryptophan concentrations of the medium were much larger, concomitant and repeated (Fig. 3). When serine was also included in the medium (Fig. 4), the losses of L-tryptophan were still larger, but the corresponding indole peaks were decreased and alternated

with those of the L-tryptophan. The cells from this experiment had a higher tryptophanase activity than those grown on the same medium without serine, presumably in response to the extra L-tryptophan synthesized under the influence of the serine.

It is concluded that, in the presence of exogenous L-tryptophan, *E. coli* is able to employ three mechanisms for controlling the intracellular concentrations of free L-tryptophan, i.e. loss of excess of L-tryptophan from the cell, 'detoxication' of L-tryptophan by induced tryptophanase and a decrease in the activity of its own tryptophan-synthesizing mechanism.

Two possibilities are suggested for the observed effects of L-tyrosine. It could cause an accumulation of intermediates on the pathway of aromatic biosynthesis (Davis, 1955, 1958), backed up by a ready supply of the necessary carbohydrate intermediates from the glucose. This could result in an increased L-tryptophan synthesis and the observed increased loss of L-tryptophan from growing cells in the presence of L-tyrosine. However, this explanation cannot be reconciled with the fact that L-tyrosine inhibits tryptophanase synthesis. Alternatively, L-tyrosine could compete with L-tryptophan for a place in the amino acid pool and prevent the L-tryptophan concentration reaching a high enough level to promote tryptophanase synthesis. The basis of such a specific competition of amino acids is difficult to explain, but is now experimentally verified for the aromatic amino acids (Scott & Happold, 1962) and has also been reported for leucine and valine (Roberts, 1959).

SUMMARY

1. The Michaelis constant of tryptophanase is about 0.255 mM and is the same for whole cells and cell-free extracts of *Escherichia coli*. The results with whole cells are not influenced by the presence of other aromatic amino acids and glucose in the reaction medium.

2. The rate at which resting cells of *Escherichia coli* take up L-tryptophan is not affected by other aromatic amino acids.

3. L-Tryptophan uptake and indole production by growing *Escherichia coli* cultures has been studied. Added serine influences these patterns in a way that suggests that indole production during growth is partly due to a shift in the equilibrium: $\text{L-tryptophan} \rightleftharpoons \text{L-serine} + \text{indole}$. L-Tyrosine appears to compete with tryptophan for a place in the amino acid pool.

4. An increase in tryptophanase activity of cells in response to increased concentrations of L-tryptophan in the growth medium is paralleled by a decrease in tryptophan-synthetase activity.

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The Number of Catalytic Sites in Creatine Phosphokinase as Determined by a Study of its Reactive Sulphydryl Groups

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Rosenberg & Ennor (1955) demonstrated that creatine phosphokinase was readily inactivated by a variety of 'sulphydryl reagents', suggesting that free SH groups on the enzyme were essential for catalytic activity. Benesch, Lardy & Benesch (1955) showed that the enzyme contained six SH groups of which two are freely reactive. The presence of only two freely reactive SH groups was confirmed by Mahowald & Kuby (1960), who isolated the products, after acid hydrolysis, of the reaction with fluorodinitrobenzene and by Watts, Rabin & Crook (1961), who showed that alkylation of two reactive thiols by iodoacetate exactly parallels loss of enzyme activity. Since both these groups of workers reported that reaction of two SH groups was accompanied by complete loss of enzyme activity, it seemed reasonable to infer that creatine phosphokinase contained a maximum of two catalytic sites per molecule provided that the activity loss is not due to changes in the secondary and tertiary structure of the protein. This study of the kinetics of the reaction between creatine phosphokinase and iodoacetate was carried out to determine the number of catalytic sites per molecule of enzyme and the number of SH groups associated with each site. We have found that the properties of different crystalline preparations varied. Evidence is presented that a particular preparation of creatine phosphokinase may contain either one or two catalytic sites per molecule.

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MATERIALS AND METHODS

Abbreviation. CPK, Creatine phosphokinase.

Crystalline creatine phosphokinase. Two samples were prepared from rabbit muscle by the method B of Kuby, Noda & Lardy (1954) and the purity was checked by starch-gel electrophoresis at pH 8.9 by using the discontinuous system of Poulik (1957). Only one component and the trace of a second, which increased on storage, could be detected. A molecular weight of 81000 (Noda, Kuby & Lardy, 1954) was used to compute enzyme concentrations.

Iodoacetic acid. This was recrystallized four times from light petroleum, b.p. 60–80°, to give a white product, m.p. 78°, containing no titratable iodine.

Crystalline ATP (disodium salt). This was obtained from Sigma Chemical Co. and used without further purification.

Creatine. This was recrystallized from hot water.

p-Nitrophenylacetate. This was recrystallized first from hot 50% ethanol and then from sodium-dried ether. The final product, m.p. 80.5°, was dried over phosphorus pentoxide *in vacuo*.

Buffers. Borate buffer, 1.0 M, was prepared by adjusting 0.2 M-sodium hydroxide to pH 9.0 with boric acid and diluting the buffer to twice the original volume. The pH was checked at the final dilution. A pH 9.0 buffer from recrystallized glycylglycine was prepared by a similar method.

Ionic strength. Where necessary, this was adjusted by the addition of α -potassium nitrate.

Assay procedures. The reaction of iodoacetate with CPK was followed by measuring the production of iodide ions with the silver-silver iodide electrode. Kinase activity was determined by measuring the phosphate transferred from ATP to creatine in a standard reaction mixture in 4 min. at 37°. The details of these assays were exactly as set out by Watts, Rabin & Crook (1961).