Studies on Tryptophan Synthetases from Various Strains of *Bacillus subtilis*

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1. Tryptophan synthetase B of three strains of *Bacillus subtilis* was prepared from 'exo-protoplasmic' and 'endo-protoplasmic' fractions; the enzyme from 'exo-protoplasmic' fraction was purified 30- to 120-fold by ammonium sulphate precipitation and DEAE-cellulose column chromatography; the latter step separated this enzyme from tryptophan synthetase A, tryptophanase and proteolytic enzymes, but the purified preparations were not stable. 2. The activity of tryptophan synthetase B did not depend on the presence of tryptophan synthetase A. 3. Tryptophan synthetases B of the strains tested differed in their utilization of 2- and 7-methylindole as compared with indole; this suggests that these tryptophan synthetases B are not identical.

The tryptophan synthetases of *Escherichia coli*, *Neurospora crassa* and a few other organisms have been the subject of considerable research (see reviews by Yanofsky, 1960; Smith & Yanofsky, 1962; Bonner, DeMoss & Mills, 1965; Creighton & Yanofsky, 1966). However, little is known about the tryptophan synthetases of *Bacillus subtilis*.

Schwartz & Bonner (1964) attempted a partial purification of tryptophan synthetases of *B. subtilis* on a Sephadex column. In a preliminary communication (Meduski, Heldenmuth & Zamenhof, 1967) we reported purification of tryptophan synthetase B by means of a DEAE-cellulose column.

One purpose of our work was the development of methods of purification and the study of properties of tryptophan synthetase B; the other purpose was the development of a simple method by which different tryptophan synthetases could be discerned. The proposed method is based on the comparison of the utilization of indole with the utilization of its methyl analogues (Meduski *et al.* 1967). It has been observed (Smith & Yanofsky, 1962; Hall, Lea & Rydon, 1962) that tryptophan synthetases of *E. coli* and *Neurospora* can indeed utilize methylindoles, but such a problem has not been studied quantitatively.

Tryptophan synthetase B (EC 4.2.1.20) has the systematic name L-serine hydrolyase (adding indole); new proposed nomenclature is α sub-unit, for tryptophan synthetase A, and β_2 sub-unit, for tryptophan synthetase B (Creighton & Yanofsky, 1966).

EXPERIMENTAL

Bacterial strains and cultures. To obtain increased production of tryptophan synthetase B on tryptophan-

containing full media, we used regulatory mutants (resistant to 1 mg. of 5-methyltryptophan/ml.; producing high activities of tryptophan synthetase) isolated in this Laboratory. Of these, strain 23 *try⁺/mtr* was described by Zamenhof & Heldenmuth (1964); strains 6051 *try⁺/mtr* and 10774 *try⁺/mtr* were derived from strains A.T.C.C. 6051 and A.T.C.C. 10774 respectively. Sulphanilamide-resistant (*/mtr, S*) and azide-resistant (*/mtr, azi*) derivatives of these strains were isolated as described previously (Ephrati-Elizur, Srinivasan & Zamenhof, 1961; Zamenhof & Eichhorn, 1967). Strain 168 *ind⁻* was the one used normally for transformation in this Laboratory (Ephrati-Elizur *et al.* 1961).

For the preparation of the enzyme the above strains were grown at 37° with agitation in Penassay (Difco Laboratories Inc., Detroit, Mich., U.S.A.) broth, supplemented with glucose (final concn. 0.6%), the depth of the liquid in Fernbach flasks was not more than 35 mm. After 16 hr. the cells were harvested by centrifugation for 15 min. at 8000g and washed three times, each time with one-tenth of the original volume of the culture, with the following solution: aqueous 0.15 M-KCl-0.05 M-EDTA neutralized with KOH to pH 7.8 (hereafter called EDTA, pH 7.8). Washed cells were frozen and stored at -30°.

For the study of growth and inhibition, the cells of each strain tested were grown in Penassay broth for 16 hr. at 37°, washed three times as described above and then resuspended in basal broth (Spizizen, 1958) supplemented with 0.5% of glucose and with indole or methylindoles. The final concentrations at the beginning of experiments were 1×10^8 cells/ml. The cultures (5.5 ml. each) were incubated with shaking at 37°; the growth after 24 hr. was assessed by extinction readings at 450 m μ .

Transformation experiments were performed as described by Ephrati-Elizur *et al.* (1961).

Materials. The sources of several substances and reagents were listed previously (Meduski & Zamenhof, 1968). Others included pyridoxal phosphate monohydrate (A grade), L-serine, azocasein, crystalline lysozyme and EDTA (Calbiochem, Los Angeles, Calif., U.S.A.), and crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill., U.S.A.),

DL-glyceraldehyde-3-phosphoric acid (Sigma Chemical Co., St Louis, Mo., U.S.A.) and dimedone (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.).

The 'saturated $(\text{NH}_4)_2\text{SO}_4$ solution' (saturated at 0° ; 4.05M) was prepared by dissolving 160g. of $(\text{NH}_4)_2\text{SO}_4$ in 190ml. of 0.1M-potassium phosphate buffer, pH 7.8, and adding 1.9ml. of 0.1M-EDTA, pH 7.8 and water to 200ml.

For column chromatography, Serva DEAE-cellulose ion-exchanger, 0.68m-equiv./g. (Gallard Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.) was used as such except for removal of fine particles by repeated sedimentation and decantation. For elution of the fractions from the column the following solutions were used: eluent I, 0.02M-potassium phosphate buffer-1mM-EDTA, pH 7.8; eluent II, 0.125M-KCl-3mM-potassium phosphate buffer-1mM-EDTA, pH 7.8; eluent III, 0.18M-KCl-5mM-potassium phosphate buffer-1mM-EDTA, pH 7.8; eluent IV, 0.36M-KCl-0.01M-potassium phosphate buffer-1mM-EDTA, pH 7.8; eluent V, 0.90M-KCl-0.02M-potassium phosphate buffer-1mM-EDTA, pH 7.8; eluent VI, 1.5M-KCl-0.02M-potassium phosphate buffer-1mM-EDTA, pH 7.8.

Extraction and purification of tryptophan synthetase. Frozen washed cells (5g.) from approx. 2l. of growth medium were suspended in 300ml. of aqueous 20% sucrose solution, which was 1mM with respect to EDTA, pH 7.8, and 0.01M with respect to potassium phosphate buffer, pH 7.8. To the solution 75mg. of lysozyme was added and the suspension was incubated in a water bath at 37° for 2hr. This treatment converted the cells into osmotically stabilized protoplasts, owing to the presence of concentrated sucrose.

All subsequent steps were carried out at 4° . The suspension was centrifuged at 15000g for 30min. and the supernatant fluid, containing the 'exo-protoplastic' fraction, was removed and preserved at -70° . The pellet of protoplasts was resuspended in 250ml. of water and stirred for 25min. The resultant lysate was then centrifuged at 35000g for 30min., and the supernatant containing the 'endo-protoplastic' fraction was supplemented with potassium phosphate buffer, pH 7.8, to a final concentration of 0.02M, and with EDTA, pH 7.8, to a final concentration of 1mM. This fraction was preserved at -70° .

The terms 'exo-protoplastic' and 'endo-protoplastic' fraction are derived merely from the method of preparation; however, specific distribution of the enzyme within the cell may have a regulatory role (compare Willstätter & Rohdewald, 1934; Heppel, 1967). Other terms that have been used to denote similar fractions are 'lyo-', 'endo-' and 'desmo-enzyme' (Willstätter & Rohdewald, 1934) or 'cell-wall fraction' and 'protoplasm fraction' (Vorbeck & Marinetti, 1965).

The pellet containing cell membranes and entrapped cytoplasmic material was washed four times with eluent I by resuspension and centrifugation at 35000g for 30min. The washed pellet consisting of cell membranes was preserved at -70° .

For purification of 'exo-protoplastic' and 'endo-protoplastic' fractions, to each volume of the fraction 2vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution (see under 'Materials') was added with stirring; solid $(\text{NH}_4)_2\text{SO}_4$ was then added slowly with stirring to an amount slightly in excess of saturation at 0° . After being stirred for 30min. the mixture was left for 3hr. at 4° ; the precipitate, referred to below as

100% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate, was collected by centrifugation at 35000g for 45min. at 4° .

Further purification was achieved by column chromatography. DEAE-cellulose (20g.) was equilibrated with 600ml. of eluent I and the slurry was used to fill the column. Each 20mg. of protein to be purified was dissolved in 2ml. of eluent I and applied to the 50ml. column by the usual procedure.

Elution was effected by stepwise increases in ionic strength at a constant pH 7.8 at 4° , with eluents I-VI (see under 'Materials'). A gravity flow rate of 1ml./min. was used, and fractions (10ml.) were collected. The course of elution of proteins was followed by using a Beckman model 132 fraction collector and LKB 4701 A Uvicord ultraviolet absorptiometer.

Concentration of eluate. Eluates were concentrated by freeze-drying and stored at -70° ; in another procedure water was removed by low-pressure ultrafiltration (Everall & Wright, 1958; Berggård, 1961), with 1000ml. flasks, $\frac{1}{2}$ in. dialysis tubing and a partial vacuum of -66 cm. Hg. Both procedures gave satisfactory stability of the enzyme; the latter procedure was preferred for immediate use of concentrates.

Ultrasonic treatment of cells. For the purpose of estimation of yield, the tryptophan synthetase B activity was also assayed in the entire cells. To this end, whole cells were subjected to ultrasonic treatment in the Branson Sonifier for 4min. at 4A. This unpurified preparation is referred to as 'Whole cells, ultrasonically treated' (Table 2). Ultrasonic treatment was used because it exposes all cell proteins that may be sources of enzymic activity; this excludes the uncertainty about cell permeability.

Assays of enzymic activities. Tryptophan synthetase B was assayed by the disappearance of indole (or its methyl analogues), by using the colorimetric methods described previously (Meduski & Zamenhof, 1968). The incubation mixture contained 200 μ moles of potassium phosphate buffer, pH 7.8, 40 μ moles of L-serine, 0.08 μ mole of pyridoxal phosphate, 2 μ moles of EDTA, pH 7.8, 0.1-0.2 μ mole of indole or its methyl analogue, and a sample of the enzyme, in a total volume of 1.8ml. After 60min. incubation at 37° with gentle shaking, 0.2ml. of ethanolic 1% dimedone solution was added, and, subsequently, 8ml. of 6.25% (w/v) trichloroacetic acid. After centrifugation at 4000g for 15min., a sample of the supernatant was diluted to 10ml. with 5% (w/v) trichloroacetic acid, and used for indole or methylindole determination (Meduski & Zamenhof, 1968). The standard curve in each case was obtained by using appropriate concentrations of indole or methylindole, and a heat-inactivated enzyme in place of the active one.

Because of the well-known acid-sensitivity of indole, the initial concentration of the trichloroacetic acid solutions never exceeded 6.25% (final concn. 5%). All steps after the addition of trichloroacetic acid were carried out at 0° . This procedure in our hands was more satisfactory than the usually employed 1M-NaOH or heat to stop the enzymic reactions involving indole (Smith & Yanofsky, 1962; Garriek, Elberfeld & Suskind, 1964). Such practices may involve a danger of enhancing the volatility of indole from aqueous solution (Zoller, 1920).

The use of trichloroacetic acid in the presence of pyridoxal phosphate was made possible by the addition of dimedone before the addition of trichloroacetic acid. Dimedone combines with pyridoxal phosphate and renders it un-

available for the reaction with the remaining indole under acid conditions (Scott, 1961).

To secure both the optimum activity and the stability of the enzyme, all solutions used in our studies contained K^+ in agreement with the observations of Schwartz & Bonner (1964), confirmed in our preliminary experiments.

One unit of tryptophan synthetase B was defined as the amount that catalyses the utilization of $0.1 \mu\text{mole}$ of indole/hr. at 37° at pH 7.8, with $55.6 \mu\text{M}$ -indole in the reaction mixture. Specific activity was expressed as units of enzyme/mg. of protein.

Tryptophanase activity of the fractions was determined in incubation mixtures (1.8 ml.) containing $2.8 \mu\text{moles}$ of L-tryptophan, $200 \mu\text{moles}$ of potassium phosphate buffer, pH 7.8, $0.08 \mu\text{mole}$ of pyridoxal phosphate and a sample of the enzyme source. The reaction was followed by the appearance of indole, as described above (Meduski & Zamenhof, 1968).

The formation of 1-(indol-3-yl)glycerol 3-phosphate from indole and D-glyceraldehyde 3-phosphate [called the tryptophan synthetase 2 reverse reaction and catalysed, as stated by Schwartz & Bonner (1964), by component A of tryptophan synthetase, hereafter called tryptophan synthetase A] was measured in the absence of pyridoxal phosphate by following indole disappearance by the above-mentioned method (Meduski & Zamenhof, 1968). The incubation mixture (1.4 ml.) contained $350 \mu\text{moles}$ of potassium phosphate buffer, pH 7.8, $29.4 \mu\text{moles}$ of D-glyceraldehyde 3-phosphate, $0.12 \mu\text{mole}$ of indole and a sample of the enzyme source. One unit of tryptophan synthetase A was defined as the amount that catalyses the utilization of $0.1 \mu\text{mole}$ of indole/hr. at 37° with $85.7 \mu\text{M}$ -indole in the reaction mixture.

In the preliminary experiments with washed cells and total lysates of *B. subtilis* we observed that indole acts as an inhibitor of tryptophan synthetase B activity. In purified tryptophan synthetase B preparations, $67 \mu\text{M}$ -indole corresponded to a half-maximal concentration where no deviation from the Michaelis-Menten equation was observable. This concentration was afterwards adopted in all our experiments. The maximal value was at 0.134 mM . At the concentration of 0.8 mM we observed approximately 20% inhibition.

The mechanism of this phenomenon was not further

studied. The phenomenon may be basically similar to the inhibition of lysozyme by indole derivatives (Shinitzky, Katchalski, Grisaro & Sharon, 1966). In the presence of high concentration of indole, not only is the active site of the enzyme involved in the reaction with indole (activity of tryptophan synthetase), but also other groups of enzyme protein (protonated imidazole residues) may react with indole leading to the formation of complexes of the charge-transfer type. Such complexes may be the cause of the substrate inhibition observed with tryptophan synthetase B but expected also with tryptophan synthetase A. Thus we chose to work at a lower range of indole concentration, where this inhibition is negligible, using at the same time a large excess of the other substrate (i.e. serine).

In experiments with tryptophan synthetase B the concentrations of 2-methylindole or 7-methylindole were 0.13 mM or $65 \mu\text{M}$ respectively, whereas the concentration of serine was 0.222 M . In experiments with tryptophan synthetase A the concentrations of indole and D-glyceraldehyde 3-phosphate were $86 \mu\text{M}$ and 0.86 mM respectively. The concentrations of indole or methylindole substrates were of the same order of magnitude as the concentration reported as K_m in the studies with *N. crassa* (DeMoss, 1962).

Proteolytic activity was assayed by the method of Michel (1966) with azocasein as a substrate, by determining the coloured digestion products soluble in trichloroacetic acid.

Other assays. Protein content of the enzyme preparations was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Distribution of activities in column eluents. The distribution of tryptophan synthetase B and A in various fractions and the DEAE-cellulose-column elution patterns are shown in Table 1 and on Fig. 1. Tryptophan synthetase B activity is found both in the 'exo-protoplasmic' and in the 'endo-protoplasmic' but not in the 'membrane' fraction; the 'exo-protoplasmic' fraction is considerably richer in this activity than the 'endo-protoplasmic'. On the

Table 1. *Tryptophan synthetase B and tryptophan synthetase A activities in cellular fractions from three strains of B. subtilis*

All activities are expressed in units/g. of original wet (packed) cells.

Strain	...	...	Activity								
			23 try ⁺⁺ /mtr			6051 try ⁺⁺ /mtr			10774 try ⁺⁺ /mtr		
			B	A	Ratio B/A	B	A	Ratio B/A	B	A	Ratio B/A
Fraction											
'Exo-protoplasmic'*			23.1	0.257	90	7.52	2.61	2.9	20.4	1.2	17
'Endo-protoplasmic'*			2.58	0.63	4.1	1.44			4.07	1.29	3.15
Ratio 'exo-protoplasmic'*			8.9	0.4	22	5.2			5	0.93	5.4
'endo-protoplasmic'											
'Membranes'†			0			0			0		

* 100% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate.

† Resuspended.

Fig. 1. DEAE-cellulose chromatography of 100% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate of 'exo-protoplastic' fraction from three strains of *B. subtilis*. A sample (2 ml.) was applied to a 50 ml. column. (a) Strain 23 *try*⁺⁺/*mtr*: 17.5 mg. of protein applied, 61% recovered; (b) strain 6051 *try*⁺⁺/*mtr*: 30.75 mg. of protein applied, 46% recovered; (c) strain 10774 *try*⁺⁺/*mtr*: 39.75 mg. of protein applied, 31% recovered. The abscissae represent tube number (10 ml./tube), fraction number and eluent used (see the Experimental section) and the ordinates E_{280} (continuous line) and specific activity of tryptophan synthetase B (○) of each fraction. ↓, Location of the tryptophan synthetase A activity; ∇, location of tryptophanase activity; ▽, location of proteolytic activity.

other hand, tryptophan synthetase A activity is found mostly in the 'endo-protoplastic' rather than in the 'exo-protoplastic' fraction. Thus, unlike those of *E. coli* (Creighton & Yanofsky, 1966;

Bonner *et al.* 1965), the activities of tryptophan synthetase B and tryptophan synthetase A of *B. subtilis* do not appear to be intimately related (Schwartz & Bonner, 1964). The ratios of the two activities in the same or in different fractions are different for each of the strains studied.

Fig. 1 further demonstrates that, especially with strains 23 *try*⁺⁺/*mtr* and 6051 *try*⁺⁺/*mtr*, one can obtain a fraction with tryptophan synthetase B activity that has no detectable tryptophan synthetase A activity. Thus, again unlike that of *E. coli*, the tryptophan synthetase A activity of *B. subtilis* does not seem to be needed for tryptophan synthetase B activity, and the two enzymes appear to be functionally independent. A similar situation appears to exist for tryptophan synthetases from *Pseudomonas putida* (Enatsu & Crawford, 1968). Our results also make it unlikely that the differences in tryptophan synthetase B activities of various fractions from various strains could be due to different contents of tryptophan synthetase A.

Progress of purification. The progress of purification of tryptophan synthetase B is shown in Table 2. Though 30- to 120-fold purification can be achieved, the final yield is low. This is in agreement with the reported observations of the instability of tryptophan synthetase B in both *E. coli* (Wilson & Crawford, 1965) and *B. subtilis* (Schwartz & Bonner, 1964). We observed that the more purified preparations of tryptophan synthetase (after DEAE-cellulose chromatography) undergo inactivation even when stored at -70° . For example, fraction 1 of the 'exo-protoplastic' fraction of strain 23 *try*⁺⁺/*mtr* when analysed immediately after preparation had a specific activity of 2.18 units/mg. with indole, 1.92 with 2-methylindole and 0.62 with 7-methylindole. After 2 months' storage the corresponding values were 0.25, 0.4 and 0.11. This inactivation occurred despite the precaution taken, to avoid the activity of proteolytic enzymes (Fig. 1), during the handling of cells of *B. subtilis* and their fractions by using EDTA in all solutions for washing, fractionation and column chromatography. An additional advantage of EDTA is the prevention of the metal-catalysed oxidation of some proteins (Ray, Davisson & Crespi, 1954).

The use of DEAE-cellulose as the main tool for fractionating 'exo-protoplastic' tryptophan synthetases was adopted after preliminary experiments that showed it to be in general the most satisfactory for separation of various enzymes from both 'exo-protoplastic' and 'endo-protoplastic' materials. The disadvantage of DEAE-cellulose is that small amounts of the enzyme sometimes continue to emerge with the subsequent fractions after the main peak, a feature also observed with DEAE-cellulose and serum proteins (Schultze & Heremans, 1966).

Table 2. *Purification of tryptophan synthetase B from three strains of B. subtilis*

Strain ...	Tryptophan synthetase B activity								
	23 <i>try</i> ⁺⁺ / <i>mtr</i>			6051 <i>try</i> ⁺⁺ / <i>mtr</i>			10774 <i>try</i> ⁺⁺ / <i>mtr</i>		
	Activity [units/g. of orig. wet (packed) cells]	Specific activity (units/mg. of protein in the fraction)	Yield (% of activity in whole cells)	Activity [units/g. of orig. wet (packed) cells]	Specific activity (units/mg. of protein in the fraction)	Yield (% of activity in whole cells)	Activity [units/g. of orig. wet (packed) cells]	Specific activity (units/mg. of protein in the fraction)	Yield (% of activity in whole cells)
Fraction									
(i) Whole cells, ultra- sonically treated	15.9	0.16	100	2.0	0.02	100	24.4	0.29	100
(ii) 100% satd. (NH ₄) ₂ SO ₄ precipitate of 'exo-protoplasmic' fraction	10.6	0.41	69	1.18	0.16	59	20.2	0.68	83.6
(iii) Fraction (ii) passed through DEAE- cellulose column (most active peak)	1.65	8.11	10.4	0.44	2.4	22	0.62	8.8	2.6
Final purification factor		50.7			120			30.3	

Table 3. *Ratios of utilization of indole and its analogues by tryptophan synthetase B from three strains of B. subtilis*

Methods are described in the text. Ratios of utilization measured are: indole/2-methylindole (a); indole/7-methylindole (b); 7-methylindole/2-methylindole (c).

Fraction	Ratio of utilization measured	Strain ...	Ratios		
			23 <i>try</i> ⁺⁺ / <i>mtr</i>	6051 <i>try</i> ⁺⁺ / <i>mtr</i>	10774 <i>try</i> ⁺⁺ / <i>mtr</i>
100% sat.					
(NH ₄) ₂ SO ₄ ppt.					
'Exo-protoplasmic'	a		0.56	1.6	0.97
'Endo-protoplasmic'	a		0.46	1.7	1.17
'Exo-protoplasmic'	b		1.7	3.25	1.1
'Endo-protoplasmic'	b		0.89	2.9	1.9
'Exo-protoplasmic'	c		0.32	0.49	0.88
'Endo-protoplasmic'	c		0.52	0.58	0.62
Most active tryptophan synthetase B fraction from DEAE-cellulose column					
'Exo-protoplasmic'	a		1.13	0.1	0.9
	b		3.5	1.44	2.1
	c		0.32	0.07	0.43

The splitting of B protein into two sub-units has been offered as a possible explanation of 'trailing' (Ray *et al.* 1954); in our work such a possibility is unlikely because of the high concentration of salts in the eluents.

The overall protein-elution profile of the preparation from strain 23 *try*⁺⁺/*mtr* resembles that of strain 6051 *try*⁺⁺/*mtr*, but not that of strain 10774 *try*⁺⁺/*mtr*, whereas the elution profile of tryptophan synthetase B of the first strain resembles that of strain 10774 *try*⁺⁺/*mtr* rather than strain 6051 *try*⁺⁺/*mtr*.

Tryptophanase is present in 'exo-protoplasmic' material separate from both tryptophan synthetase

B and A. With strain 23 *try*⁺⁺/*mtr* most of the tryptophanase is found in fractions 2 and 3; fractions 1, 4 and 5 did not have any detectable tryptophanase activity. The complete separation of tryptophan synthetase B and tryptophanase is therefore achieved in the last two fractions.

The activity of the inducible tryptophanase of *E. coli* resembles activity of tryptophan synthetase B (Newton & Snell, 1964). It is of importance to assess the extent to which tryptophanase could interfere with the results of assay for tryptophan synthetase B by reconvertng the tryptophan formed into indole. The concentration of substrates used (excess of serine) would not favour this

reaction. Nevertheless, the results obtained with column-purified tryptophan synthetase B must be considered more reliable than those with preparations from which tryptophanase has not been removed (100% satd. ammonium sulphate precipitates, and especially crude cell lysates).

Ratios of utilization of indole and its analogues. The ratios of the utilization of indole and its analogues by tryptophan synthetases B from three strains are represented in Table 3. It can be seen that tryptophan synthetases B, at various stages of purification, utilize 2-methylindole better than 7-methylindole.

7-Methylindole was chosen since this analogue has been reported to be better utilized by *Neurospora* tryptophan synthetase (Hall *et al.* 1962) and to be less inhibitory for *Salmonella* (Fildes & Rydon, 1947) than the analogues with a methyl group in other positions on the benzene ring. The analogue with a methyl group in the 2-position on the pyrrole ring should be even more utilizable, as this substitution can be considered to favour the negative charge in the 3-position. [For a discussion of resonance structures of indole, see Badger (1961); for a discussion of the influence of substituents at position 2 on the reactivity of position 3 in indole, see Albert (1959)]. In the synthesis of tryptophan, such a charge is required for the condensation of the indole ring with the Schiff's base formed between pyridoxal phosphate and serine (Pullman, 1963; Fasella, 1967).

Table 3 further shows that the ratios of utilization of indole and its methylated analogues are different for tryptophan synthetase B of each strain. These results are consistent with the suggestion that the tryptophan synthetases B of the three strains are not identical and that the differences in activities towards indole and its analogues can be used for convenient demonstration of this fact. Such a conclusion must eventually be corroborated by amino acid analysis of the purified enzymes. Amino acid analysis did indeed reveal considerable differences in another enzyme, subtilisin, obtained from different strains of *B. subtilis* (Smith *et al.* 1966; Markland & Smith, 1967).

As can also be seen from Table 3, both 'exo-protoplasmic' and 'endo-protoplasmic' fractions appear to contain essentially the same tryptophan synthetase B, as judged by the similarities of the ratios of activities towards indole and its analogues. Occasional deviations in the ratios cannot be explained at present; they do not invalidate the general pattern.

Relationships between strains. The study of the ratios of utilization of indole and its analogues (Table 3) reveals differences between tryptophan synthetases B from the three strains, but it does not permit the establishment of 'relationships' between

these strains. The specificity of tryptophan synthetase B of strain 6051 *try⁺⁺/mtr* is somewhat closer to that of strain 23 *try⁺⁺/mtr* than to that of strain 10774 *try⁺⁺/mtr* (especially as far as the ratio of utilization of 7-methylindole to that of 2-methylindole is concerned), but this is not pronounced. Obviously, the study of relationship of only a few enzymes cannot serve as a taxonomic tool.

Fig. 2. Growth of *B. subtilis* strains, measured by E_{450} , in 24 hr. at 37° at various concentrations of indole (O), 2-methylindole (Δ) or 7-methylindole ($\square$). (a) Growth of strain 23 *try⁺⁺/mtr*; (b) growth of strain 6051 *try⁺⁺/mtr*; (c) growth of strain 10774 *try⁺⁺/mtr*.

Table 4. *Cross-transformability of three strains of B. subtilis*

/S, sulphanilamide resistance; /azi, sodium azide resistance; ind⁺, indole non-requirement. Percentage transformation is given as compared with homologous transformation; in lines 5 and 6, similar strains were used as DNA donor and as recipient.

DNA donor	Recipient	Characters transformed into	Transformation (%)
23 try ⁺⁺ /mtr, S	6051 try ⁺⁺ /mtr	/S	65-100
23 try ⁺⁺ /mtr, S	168 ind ⁻	/S	100
23 try ⁺⁺ /mtr, S	168 ind ⁻	ind ⁺	100
6051 try ⁺⁺ /mtr, S	168 ind ⁻	/S	100
6051 try ⁺⁺ /mtr, S	6051 try ⁺⁺ /mtr	/S	100
6051 try ⁺⁺ /mtr, azi	6051 try ⁺⁺ /mtr	/azi	100
10774 try ⁺⁺ /mtr	168 ind ⁻	ind ⁺	0
10774 try ⁺⁺ /mtr, azi	6051 try ⁺⁺ /mtr	/azi	0

Other criteria for the evaluation of relationships between the three strains did not provide a consistent picture either. The appearances of the colonies of the three strains grown on Penassay-agar for 24 hr. at 37° are entirely different. Strain 23 try⁺⁺/mtr forms small rough colonies with jagged peripheries, strain 6051 try⁺⁺/mtr small round compact colonies, and strain 10774 try⁺⁺/mtr large and thinly spreading colonies, with compact raised centres of different textures. The growth of the three strains is differently inhibited or stimulated by indole and its two methyl analogues (Fig. 2). On the other hand, transformation studies (Table 4) suggest that, at least as far as the genes studied are concerned, strains 23 try⁺⁺/mtr and 6051 try⁺⁺/mtr are related because they easily cross-transform (Leidy, Hahn & Alexander, 1956; Marmur, Seaman & Levine, 1963; Marmur, Falkow & Mandel, 1963), whereas strain 10774 try⁺⁺/mtr is unrelated to them.

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