ANTHRANILIC ACID AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF TRYPTOPHAN BY *BACT. TYPHOSUM.*

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ANTHRANILIC acid has recently been shown, by a number of workers, to be implicated in the biosynthesis of tryptophan by micro-organisms. Thus, Snell (1943) found that anthranilic acid, like indole, could replace tryptophan for the growth of *Lactobacillus arabinosus* and *L. casei* ; similar, but less detailed observations have been made by others (Greene and Black, 1943 ; Wright and Skeggs, 1945 ; Schweigert, Säuberlich, Baumann and Elvehjem, 1946 ; Schweigert, 1947). Snell (1943) postulated anthranilic acid as a precursor of indole in the synthetic chain leading to tryptophan, but was careful to point out that his results did not suffice to distinguish between this and the alternative, but unlikely, possibility that anthranilic acid was an intermediate in the synthesis of tryptophan from indole. More direct evidence in the case of the mould *Neurospora crassa* was afforded by Tatum, Bonner and Beadle (1944), who obtained, by X-irradiation, two mutants, one of which (10575) grew well on indole, but not on anthranilic acid, while the other grew well on both substances ; furthermore, they showed that anthranilic acid accumulated in relatively large amount in the medium in which the former of these two mutants was growing. These results clearly indicate that the (single) genetic block in the mutant 10575 was in the conversion of anthranilic acid into indole and thus establish that anthranilic acid is indeed the precursor of indole, at any rate in the case of this particular mould. Finally, Lemberg, Tandy and Goldsworthy (1946) identified anthranilic acid in small amount (*ca.* 2 mg. l) in the culture filtrate from *Bact. coli* grown both in the presence and absence of sulphathiazole ; in these experiments the possibility that the anthranilic acid was really a degradation product of tryptophan was not excluded.

The present work had as its objective the establishment of the role of anthranilic acid as an intermediate in the biosynthesis of tryptophan by *Bact. typhosum*. Two complementary lines of investigation were followed : First, the investigation of the accumulation of anthranilic acid in growing cultures of suitably modified, "trained," strains of the organism and, second, an attempt to find inhibitors which should be reversed specifically by anthranilic acid, indole and tryptophan.

THE PRODUCTION OF ANTHRANILIC ACID BY TRAINED STRAINS OF *Bact. typhosum.*

Three strains were used in this investigation, viz. 3390*a*, which will grow on a simple ammonia medium, 3390*d*, which will grow on an ammonia medium supplemented with indole or tryptophan, and 3390, from which the other two

were obtained by training (Fildes, 1945), which required an amino-acid medium with either indole or tryptophan. It was hoped that at least one of the trained strains would have a genetic block, between anthranilic acid and indole, leading to the accumulation of anthranilic acid in the culture medium.

Experimental Methods.

Since it was desired to follow the production of anthranilic acid in the medium during growth, it was necessary to devise some analytical method which could be applied to fairly small volumes of liquid while being sufficiently sensitive to enable concentrations of anthranilic acid of the order of 1 μg. c.c. to be estimated. It may be said at once that this objective has only been partially achieved and a really satisfactory method for the estimation of anthranilic acid is still required.

The first method investigated was that of Kotake, Otani, Nishino and Imai (1941), which depends on reaction with a modified Ehrlich reagent followed by oxidation with hydrogen peroxide; in our hands the method was too insensitive and non-specific, and too much affected by small variations in the reaction conditions to be of any real value. The use of the first stage of the reaction, omitting the peroxide treatment (Tauber and Laufer, 1941) was more satisfactory but, although some preliminary unreported results were obtained with its aid, was too insensitive for convenience.

Recourse was finally made to diazotization and coupling with dimethyl-α-naphthylamine, the resulting red-violet colour being measured with an absorptiometer; interference by non-acidic aromatic amines was avoided by first extracting such substances at pH 7·5 and applying the coupling reaction only to material extracted from the medium when subsequently brought to pH 3·8. *p*-Aminobenzoic acid gives a similar, and more intense, colour, but its presence is readily detected since the colour develops rapidly, in the course of a few minutes, whereas that from anthranilic acid requires several hours for development. As a final check, the dyestuff was extracted from the combined coupled solutions from each experiment and subjected to chromatography, following Lemberg, Tandy and Goldsworthy (1946). The detailed procedure was as follows:

An aliquot of the growing culture (usually 25 or 50 c.c.) was removed and autoclaved. Bacterial bodies were removed by centrifugation and the clear supernatant brought to pH 7·5 and thoroughly extracted with ether; the residual aqueous layer was then brought to pH 3·8 with acetic acid and again thoroughly extracted with ether. This second etherial extract was dried over sodium sulphate and evaporated to dryness, finally for at least 24 hours in a vacuum desiccator over silica gel and sodium hydroxide. The residue was dissolved in N HCl (2 c.c.) and cooled in ice; 10 per cent sodium nitrite (1 c.c.) was added and the mixture kept in ice for 30 minutes. Twenty-five per cent urea (2 c.c.) was then added and the mixture kept in ice for a further 30 minutes. Finally, 50 per cent acetic acid (4 c.c.) and 2 per cent alcoholic dimethyl-α-naphthylamine (1 c.c.) were added and the well mixed solution kept at 0–2° overnight. Next day, the colour developed was measured with the Hilger "Spekker" absorptiometer using 1 cm. cells and filters H503 ("Calorex") and 605 (Ilford "Spectrum yellow-green"). The anthranilic acid concentration was then read off from a calibration curve, which was approximately linear up to a (final) concentration of 80 μg./c.c.; a reading of 0·200 corresponded to 20 μg./c.c. (final).

For chromatography the combined coloured solutions were treated with one-half to one-quarter their volume of M sodium acetate and the colouring matter extracted with ether. The ether extract was then shaken with 5 c.c. portions of 2 N sodium hydroxide, any colourless alkali extracts being discarded; the coloured alkaline extract was treated with M acetic acid (10 c.c.) and the dyestuff extracted with a little ether. This extract was then run, with gentle suction, through a column (200 mm. × 10 mm.) of activated alumina (B.D.H. "for chromatographic analysis") made up with 2 per cent acetic acid in ether, and developed with the same solvent. Contrary to the findings of Lemberg, Tandy and Goldsworthy (1946) we found that the anthranilic acid dyestuff gave a fast-running purple band (R = *ca.* 0·8) which was easily separable from the

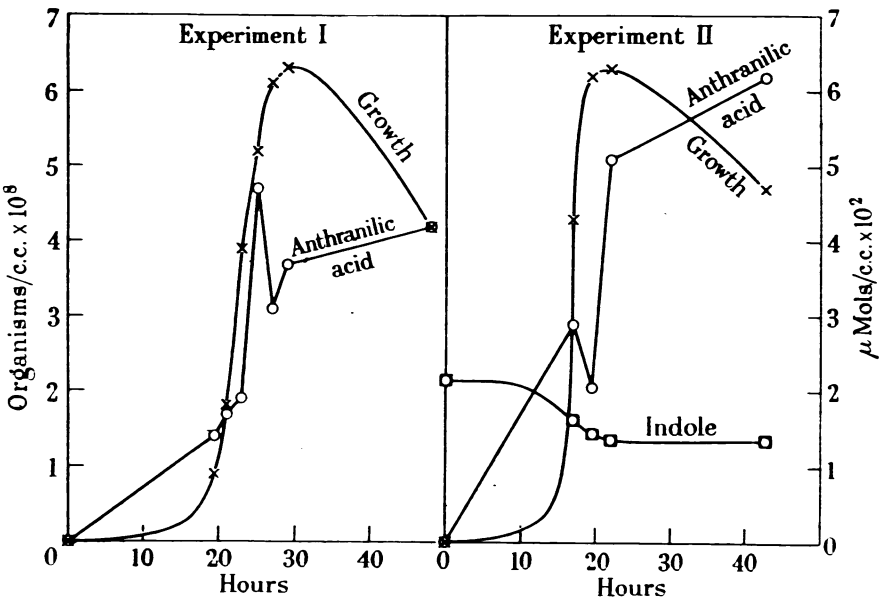


FIG. 1.—Production of anthranilic acid and utilization of indole; strain 3390*M*.

orange band of the *p*-aminobenzoic acid dyestuff which moved only about half as fast (R = *ca.* 0·4); the difference between these results, which were repeatedly confirmed, both by using the purified dyestuffs and by subjecting mixtures of the two amino-acids to the coupling procedure, and those of Lemberg, Tandy and Goldsworthy are no doubt to be ascribed to differences in the alumina used. Final confirmation of the identity of the products was obtained by mixing some of the eluate with an equal volume of a solution of the pure anthranilic acid dyestuff and chromatographing the mixture; failure to obtain two bands affords convincing evidence of the identity of the dyestuffs.

The organisms were grown, stagnant, at 37° in shallow layers in conical flasks (500 c.c. in a 2 l.; 300 c.c. in a 1 l.) in an atmosphere of air containing 5 per cent of carbon dioxide, from an inoculum sufficient to give, initially, 3,000 organisms/c.c. The media used were those of Fildes (1945), 3390 being grown

in the amino-acid medium in the presence of a final concentration of indole of M/50,000, 3390*a* in the ammonia medium, and 3390*d* in the same with the addition of indole to a final concentration of M/50,000. Bacterial growth was estimated by turbidity and indole with the Ehrlich reagent, after centrifuging, using in both cases the Hilger "Spekker" absorptiometer.

Experimental Results.

Strain 3390*d*. The results of two experiments are shown in Fig. 1; the fluctuations in the anthranilic acid values are no doubt to be ascribed to the inadequacy of the analytical method. In both experiments the dyestuff was extracted from the solutions after estimation of the colour and examined chromatographically; it proved to be homogeneous and inseparable from the authentic dyestuff prepared from pure anthranilic acid; the amount of *p*-aminobenzoic acid present in the solutions was certainly much less than 5 per cent of the total aminobenzoic acid present.

Strains 3390 *and* 3390*a*. The results obtained are shown in Table I.

TABLE I.—*Production of Anthranilic Acid by* Bact. typhosum 3390 *and* 3390*a*.

	Time (hrs.).	Organisms/c.c. $\times 10^8$.	Anthranilic acid µmols./c.c. $\times 10^2$.	Indole µmols./c.c. $\times 10^2$.
Strain 3390	0	0	0	2·1
	17·5	0·7	0·2	2·0
	19·5	1·7	0·2	1·5
	21·5	4·5	1·0	1·1
	23·5	6·2	0·7	1·0
	25·5	8·3	0·7	0·9
	27·5	8·9	1·3*	0·75
	29·5	8·4	1·0*	0·7
	48	6·4	1·2*	0·75
Strain 3390*a* (*no indole*)	0	0	0	
	22	0·8	1·0	
	24	1·0	0·8	
	26	2·5	0·8	
	28	5·3	0·7	
	30	5·7	0·7	
	32	6·1	0·6	
	36	5·2	..	
	54	5·1	0·8	
Strain 3390*a* (*M*/40,000 *indole*)	0	0	0	2·5
	20	0·9	1·6*	2·4
	23	3·0	1·5*	2·2
	26	5·7	1·1*	1·6
	28	6·4	0·4	1·4
	50	5·0	0·7	1·3

* Rapid colour development in these cases showed that much of the chromogenic material was *p*-aminobenzoic acid.

In a further experiment with strain 3390 two separate 500 c.c. batches were allowed to grow for 65 hours and then worked up as usual ; colorimetric estimation in a final volume of 10 c.c. in each case, showed that the final concentrations of anthranilic acid in the two runs were 0·2 μmol. c.c. and 0·35 μmol. c.c. (0·3 μg. c.c. and 0·5 μg. c.c.) ; chromatographic examination showed the presence of much anthranilic acid accompanied by a small, but definite, quantity of *p*-aminobenzoic acid.

Discussion.

It is clear from these results that anthranilic acid is produced and excreted into the medium by all three strains of *Bact. typhosum* investigated. In only one case, however, viz. the partially trained strain 3390*d*, is the amount large. It is important to note that, in this case, the amount of anthranilic acid produced is very much greater than the amount of indole used up, the ratio of these two amounts, at full growth, being about 7·5:1. It is thus certain that the anthranilic acid cannot have arisen by degradation of indole and must therefore have been synthesized, but not used, by the organism. It seems, then, that the genetic block which distinguishes 3390*d* from 3390*a* and makes the former dependent on indole is between anthranilic acid and indole. The present results thus provide evidence in favour of the view that anthranilic acid is the precursor of indole in the biosynthetic chain leading to tryptophan.

The Reversible Inhibition of *Bact. typhosum* by the Methyl-Anthranilic Acids

The methyl-anthranilic acids were chosen for investigation in consequence of the earlier finding (Fildes and Rydon, 1947) that the methyl-indoles and methyl-tryptophans were potent inhibitors of *Bact. typhosum*, interfering with the utilization of indole and of tryptophan, respectively. As test organism it was necessary to use a strain which could use anthranilic acid and strain 3390*a* was adopted since the work outlined above showed that, of our three available strains, only this one could fulfil this requirement ; it would have been better to have employed a strain which could use, but not synthesize, anthranilic acid, but it has so far not proved possible to obtain such a strain by training (private communication from Sir Paul Fildes).

Preparation of the Methyl-Anthranilic Acids.

N-Methyl-anthranilic acid was a specimen prepared by Dr. A. G. Lidstone with m.p. 178–179° (Wilstätter and Kahn, 1904, give m.p. 179°).

3-*Methyl-anthranilic acid* (3-methyl-2-aminobenzoic acid) was prepared by hydrogenating 3-methyl-2-nitrobenzoic acid (Müller, 1909) in alcoholic solution with Adams' catalyst. The product, after taking through acid to remove any unchanged nitro-acid, was recrystalized from water, forming needles, m.p. 168–169° (lit. m.p. 172°).

4-*Methyl-anthranilic acid* (4-methyl-2-aminobenzoic acid) was prepared by the method of Mayer and Günther (1930) and recrystallized from water, forming flat prisms, m.p. 174° (lit. m.p. 177°).

5-*Methyl-anthranilic acid* (5-methyl-2-aminobenzoic acid) was prepared from 5-methyl-2-nitrobenzoic acid (Müller, 1909) as described for the 3-methyl-compound and recrystallized from water, forming needles, m.p. 172–173° (lit. m.p. 172·5°).

6-*Methyl-anthranilic acid* (6-methyl-2-aminobenzoic acid) was prepared by the method of Gabriel and Thieme (1919) and recrystallized from benzene, forming leaflets m.p. 130–131° (Gabriel and Thieme, 1919, give m.p. 125–126°).

Methyl-Anthranilic Acids as Bacterial Inhibitors.

The test organism was *Bact. typhosum* 3390*a* and the tests were carried out as described by Fildes (1945) except that the inoculum (10,000 organisms per flask of 10 c.c.) was prepared by dilution of a 48-hour culture, the strength of which had been estimated by turbidity, using the "Spekker" absorptiometer.

In the first series of experiments (Table II) the five methyl-anthranilic acids were examined for their anti-bacterial activity, anthranilic acid itself being included as a control.

Attention may first be drawn to the inhibitory activity of anthranilic acid itself; this is most marked in the higher concentrations (M/100 and M/500), but some inhibitory activity persists down to concentrations as low as M/12,500. This finding cannot be regarded as inconsistent with the function of anthranilic acid as an intermediate in tryptophan synthesis since indole, which is generally accepted as an intermediate in this synthesis, was found by Fildes (1941) to be inhibitory at concentrations higher than M/4,000. The mechanism of this inhibitory activity of anthranilic acid remains to be elucidated; it is not markedly reversed by riboflavin (M/100,000).

Turning to the methyl-anthranilic acids it will be seen that two of these (the N-methyl- and 3-methyl-compounds) have inhibitory activities similar to that of anthranilic acid itself and one (the 6-methyl-compound) is completely non-inhibitory, while the other two (4- and 5-methyl-anthranilic acids) are much more inhibitory than anthranilic acid itself, the 5-methyl-compound being much the more potent inhibitor of the two. Further work was restricted to these two most inhibitory compounds.

Reversal of Growth Inhibition by 4- and 5-Methyl-anthranilic Acids.

The main object of the present work being to determine whether or not anthranilic acid was a precursor of tryptophan, experiments were carried out with four potential reversers of the inhibition, viz. anthranilic acid, indole, tryptophan and *p*-aminobenzoic acid, the argument being that, if anthranilic acid was in fact involved in the synthesis of tryptophan its reversing activity should resemble those of indole and tryptophan, rather than that of *p*-aminobenzoic acid which, so far as is known, plays no part in tryptophan synthesis. The results of these experiments are shown in Tables III and IV. The concentrations of the inhibitors were such as to minimize inhibition of the type shown by anthranilic acid itself.

TABLE II.—*Inhibitory Action of Anthranilic Acid and the Methyl-Anthranilic Acids.*

Inhibitor (Molarity × 10^6)	Growth in hours or days.*						
	22	28	48	3	4	5	7
Anthranilic acid							
10,000	0	0	0	0	0	++	++
2,000	0	0	++	++	++	++	++
400	0	Tr.	++	++	++	++	++
80	0	+	++	++	++	++	++
16	0	++	++	++	++	++	++
0	0	+±	++	++	++	++	++
N-Methyl-anthranilic acid							
10,000	0	0	+	++			
2,000	0	0	+±	++			
400	0	0	+±	++			
80	Tr.	+	++	++			
16	±	++	++	++			
0	±	++	++	++			
3-Methyl-anthranilic acid							
10,000	0	0	0	+±			
2,000	0	0	++	++			
400	0	0	++	++			
80	0	+	++	++			
16	0	+	++	++			
3·2	Tr.	++	++	++			
0	Tr.	+±	++	++			
4-Methyl-anthranilic acid							
10,000	0	0	0	0	0	0	0
2,000	0	0	0	0	0	0	0
400	0	0	0	0	++	++	++
80	0	0	++	++	++	++	++
16	±	+	++	++	++	++	++
3·2	+	+±	++	++	++	++	++
0	+	+±	++	++	++	++	++
5-Methyl-anthranilic acid							
10,000	0	0	0	0	0	0	0
2,000	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0
16	0	0	0	++	++	++	++
3·2	0	Tr.	++	++	++	++	++
0	±	++	++	++	++	++	++
6-Methyl-anthranilic acid							
10,000	+±	++					
2,000	+±	++					
400	+±	++					
80	+±	++					
16	+±	++					
3·2	+±	++					
0	+±	++					

* In this and the other tables + signs are proportional to the amount of growth; Tr. = trace; ++ = full growth.

Inspection of Tables III and IV shows that anthranilic acid, indole and tryptophan are all effective in reversing the inhibition of the growth of *Bact. typhosum* 3390*a* brought about by 4- and 5-methyl-anthranilic acids ; *p*-aminobenzoic acid, on the other hand, is ineffective in this respect. Since the inhibitory action of the methyl-anthranilic acids is reversed by both indole and tryptophan it is clear that these acids act by inhibiting some prior process in the synthetic chain leading through indole to tryptophan. That this inhibitory action is also reversed by anthranilic acid, but not by *p*-aminobenzoic acid indicates strongly that anthranilic acid is either the product or one of the reactants in the process inhibited by the methyl-anthranilic acids ; it thus follows that anthranilic acid must itself be involved in the biosynthetic chain leading to tryptophan.

TABLE III.—*Reversal of Inhibitory Action of 4-Methyl-Anthranilic Acid.*

Reverser (2×10^{-5}M).	4-Methylanthranilic acid (Molarity $\times 10^{6}$).	Growth in hours or days.			
		22	28	48	3
Anthranilic acid .	400	0	0	++	
	200	0	Tr.	++	
	100	±	+±	++	
	50	Tr.	+	++	
	25	Tr.	+	++	
	0	Tr.	+	++	
Indole . . .	400	0	±	++	
	200	±	+±	++	
	100	±	+±	++	
	50	±	+±	++	
	25	±	++	++	
	0	Tr.	+±	++	
Tryptophan . .	400	0	0	++	
	200	0	Tr.	++	
	100	0	+	++	
	50	0	+±	++	
	25	+	+±	++	
	0	0	+	++	
p-Aminobenzoic acid	400	0	0	Tr.	++
	200	0	0	0	++
	100	0	0	++	++
	50	0	0	++	++
	25	0	0	++	++
	0	Tr.	+	++	++
None . . .	400	0	0	0	++
	200	0	0	0	++
	100	0	0	+±	++
	50	0	0	++	++
	25	0	0	++	++
	0	0	+	++	++

TABLE IV.—*Reversal of Inhibitory Action of 5-Methyl-Anthranilic Acid.*

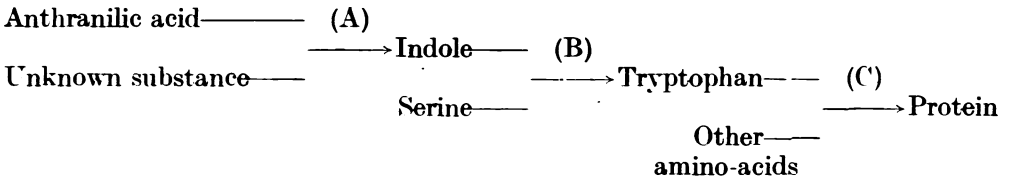
Reverser (2×10^{-5}M).	5-Methylanthranilic acid (Molarity $\times 10^6$).	Growth in hours or days. 22	28	48	3	5	7
Anthranilic acid	400	0	Tr.	++			
	200	0	++	++			
	100	Tr.	++	++			
	50	±	++	++			
	25	Tr.	+±	++			
	0	Tr.	+±	++			
Indole	400	0	±	++			
	200	±	++	++			
	100	±	++	++			
	50	±	++	++			
	25	±	+±	++			
	0	Tr.	±	++			
Tryptophan	400	0	0	++			
	200	Tr.	+±	++			
	100	Tr.	+±	++			
	50	Tr.	+±	++			
	25	±	++	++			
	0	0	±	++			
p-Aminobenzoic acid	400	0	0	0	0	0	0
	200	0	0	0	0	0	0
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
	25	0	0	0	++	++	++
	0	+±	++	++	++	++	++
None	400	0	0	0	0	0	0
	200	0	0	0	0	0	0
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
	25	0	0	0	++	++	++
	0	0	+±	++	++	++	++

The probable persistence down to low concentrations of some inhibitory action of the type shown by anthranilic acid itself makes it unwise to draw any further conclusions from these results. In particular it is not possible to decide whether the methyl-anthranilic acids act by interfering with the synthesis or with the utilization of anthranilic acid, and this question remains unanswered; the production of a strain of *Bact. typhosum* requiring anthranilic acid would go far towards solving this particular problem.

It would not be difficult to draw conclusions relating to the mode of combination of anthranilic acid with the bacterial enzyme involved in the reaction inhibited by the methyl-anthranilic acids, using arguments similar to those employed in the case of *p*-aminobenzoic acid (Rydon, 1947); the evidence, however, is slight and this aspect of the results will be fully discussed when experiments have been carried out with some other substituted anthranilic acids.

The Tryptophan Series of Biosyntheses.

The experiments described in this paper have afforded two independent lines of evidence leading to the conclusion that anthranilic acid is a precursor of indole in the biosynthesis of tryptophan by *Bact. typhosum* and, taken together, they may be regarded as establishing this point beyond reasonable doubt. We may summarize our present knowledge of the synthetic processes as follows :



In this scheme, process (A) is, probably, inhibited by the methyl-anthranilic acids, process (B) is inhibited by the methyl-indoles and indole-acrylic acid and process (C) by the methyl-tryptophans. The outstanding point now left for investigation is the nature of the substance which reacts with anthranilic acid to form indole.

SUMMARY AND CONCLUSIONS.

1. *Bact. typhosum* is capable of synthesizing anthranilic acid, not merely of producing it by degradation from indole or tryptophan.

2. 4- and 5-Methyl-anthranilic acids are potent inhibitors of the growth of *Bact. typhosum* ; this inhibition is reversed equally well by anthranilic acid, indole and tryptophan, but not by *p*-aminobenzoic acid.

3. It is concluded, from these two lines of evidence, that anthranilic acid is the precursor of indole in the biosynthesis of tryptophan by bacteria.

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