Studies on the Biosynthesis of Pyocyanine. (VII)
On the Effect of Anthranilic Acid. (1)

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From a reexamination on the mechanism of inhibitory action of aromatic compound on pyocyanine formation, there has arisen the new possibility that anthranilic acid is an intermediate product in pyocyanine synthesis. As an evidence supporting the above concept, besides its effect on pigmentation independent of bacterial growth, it was found that according to the cultural condition, such an accumulation product as regarded to be derived from anthranilic acid was recognized in a culture medium of the bacteria. On the other hand, an interrelationship in metabolism has been pointed out between anthranilic acid and methionine, in expectation that the product derived from anthranilic acid might be assigned a role of the methyl acceptor.

INTRODUCTION

In the preceding paper[1], it has been emphasized that biosynthesis of pyocyanine was not performed through such a way as the chemical synthetic process presented by Wrede *et al.*,[2] and that the mechanism of this synthesis might be so complicated that a possible intermediate was hardly postulated in the known compound. From these facts, recognition of the intermediate has been obliged to depend throughout on the bacterial culture, and every effort was directed toward this work. In practice, however, it was a very difficult matter to obtain the intermediate from the bacterial cultures, owing to an extremely unstable property, as has been experienced in the preceding work on the product derived from pyocyanine by fungi[1]. On the other hand, it has been shown that an intermediate in the process of the chemical synthesis by the method of Wrede *et al.*, gave rather inhibitory effect on pyocyanine formation even in the concentration where the bacterial growth was not affected. This phenomenon was observed with major aromatic amines or polyphenols.

If one might become aware of the mechanism of their inhibitory effect on pigmentation to be based on the antagonistic action, then one would be able to introduce a concept of "metabolite analogue" and to point out that, at least, any one of the intermediate products in pyocyanine synthesis might belong to an aromatic compound. According to these concepts the effect of several aromatic compounds was once more reexamined and it was found that anthranilic acid, which had been disregarded from a negative effect of tryptophan, revealed an increasing effect on pyocyanine formation, whereas every other compound has not given any positive effect on pigmentation. On the other hand, anthranilic acid was found to neu-

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tralize competitively the inhibitory action of the other aromatic compound on pyocyanine formation.

In the present paper, the effect of anthranilic acid will be presented, being compared with other aromatic compound, and some discussions on its metabolism will be brought up.

EXPERIMENTAL AND DISCUSSION

Effect of Anthranilic Acid

In order to serach for the effective matter for pigmentation among the aromatic compounds, the following experiment was carried out : to the medium composed of 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$, pH 7.4, were added the aromatic compounds dissolved in alcohol and incubated at 37° for 4 days. Table 1 shows that increasing effect on pyocyanine formation was noticeably revealed with anthranilic acid, while rather inhibitory action was observed with other compounds.

As was already presented, among various amino acids constituting casein, only

Table 1. Effect of aromatic compound on pyocyanine formation.

Compound(M)		2×10^{-2}	10^{-2}	5×10^{-3}	2×10^{-3}	10^{-3}	5×10^{-4}	2×10^{-4}	10^{-4}
Phenol	p	×	−	−	Trace	+	###	###	###
	g		++	###	###	###	###	###	###
Resorcinol	p	×	−	−	−	−	+	++	###
	g		++	###	###	###	###	###	###
Guajacol	p	×(×)	×	−	−	++	+	###	###
	g			++	++	###	###	###	###
Pyrogallol	p	×(×)	−(×)	−(×)	−(×)	+(−)	##(###)	###(###)	##(###)
	g		+(×)	++(×)	++(×)	###(++)	###(###)	###(###)	###(###)
p-Aminophenol	p	×(×)	−(×)	−(×)	Trace	−(−)	++(++)	###(###)	##(###)
	g		++(×)	###(×)	###	###(++)	###(###)	###(###)	###(###)
Acetanilide	p	−(×)	−(×)	−(−)	++(−)	###(++)	###(###)	###(++)	##(###)
	g	++(×)	++(×)	###(+)	###(###)	###(###)	###(###)	###(###)	###(###)
o-Phenylene-diamine	p	−(×)	−(×)	−(−)	+(−)	++(+)	###(++)	###(++)	###(###)
	g	+(×)	++(×)	###(+)	###(++)	###(###)	###(###)	###(###)	###(###)
Aniline	p	(−)	(−)	+	(++)	(###)	(###)	(###)	(###)
	g	(++)	(###)	(###)	(###)	(###)	(###)	(###)	(###)
Brenzcatechine	p	(×)	(×)	(×)	(+)	(+)	(###)	(###)	(###)
	g				(++)	(###)	(###)	(###)	(###)
Hydroquinone	p	(×)	(×)	(×)	(×)	(×)	(−)	(###)	(###)
	g						(++)	(###)	(###)
Anthranilic acid	p	(###)	(###)	(###)	(###)	(###)	(###)	(###)	(###)
	g	(###)	(###)	(###)	(###)	(###)	(###)	(###)	(###)
Control	p	###(###)							
	g	###(###)							

Basal medium : 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$, pH 7.4. (+), (++), (###), (###) Represent the amount of pyocyanine of 0.002%, 0.005%, 0.01% and 0.02%, and the bacterial cell number of 10^8, 2×10^8, 5×10^8 and 10^9 per ml in rough estimation, respectively ; +, ++, ###, ###, the same amount of pyocyanine and the bacterial cell number as above in the case of peptone-containing media. (×), ×No multiplication. g, Bacterial growth ; p, pyocyanine. Strain By was incubated at 37°C for 4 days.

methionine was pointed out to affect the formation of pyocyanine. Thus, the effect of tryptophan could not be recognized in the work hitherto done.[3] This result is shown to expatiate in Table 2.

Table 2. Effect of aromatic compound on pyocyanine formation

Compounds (*M*)	5×10^{-3}	2×10^{-3}	10^{-3}	5×10^{-4}	2×10^{-4}	10^{-4}	Nil
Anthranilic acid	7.2	14.2	16.2	10.0	6.2	4.8	5.0
p-Aminobenzoic acid	—	Trace	4.4	7.2	3.8	5.6	4.8
Benzoic acid	2.4	4.8	4.2	6.8	4.2	4.0	4.0
Salicylic acid	—	—	2.0	6.4	6.2	5.0	5.2
DL-Tryptophan	6.2	6.6	5.4	6.8	4.8	4.4	4.6
L-Phenylalanine	4.2	4.4	4.4	4.2	4.6	5.2	5.0
DL-Tyrosine	5.6	3.6	3.6	5.2	6.2	3.8	4.2
Indole	—	3.2	6.4	5.4	4.6	5.6	5.8
Indoleacetic acid	—	2.4	6.0	6.0	3.8	4.4	4.6

Bacterial strain B_1 was incubated at 37° for 4 days. Basal medium: 2.5% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_3(SO_4)_3$; pH 7.4. Pyocyanine was expressed in mg%.

For the reason why anthranilic acid reveals an increasing effect on pigmentation whereas the effect of tryptophan is hardly recognized, the following explanation will be brought up: The present strain can grow on the synthetic medium containing urea as a sole source of nitrogen, so that tryptophan should be synthesized from inorganic nitrogen, together with other amino acids. Therefore, anthranilic acid must also be synthesized by the bacteria as a precursor of tryptophan, hence the effect of anthranilic acid on pigmentation may be due to such a capacity of the bacteria for the synthesis of anthranilic acid, as does not allow to form beyond a level of sustaining the growth.

Based on these concepts, the strain unresponsive to anthranilic acid may exist according to the bacterial capacity for the formation of anthranilic acid. Similarly, no effect of tryptophan may be illustrated by considering the mechanism mentiond above, or by the less capacity to convert tryptophan to anthranilic acid.

Antagonism between Anthranilic Acid and Other Aromatic Substance

As was shown in Table 1, there was usually observed a discrepancy between the limiting concentrations of inhibitors of pyocyanine formation and of bacterial growth. It is suggested from this fact that not only major inhibiting substances but anthranilic acid is also concerned with both systems. However, it remains obscure whether the inhibitory effect will be ascribable to antagonistic action or not. To this question the result shown in Table 3 would respond: among the substances showing the inhibitory action, some aromatic amines are regarded as an antimetabolite of anthranilic acid, although the inhibitory effect of many of polyphenols is not necessarily attributable to the antagonistic inhibition, because the inhibition shown by the amines such as aniline, acetanilide or *o*-phenylenediamine is neutralized by the addition of anthranilic acid, while polyphenols, with

the exception of resorcinol, are not suppressed to inhibit both pigmentation and bacterial growth even at a high level of anthranilic acid administered. Resorcinol was observed to resemble the amine mentioned above, surffering the effect of anthranilic acid, although on this mechanism none is known. Even in this experi-

Table 3. Antagonistic action between anthranilic acid and some aromatic compounds.

Inhibiting matters (M)		5×10^{-3}	2×10^{-3}	10^{-3}	5×10^{-4}	2×10^{-4}	10^{-4}
Resorcinol	U	×	−	3.2	4.4	5.0	6.8
			+	++	+++	+++	+++
	UA	−	4.2	8.6	14.2	12.8	12.4
		++	+++	+++	+++	+++	+++
p-Aminophenol	U	×	×	×	−	4.8	6.4
					+	++	+++
	UA	×	×	−	−	5.6	10.2
				+	++	+++	+++
Hydroquinone	U	×	×	×	−	−	3.0
					+	++	+++
	UA	×	×	×	−	−	6.4
					++	+++	+++
Aniline	U	−	−	3.2	6.6	4.2	5.6
		++	+++	+++	+++	+++	+++
	UA	4.2	6.2	12.2	8.6	14.2	16.0
		+++	+++	+++	+++	+++	+++
Acetanilide	U	−	−	2.0	4.6	4.6	5.2
		+	+++	+++	+++	+++	+++
	UA	3.0	6.4	10.2	16.0	12.2	16.0
		+++	+++	+++	+++	+++	+++
o-Phenylenediamine	U	−	−	3.6	5.4	4.2	5.8
		+	++	+++	+++	+++	+++
	UA	4.0	6.4	8.2	14.2	16.2	10.2
		+++	+++	+++	+++	+++	+++

U : medium containing 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$; pH 7.4. UA : U+$2\times10^{-2}M$ anthranilic acid. Pyocyanine was expressed in mg%. Expression of the bacterial growth was the same as in Table 1.

ment, there has also existed, as shown in Table 3, a discrepancy between limiting concentrations of the inhibitors of pyocyanine formation and of bacterial growth, even in the presence of anthranilic acid. This phenomenon may be interpreted to be based on the difference between the capacities for the combinations of anthranilic acid and of inhibitor with enzymes in both systems.

On the other hand, it has been noted that in the medium containing peptone, the inhibitory action was hardly recognized at a fairly high level of the inhibitors, in comparison with the case of synthetic medium (Table 1). This reason may be due to the possibility that such an amino acid or other growth factor exists in peptone, as assigns its precursor to anthranilic acid.

Effect of Anthranilic Acid on Pyocyanine Formation and Bacterial Growth

It was observed that the present bacteria could grow to some extent by feeding anthranilic acid as a sole source of carbon and nitrogen, and that when glycerol was added to it, bacterial growth was satisfactory and pyocyanine was produced. This facts signify that anthranilic acid is cleaved to carbon and nitrogenous compounds to be available for the bacterial growth through an aromatic

pathway.[4-10] However, the anthranilic acid added to the medium prepared with favorable carbon and nitrogen sources is considered to be directly utilized as a possible precursor of pyocyanine through an anabolic pathway, and its effect will be revealed independently of the bacterial growth, so that pyocyanine formation may be expected even by the addition at a stationary growth phase. Table 4 and Fig. 1 show the result of the experiment on the effect of anthranilic acid

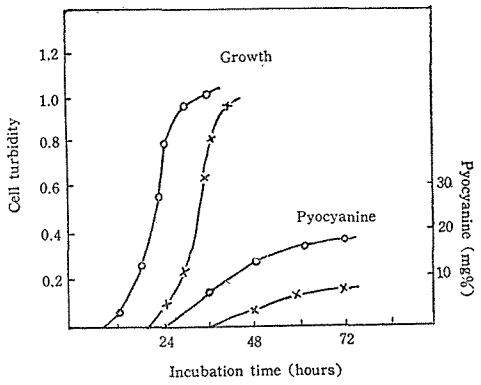

Fig. 1. Effect of anthranilic acid on pyocyanine formation.
(×—×—×) Results with the same medium as U in Table 3 ; (○—○—○) with the same medium as UA in Table 3.

Table 4. Effect of anthranilic acid on pyocyanine formation.

Medium	Pyocyanine (mg%) Strains					Glycerol remained (%) Strains					Bacterial cell number ($\times 10^8$/ml) Strains				
	B_Y	B_n	B_K	B_A	B_t	B_Y	B_n	B_K	B_A	B_t	B_Y	B_n	B_K	B_A	B_t
U	12.0	5.2	4.0	3.6	3.8	1.20	1.14	1.00	1.20	1.14	10.8	11.0	9.6	10.2	11.8
UA	23.0	11.2	9.8	4.0	7.2	1.18	1.18	0.99	1.12	1.04	11.2	10.6	9.6	12.3	12.0
UM	16.2	11.0	5.0	9.6	6.6	1.08	1.02	1.06	1.03	1.12	12.0	10.4	—	11.6	12.0
UAM	24.6	21.0	9.0	11.6	16.0	1.16	1.15	1.10	1.18	1.08	11.8	9.8	11.0	12.3	11.0

U : the medium composed of 2.5% glycerol, 0.2% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$; pH 7.4.
UA : U+0.002*M* anthranilic acid ; UAM : UA+0.001*M* methionine. Incubated at 37° for 3 days.

designed in consideration of the bacterial growth. As shown in the table or the figure, a noticeable effect of anthranilic acid has been shown on pyocyanine formation, whereas on bacterial growth its effect was not appreciable. However, as a suggestion for the precursor of anthranilic acid in the growth system, its lag phase was observed to be shortened as in the case of the pigmentation.

In spite of a remarkable effect of anthranilic acid administered at an initial stage, its effect was not so satisfactory in the case of the addition after 48 hours' incubation, although a considerable effect was expected by the addition within 24 hours of the incubation (Table 5).

As mentioned before, the strain revealing a remarkable pigmentation even in the synthetic medium prepared with urea as a sole source of nitrogen, must be

Table 5. Effect of anthranilic acid added at a different growth stage.

Strains	Growth stages (incubation hours after when AA was added)				
	initial	24	48	72	Control
B_Y	24.2	21.6	12.6	12.0	12.0
B_1	11.2	11.0	6.8	5.6	6.0

Medium was the same as in Table 1. Pyocyanine was measured after 84 hours of total incubation times, and expressed in mg%. AA : Anthranilic acid.

regarded to have the ability to synthesize fully the intermediate product at every step in pyocyanine formation system only from glycerol and urea, so that anthranilic acid as well as every other organic matter is not required to be administered. Similarly, even among the strains which do not fully produce pyocyanine in synthetic medium, such a strain which does not respond to anthranilic acid will exist according to its synthesizing capacity. In fact, the strain as described above has been isolated, of which the majority was observed to be responsive to methionine.

On the other hand, such a strain as responds to both anthranilic acid and methionine was isolated. These facts may suggest that methionine and anthranilic acid are not only concerned with the synthesis of pyocyanine but also there exists an interrelationship in role between both substances.

Effect of Anthranilic Acid on Pigmentation in Resting System

In order to test the effect of anthranilic acid on pyocyanine formation in resting system, the following experiments were attempted.

Preparation of resting cells. Resting cells free from pyocyanine were prepared according to the method presented in the previous paper[1].

Determination of anthranilic acid. Anthranilic acid was determined by

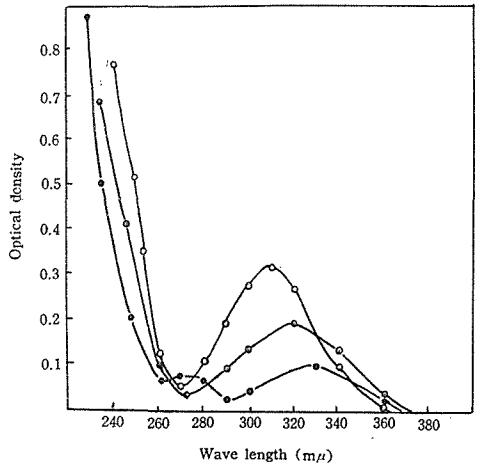

Fig. 2a. Absorption spectra of anthranilic acid. (○—○—○) With $5\times10^{-3}M$ NaOH solution, (◎—◎—◎) aqueous solution, (●—●—●) $5\times10^{-3}M$ HCl solution. Concentrations of anthranilic acid were approximately $10^{-4}M$

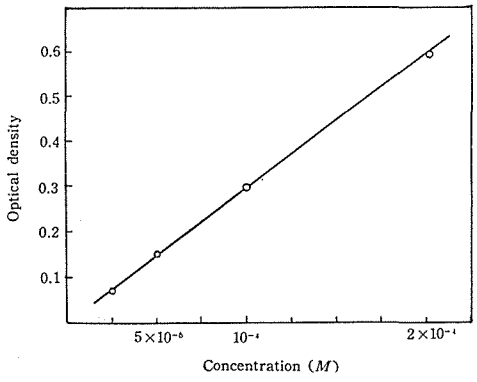

Fig. 2b. Relation between the concentration of anthranilic acid and the value of optical density at 310mμ with neutral aqueous solutions.

measuring the value of optical density at 310 mμ with neutral sample solution (Fig. 2b). In this case, it should be noted that absorption peak of anthranilic acid moves according to the pH value. The absorption maximum shown at 310 mμ with neutral or alkaline aqueous solution, is indicated to move toward 330 mμ with acidified solution, and its extinction is measured in lower value in proportion to the fall in pH (Fig. 2a). On the other hand, if the measurement was performed at 520 mμ with the sample through a diazotization procedure, the product derived from anthranilic acid which also revealed diazotization reaction, would affect the result of determination.

Preparation of reaction mixture. The following respective materials were dissolved in 100 ml of distilled water containing 0.025 % K_2HPO_4 and 0.025 % $MgSO_47H_2O$, and resting cells were suspended at a level of 0.02 g per ml of the solution : (1) 0.0137 g of anthranilic acid ; (2) 0.0137 g of anthranilic acid and 0.1g of sodium succinate ; (3) 0.0137 g of anthranilic acid, 0.0149 g of methionine and 0.1 g of sodium succinate ; (4) 0.1 g of sodium succinate.

Treatment of reaction mixture. After the incubation of desired period, reaction mixture was treated with calcium phosphate gel to remove the bacterial cells, washed down with distilled water through a filter and adjusted to definite volume.

In this case $ZnSO_4$ must not be used as a protein precipitant, for a green colored product regarded as a complex of anthranilic acid and Zn is formed, which shows absorption maximum at 610 mμ.

Absorption spectrum of reaction mixture. In the present experiment, pyocyanine could hardly be formed and the result shown in Fig. 3 was obtained. With the sample incubated for 6 hours, absorption curve appeared to be of anthranilic acid. However, if this sample was treated with ethyl ether to remove anthranilic acid remaied, the absorption curve of the residue would be given as in the case of 16 hours' incubation, indicating that absorption peak moves from 310 mμ to 260 mμ in proportion to the consumption of anthranilic acid. As shown

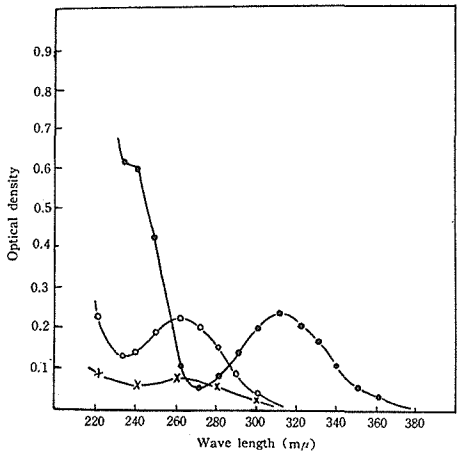

Fig. 3. Absorption spectrum of reaction mixture of anthranilic acid. (●—●—●) Anthranilic acid, (○—○—○) reaction product, (×—×—×) control.

Table 6. Effect of various substances on anthranilic acid metabolism.

Substrates	Optical density at 260 mμ	Anthranilic acid remained (mg%)	pH (final)
A	0.210	2.9	7.8
A+M	0.260	0.8	7.6
S	0.200	—	8.4
A+S	0.570	Trace	8.8
A+M+S	0.610	—	8.8
M+S	0.320	—	8.2
G	0.184	—	7.6
A+G	0.388	Trace	7.6
A+M+G	0.404	—	7.4
A+(G)	0.178	12.0	4.5
Nil	0.180	—	7.4

A, $10^{-3}M$ anthranilic acid ; M, $10^{-3}M$ methionine ; S, $10^{-2}M$ succinate ; G, $10^{-2}M$ glycerol ; (G), $10^{-2}M$ glucose. Incubation, at 37° for 16 hours.

in Table 6, it was found that samples Nos. 2 and 3 containing respectively 0.1 % succinate were highly measured in value of optical density at 260 mμ as compared with No. 1.

In this case, when glucose was used as a respirating substrate, anthranilic acid could hardly be consumed and reaction product was not formed owing to the fall in pH. Similar phenomenon has also been observed in the incubation under an anaerobic condition.

It has been found that the consumption of anthranilic acid was accelerated by the administration of methionine with the appreciable increase in the amount of the product showing the absorption maximum at 260 mμ, although the relation between both fluctuations was not always proportional (see Table 6). On the other hand, it was ascertained that the absorption band could not be ascribed to

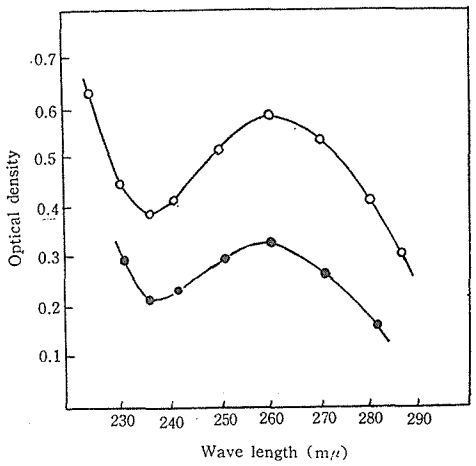

Fig. 4. Absorption spectrum of accumulation product in bacterial culturers. (○—○—○) Accumulation product, (●—●—●) reaction product from anthranilic acid.

the aromatic amino acid due to the autolysis of the cells, since the detection of amino acid was negative except the sample No. 3.

In connection with the results shown above, however, another question has come to arise : the present product might be attributed to the secondary growth of the bacteria due to the administrations of anthranilic acid and succinate, because the same absorption spectrum as with the reaction product from anthranilic acid was found to be revealed with the cultured solution of the bacteria grown especially on the excess of phosphate used (Fig. 4).

And so, the following experiment was carried out to answer the above question : 0.0005*M* urea, 0.0005*M* $(NH_4)_2SO_4$ or 0.001*M* glutamic acid was respectively substituted for 0.001*M* anthranilic acid in reaction mixture. In parallel with this, the synthetic media of 0.1*M* succinate which contained respectively one of the following nitrogenous matters, were prepared : 0.001*M* anthranilic acid, 0.001*M* glutamic acid or 0.0005*M* urea.

These results are shown in Figs. 5a and 5b. From the figures, it may be clear that the product of reaction mixture can not depnd on the secondary growth of the bacteria but can originate from anthranilic acid itself.

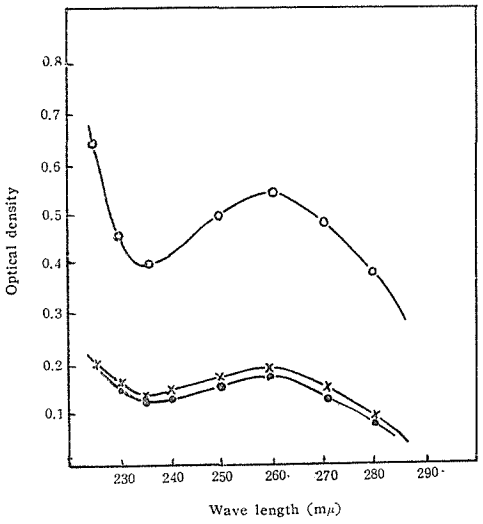

Fig. 5a. Absorption spectra of the reaction mixtures incubated in the presence and absence of anthranilic acid.
(○—○—○) 0.001*M* anthranilic acid+0.01*M* succinate ; (×—×—×) 0.0005 *M* urea+0.01 *M* succinate ; (●—●—●) 0·01*M* succinate.

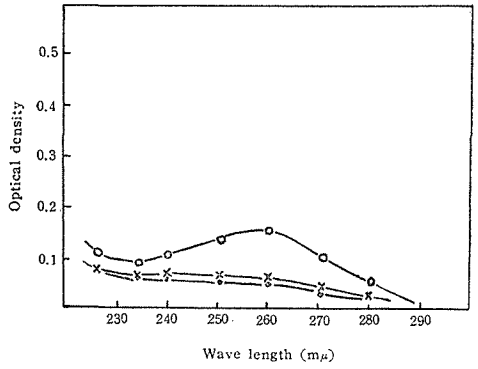

Fig. 5b. Absorption spectra of culture extract of the bacteria grown on anthranilic acid, on urea and on glutamic acid.
(○—○—○) With the samplefrom the medium containing 0.001 *M* anthranilic acid and 0.01*M* succinate, (×—×—×) from the medium containing 0.0005*M* urea and 0.01*M* succinat, (●—●—●) 0.001*M* glutamic acid and 0.01*M* succinate.

It is noteworthy that even at an extremely low level, the presence of anthranilic acid brings about the formation of the metabolite expected to be the same as the reaction product from anthranilic acid, whereas these phenomena were not observed with the other cases. These results will further suggest that what is recognizable in the culture medium of the bacteria may be identical with the product derived from anthranilic acid by the resting cells. This possibility has

also been supported in similarity of the effect on pyocyanine formation between both products, as will be illustrated later (see Table 7).

Metabolism of Anthranilic Acid

From the fact that by the addition of respirating substrate, an increment of the reaction product is brought about, and from the fact that its effect on pyocyanine formation is maintained beyond that of anthranilic acid itself, this product may be regarded as an intermediate at a reaction step in anabolic pathway.

It has widely been known that anthranilic acid is catabolized into β-ketoadipic acid *via* catechol and *cis,cis*-muconic acid by cell-free preparation of *Pseudomonas* strain[4-10]. If the present product is the intermediate in catabolic pathway, it must be one of the compounds mentioned above. However, any one of these compounds has never revealed the effect on pyocyanine formation, and was not identified with the present reaction product in the comparative experiments by Rothera test,[11] spectroscopic and paper chromatographic techniques. Fig. 6 shows

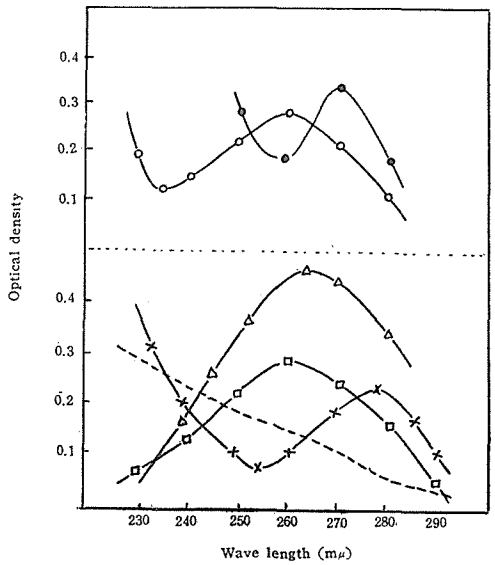

Fig. 6. Absorption spectra of the products derived from anthranilic acid.
(○—○—○) With the reaction product of anthranilic acid by resting cells, (●—●—●) auto-oxidation product of anthranilic acid, (□—□—□) *cis, cis*-muconic acid, (△—△—△) *trans, trans*-muconic acid, (×—×—×) pyrocatechol, (·····) laevlic acid.

the absorption spectra of the products which are derived from anthranilic acid by biochemical or automatic oxidation. To the contrary, however, in the experiment with use of acetone dried cells, Rothera reaction positive product was recognized in the incubation mixture of anthranilic acid, catechol or *cis, cis*-muconic acid, suggesting the formation of β-ketoadipic acid. In the experiment with use of the cells through a freezing procedure, Rothera reaction was also found to be somewhat positive in every case, although the formation of the product revealing

absorption maximum at 260mμ was simultaneously detected from anthranilic acid and catechol. But the absorption band of the latter was found to be based on the formation of *cis,cis*-muconic acid, referring to the paper chromatographic and photometric tests.

From these results, the reaction product from anthranilic acid by growing or resting cells must be regarded to depend mostly on the anabolic reaction, although negative Rothera reaction may, in part, be attributed to the further metabolism of β-ketoadipic acid into acetic, succinic and other organic acids[7]. What has hitherto been known as the metabolic pathway other than aromatic one, is tryptophan synthesis and its conversion to nicotinic acid or to quinoline dervatives (Fig. 7)[12-37]. Of the compounds shown in Fig. 7, only nicotinic acid was observed

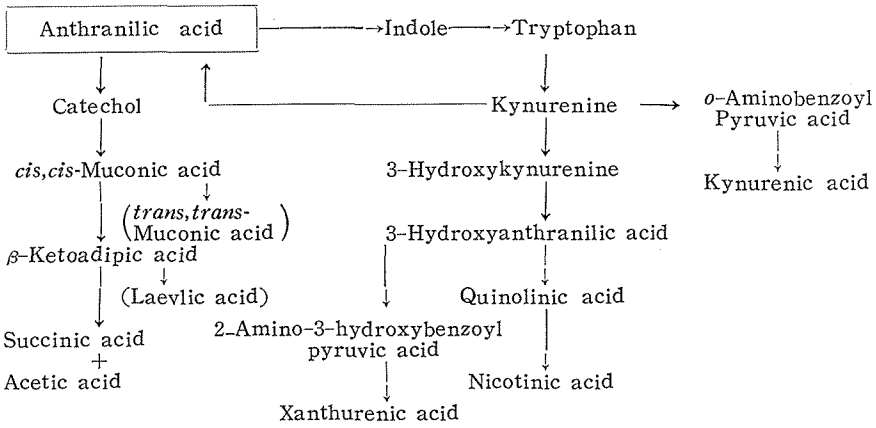

Fig. 7. Pathways of anthranilic acid metabolism.

to resemble the present product in shape of absorption curve, but its effect on pyocyanine formation was hardly recognized and phosphomolybdate test was negative against the positive reaction of the present product, as will be illustrated later. On the other hand, this product is also similar in absorption curve to nucleic acid, or more to a kind of polynucleotides of RNA type reported by Pinchot[38] as a new coenzyme of oxidative phosphorylation in *Alcaligenes faecalis*.

However, these substances may hardly be approved to be excreted in the medium of the bacteria, after the same manner as in a culture medium of yeast or major bacteria, in which the product showing a characteristic absorption bands could not be recognized, differing from the case of the present bacteria.

At any rate, any one that appeared to correspond with the present product could not be taken up from the members in reaction sequence of tryptophan catabolism, so that the reaction product must be regarded to be of the third pathway as yet unknown.

One of the reasons why the reaction product was regarded to be of the anabolic pathway of anthranilic acid, could also be found out in its greater effect on pigmentation than that of anthranilic acid itself, as in the case of the accumulation product in the medium of excess of phosphate. Thus, a possibility of the identity between both of the products mentioned above has also been pointed out

in the effect on pigmentation. When the preparation from the cultured solution of the bacteria through the extraction treatment with butanol was fed to the mutant strain which lost the ability to form pyocyanine, pigmentation was permitted to bring about in both cases. If these preparations were added to the synthetic medium of the normal strain, pyocyanine formation would remarkably be promoted rather beyond the case of peptone medium.

However, it is considerably doubtful to attribute the effect on pigmentation to the fraction revealing the absorption peak at 260 mμ, so that the following tests were carried out : the cultured solution of the bacteria and the reaction mixture were extracted with pyridine, after being saturated with ammonium sulfate and pyridine layer was transferred into a small amount of aqueous solution by shaking with excess of ethyl ether, and then concentrated under reduced pressure to be mounted on paper chromatography using the solvent system, *n*-butanol-alcohol-water (1 : 1 : 2). The paper chromatographed was sectioned to 10 fractions in order to examine the effect on pigmentation and the absorption spectrum. Results are shown in Table 7. It has apparently indicated in good agreement

Table 7. Effects of the product derived from anthranilic acid and of the accumulation product in the medium, through a chromatographic fractionation.

Fraction (Rf)	Optical density at 260mμ		Pyocyanine produced (mg%) Strains			
			B_1		C_1	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
10	0.002	0.002	5.0	5.0	—	—
9	0.003	0.002	4.8	5.2	—	—
8	0.002	0.003	6.0	4.8	—	—
7	0.008	0.005	5.4	6.0	—	—
6	0.154	0.242	6.8	12.0	4.0	1.0
5	0.675	0.680	21.8	22.4	6.8	5.0
4	0.312	0.580	14.0	21.0	3.8	5.4
3	0.222	0.250	8.6	11.0	2.0	2.0
2	0.058	0.080	6.2	7.0	—	Trace
1	0.032	0.032	6.0	5.4	—	—

Basal medium was the same as in Table 1.
No. 1: Fraction from the reaction mixture of anthranilic acid.
No. 2: From the cultured solution of the bacteria grown on the excess of phosphate.

between both cases that the fractions showing absorption maximum at 260 mμ were those that brought about the effect on pyocyanine formation.

The fact that phosphomolybdate test is given to be positive in both cases, suggests that these products possess a benzene ring and that neither condensation of two benzene rings nor closure of phenazine nucleus would as yet come to a step. For the question why the cultural solution or the reaction mixture of anthranilic acid stops in their reaction at the step mentioned above, the following discussions will be brought up :

1. In the medium containing the excess of phosphate, the formation or the

action of enzyme in pyocyanine synthesis system may be prevented at some step in reaction series. Judging from the result in the preceding work on the enzymatic synthesis of pyocyanine from the blue colored product derived from pyocyanine by fungi[1], the formation of the enzyme should be regarded not to take place, but to be also based on the result whose details will be presented in the following report : this mechanism will be supported to be rather due to the inhibition of the enzyme action.

2. A moiety may be wanted for the condensation or the closure of rings. In addition to this possibility, for the enzyme action at these steps a higher free energy may be required as compared with the other reaction steps. Hence the enzymic activity will come into question : supposedly, in the resting system, the energy required for the synthetic reaction may not so fully be satisfied as in the growing system, considering together that the free energy released by the bacterial respiration may simultaneously be put in the other reaction systems assigning its substrate to anthranilic acid.

Isolation Procedure and Characteristics of Reaction Product

Although this product has not come to be purified owing to the labile property or to the insolubility in major organic solvents, and yet some informations will be presented about its characteristics. As mentioned before, this product is insoluble in ethyl ether and many other organic solvents, so that it can easily be separated from anthranilic acid. On the other hand, this was found to be adsorbed onto active charcoal, although the elution was not necessarily easy. When $ZnSO_4$ was used as a protein precipitant of the reaction mixture, the characteristic absorption band of the product become to be lost, probably on account of its degradation.

Isolation procedure has been carried out as follows : The cleared reaction mixture through the treatment with calcium phosphate gel was adjusted to pH 5.0 and 2% charcoal was added to it. And the charged adsorbent, followed by washing with distilled water, was eluted with pyridine or lutidine. Then, the blue fluorescent eluate solution was transferred into a small amount of aqueous solution by shaking with excess of ethyl ether or chloroform. In parallel experiment, the reaction mixture was directly extracted and aqueous solution was obtained according to the procedure mentioned before. The concentrated aqueous solutions were prepared by repeating the transference between its organic solvent and aqueous solutions so as to employ on chromatography. These concentrated aqueous solutions thus obtained were developed on column of alumina using the solvent system, *n*-butanol-alcohol-water (2 : 1 : 2).

In spite of these purification procedures, it has come to be found out that the isolation of the product was a very difficult matter, because the reaction mixture regarded as one component was found to be a mixture of several substances which were considered to be derived mainly from the reaction product owing to its unstable property, as seen in Fig. 8. representing the result of paper chromatography using the slolvent system, *n*-butanol-acetic acid-water (5 : 1 : 2). The degradation of the product was caused during the purification procedure, espe-

cially when charcoal was chosen for the adsorption treatment. If the fraction soluble in butanol was concentrated under reducing pressure through air current, it would easily be broken down into the product showing an intensive fluorescence similar to that of anthranilic acid.

It has been found that this product gave a positive reaction in the phosphomolybdate test as already described, and also gave a diazotization reaction as in the case of anthranilic acid, which could be distinguished from that in anthranilic acid in solubility of the diazotization product in ethyl ether and other organic solvent. On the other hand, it was noted that both cation and anion exchange resins were used interchangeably without an apparent difference in result. From these facts, the present product may be an amphoteric compound belonging to the aromatic one.

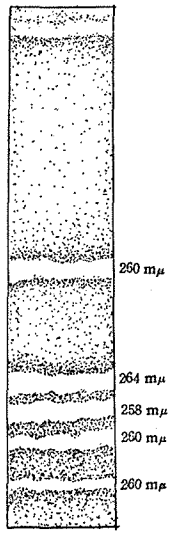

Fig. 8. Paper chromatograms of reaction mixture of anthranilic acid. Solvent system, *n*-butanol-acetic acid-water (5 : 1 : 2). Figures express respectively an wave length at which absorption maxima of each fraction are shown.

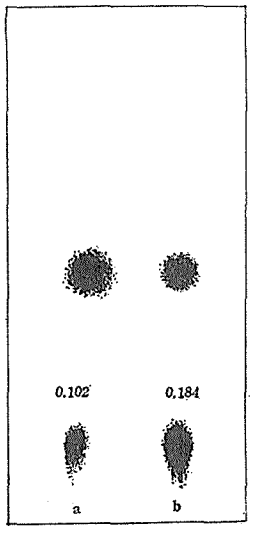

Fig. 9. Paper chromatograms of the reaction mixtures of methionine incubated in the presence and absence of anthranilic acid.

Solvent system, *n*-butanol-acetic acid-water (4 : 1 : 2). Amounts of the product corresponding to homocysteine were compared by measuring the value of optical density at 560mμ with the eluates of the spot sprayed with ninhydrin. a, Result with the incubation mixture of methionine in the absence of anthranilic acid ; b, do in the presence of anthranilic acid.

Interrelationship in Metabolism between Anthranilic Acid and Methionine

As was illustrated before, the consumption of anthranilic acid incubated with resting cells was found to be promoted by the addition of methionine. On the other hand, as reported previously, it has been found that from the paper chromatography of methionine incubated with resting cells or cell-free preparation, the spot regarded to be of homocysteine was detected[3]. Furthermore, the paper chromatographic test has been performed in the presence of anthranilic acid, according

to the concept led from the above-mendioned facts, as expressed below:

Methionine → Anthranilic acid
Homocysteine → *N*-Methylanthranilic acid

In fact, as might have been expected, it was observed that the spot corresponding in Rf value to homocysteine was noticeably detected in the presence of anthranilic acid, but the spot of *N*-methylanthranilic acid could not be recognized in the present experiment (Fig. 9). Even if an explanation for the above result might be tried to find out in further metabolism of *N*-methylanthranilic acid, the fact that this consumption seemed to be far slower than that of anthranilic acid, would hardly admit its possibility as a metabolite in the bacteria.

On the other hand, the possibility of methylating α-hydroxyphenazine cannot be expected at all, as has been put forward in the preceding paper. If the synthesis of phenazine nucleus may be accomplished through a condensation of two benzene rings, methylation will be performed at an earlier step than that of phenazine ring closure, otherwise will occur between a phenazine derivative other than α-hydroxyphenazine, and the methyl donor. In this reaction, although methyl donor may be assigned to methionine, methyl acceptor will be expected to be rather a modified product of anthranilic acid than itself.

SUMMARY

1. From an explanation for the inhibitory action of polyphenols or of aromatic amines, such a possibility has been found out as anthranilic acid is concerned with the synthesis of pyocyanine as its precursor.

2. Among the aromatic compounds showing inhibitory action on pyocyanine formation, several amines were considered to be a metabolite analogue of anthranilic acid, since the above inhibition was competitively neutralized by the addition of anthranilic acid.

3. Considering the relation between pyocyanine formation and bacterial growth, it is assumed that an extra of anthranilic acid assigned for the growth may be offered for the pyocyanine formation.

4. Although the experiment with use of resting cells was unsuccessful in the synthesis of pyocyanine from anthranilic acid, it was found that the product regarded as a moiety of the intermediate in pyocyanine synthesis was formed in its reaction mixture, and was noted that the accumulation product expected to be identical with the above product was recognized in a culture medium of the bacteria.

5. Interrelationship in metabolism was pointed out between anthranilic acid and methionine, suggesting the possibility that such a reaction may exist in pyocyanine synthesis system as methylates anthranilic acid or its derivative.

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