Studies on the Biosynthesis of Pyocyanine. (VIII)

On the Effect of Anthranilic Acid. (2)

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As an evidence supporting the possibility of anthranilic acid to be an intermediate in pyocyanine synthesis, it was found that anthranilic acid was accumulated in the medium of bacterium *Pseudomonas aeruginosa*, cultured in the presence of inhibitory agent, and that pyocyanine could be synthesized from anthranilic acid in resting system, by administering glutamic acid or glycine. It has been resulted that the inhibitory agent such as aniline, *o*-phenylenediamine and *p*-aminobenzoic acid should be a metabolite analogue of anthranilic acid.

On the other hand, anthranilic acid has been demonstrated to be formed through both anabolic and catabolic pathways, and postulated to be, in part, derived directly from indole by oxidative cleavage of its nucleus as a reverse reaction of the conversion of anthranilic acid to indole.

INTRODUCTION

In the preceding work[1] designed according to the concept that the aromatic compound revealing an inhibitory action on pyocyanine formation may be a metabolite analogue of the intermediate in pyocyanine synthesis, anthranilic acid has been pointed out to be a possible intermediate in this system. However the experiment was unsuccessful in the synthesis of pyocyanine under an enzymatic condition, and yet it has been shown that the reaction product from anthranilic acid by resting cells was also regarded as the intermediate in pyocyanine synthesis from the fact that this product was possibly identical with the one accumulated in the medium of the bacteria which has been considered to be an intermediate in pyocyanine synthesis. Provided the concept mentioned above is reasonable, the accumulation of anthranilic acid should be brought about in the presence of the inhibitory agent.

Based on the fact that the present strain can grow on the synthetic medium prepared with urea as a sole source of nitrogen, anthranilic acid must be regarded to be synthesized by the bacteria. However the work dealing with the formation of anthranilic acid by *Pseudomonas aeruginosa* had not been presented. Recently, Takeda and Nakanishi[2] have reported that anthranilic acid was isolated from the culture medium of the bacteria mentioned above.

In the present work, the experiment has succeeded in obtaining anthranilic acid from the synthetic medium containing the inhibitory agent which had preced-

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ingly been put forward, and demonstrated that the inhibitors such as aniline or acetanilide, *o*-phenylenediamine and *p*-aminobenzoic acid are metabolite analogue of anthranilic acid. On the other hand, it has been known that when phosphate was kept at an extremely low level, pyocyanine could enzymatically be synthesized from anthranilic acid in the presence of glutamic acid or glycine. This condition was found to be also applicable to the other case of the enzymatic synthesis of pyocyanine.

EXPERIMENTAL AND DISCUSSION

Formation of Anthranilic Acid

In order to find out the condition for the formation of anthranilic acid, two kinds of the medium containing the following materials were prepared: (1) 2% glycerol, 1% peptone, 0.2% urea, 0.05% $MgSO_4 7H_2O$ and 0.025% K_2HPO_4. (2) 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$.

With the above media, various strains of *Pseudomonas aeruginosa* were cultured at 37° for 48 hours. The cultured solution was acidified with dilute sulfuric acid and extracted with ethyl ether to test their fluorescence under ultraviolet light. As shown in Table 1, with the peptone medium fluorescence

Table 1. Formation of fluorescent product.

Medium	Fluorescence Strains					
	B_1	B_2	Bk	C_1	Bt	Bd
No. 1	+	+	Trace	+	+	+
No. 2	###	###	##	###	###	###

No. 1 : 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. No. 2: 1% peptone was added to No. 1. Incubation, at 37° for 48 hours. Fluorescence was observed with ether extract of the cultured solution.

was considerably revealed in major cases of the strain, while with the synthetic medium it was hardly indicated. In addition to this experiment, effects of various inhibitory agents were tested on the detection of the fluorescent product.

As was previously mentioned,[1] a discrepancy is shown in a considerably wide range between the limiting concentrations for the growth and for the pigmentation, so that the inhibitory agent was given at a maximal concentration at which the bacterial growth was satisfactory while no pyocyanine was formed. The inhibitors dissolved in alcohol were added to the synthetic medium shown above after its pasteurization and incubated at 37° for 3 days. The results are shown in Table 2. Among the cultured solutions of various inhibiting agents, it was observed that fluorescence was remarkably exhibited in the case in which aniline (or acetanilide), *o*-phenylenediamine or *p*-aminobenzoic acid was added to the medium as an inhibitor, whereas in the case of polyphenols results were negative with the exception of resorcinol, of which fluorescence was found to originate

Table 2. Formation of flurorescent product.

Inhibitory agents added	Fluorescence No. 1	Fluorescence No. 2	Spot corresponding to AA
Aniline	###	###	+
Acetanilide	###	###	+
o-Phenylenediamine	###	###	+
p-Aminobenzoic acid	###	###	+
Phenol	##	+	−
Resorcinol	###	##	−
Pyrocatechol	##	+	−
Hydroquinone	##	+	−
Guajacol	##	+	−
Control	##	+	−

Basal medium was the same as No. 1 in Table 1. No. 1, the result with butanol extract ; No. 2, ether extract.

AA, anthranilic acid.

from itself during the preservation of its solution for several days.

Paper Chromatography

In order to test whether these fluorescences will be due to anthranilic acid or not, paper chromatographic test was performed.

The results are shown in Fig. 1. It has been indicated that the fluorescence of the products in the medium containing peptone or the aromatic amine described above might be ascribable to their identity with anthranilic acid. On the

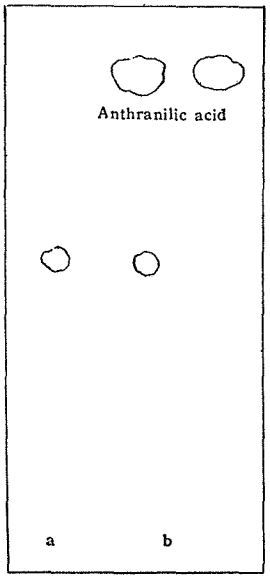

Fig. 1. Paper chromatography of culture extract of the bacteria incubated in the presence of inhibitory agent of pigmentation. a, Result with the same medium as No. 1 in Table 1; b, with the medium containing 0.05% acetanilide and the same components as in a. Solvent, *n*-butanol-acetic acid-water (4:1:2).

contrary, the fluorescence shown in the synthetic medium without peptone or inhibitory agents, could not be ascribed to anthranilic acid. It is worthy to note why anthranilic acid is detected in the peptone medium. This reason can not, of course, be attributed to its inhibitory action, but to its breaking down as will be illustrated later.

It is an interesting fact that *p*-aminobenzoic acid which is to be an essential metabolite in the bacteria, competitively inhibits the metabolism of anthranilic acid. It has been believed by the author that the key to solve the mechanism of toxic action of this compound on the bacterial growth must be found in this point.

Absorption Spectrum

As a further study to ascertain the identity of the fluorescent product with anthranilic acid, a photometric test has been done.

For the paper chromatography, the following solvent system was employed: *n*-butanol-acetic acid-water (4 : 1 : 2). Since the spot on the one-dimensional paper chromatogram using the above solvent system gave a mixed absorption spectrum of anthranilic acid and inhibitory agent, this eluate was again chromatographed with the solvent, benzene-alcohol (8 : 1). Fig. 2 represents the result in the case of acetanilide. As might have been expected, a characteristic absorption band was observed, suggesting that the accumulation product is identical with anthranilic acid itself.

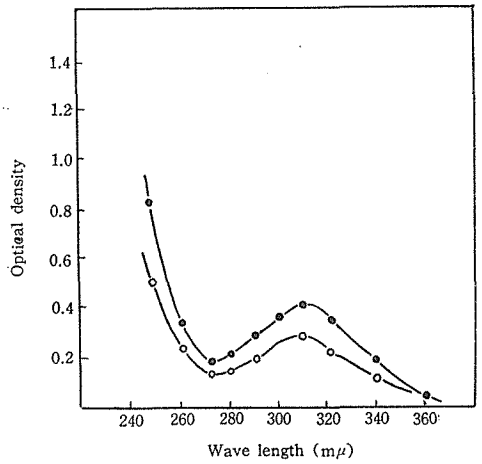

Fig. 2. Absorption curve of a culture extract of the bacteria incubated in the presence of acetanilide. Medium was the same as in the case of a in Fig. 1. (●—●—●) Anthranilic acid, (○—○—○) the culture extract. Absorbances were measured with neutral aqueous solution in each case.

Formation of Anthranilic Acid by Catabolic Reaction

As mentioned before, the formation of anthranilic acid in the culture medium prepared with peptone, has been assumed to originate from tryptophan which is to be a component of peptone. In order to verify this assumption the following experiment was carried out: 0.001*M* DL-tryptophan dissolved in 0.01*M* phosphate buffer, pH 7.6, was incubated at 37° for 12 hours, with resting bacterial cells suspended at a level of 0.02g wet cell per ml of the solution.

The paper chromatographic test has shown that tryptophan was converted to anthranilic acid by resting cells (Fig. 3).

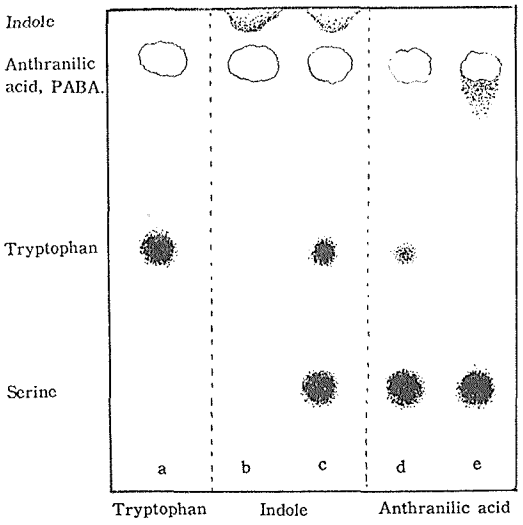

Fig. 3. Paper chromatograms of the reaction products from tryptophan, from indole and from anthranilic acid. a, Tryptophan; b, indole; c, indole+serine; d, anthranilic acid+serine; e, anthranilic acid+serine+PABA (*p*-aminobenzoic acid).

It has generally been recognized on tryptophan metabolism that there are two general pathways for the oxidative catabolism : one, *via* anthranilic acid and catechol, referred to the "aromatic pathway"; the other, *via* kynurenic acid termed the "quinoline pathway." Accumulation of anthranilic acid by way of catabolic reaction is probably attributed to the impaired enzyme activity of the resting cells. It is well known that tryptophan is synthesized from indole through a condensation with serine by tryptophan desmolase,[3,4,5,8] and that indole is formed by oxidative cleavage of tryptophan by tryptophanase,[6,7] liberating pyruvic acid and ammonia. On the other hand, although indole has been demonstrated to be derived from anthranilic acid,[8~10] the reverse reaction, conversion of indole to anthranilic acid has not yet been presented.

In the present experiment with use of resting cells to which indole was fed, the formation of anthranilic acid was recognized by paper chromatographic and spectroscopic studies. This result suggests that anthranilic acid is rather directly derived from indole through its ring splitting by the enzyme to be termed as "indolase." If anthranilic acid might have originated from kynurenine through tryptophan synthesis, serine would be required in this reaction system, and alanine had to be detected to liberate from kynurenine. To the contrary, it was found in paper chromatographic test that even in the absence of serine, the reaction readily proceeded to form anthranilic acid without detection of alanine, and that when serine was fed tryptophan was synthesized, whereas in the absence of serine any amino acids could hardly be recognized (Fig. 3).

At the oxidation step of tryptophan to kynurenine the double bond of indole ring has been suggested to be firstly oxidized into either 2-hydroxy- or 2, 3-

dihydroxytryptophan, though this is a hypothesis which remains to be proved.[11,12]

If the double bonds might also be oxidized even in the case of indole, the intermediate product would be postulated to be 2-hydroxy- or 2, 3-dihydroxyindole, although anthranilic acid might not necessarily be negated to be formed *via* kynurenine. And therefore the above-mentioned system would, at least in part, be recognizable in anthranilic acid formation, as shown in Fig. 4.

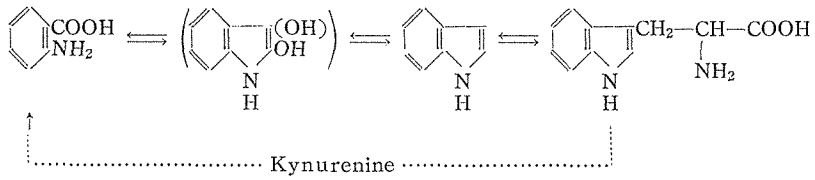

Fig. 4. Hypothetical scheme for anthranilic acid formation.

Based on the result in the present experiment, the formation of anthranilic acid in the peptone medium would be ascribable to the degradation of tryptophan existing in peptone, and yet this may, in part, be of anabolic reaction due to an abundant bacterial growth in the peptone medium, or of the accumulation due to the impaired bacterial capacity for the further metabolism of anthranilic acid.

On the other hand, there was observed in paper chromatography a faint spot corresponding to tryptophan even with the reaction mixture of anthranilic acid and serine (Fig. 3). However, in the presence of *p*-aminobenzoic acid any formation of amino acids was not recognized, suggesting an antagonistic action of the metabolisms between anthranilic acid and *p*-aminobenzoic acid. It is probable that the toxic action of *p*-aminobenzoic acid in high concentration on bacterial growth may be due to the above fact.

It is interesting to note that there exists the antagonistic action between the essential metabolites of the bacteria.

Isolation of Anthranilic Acid

In order to isolate anthranilic acid the following experiment was carried out: to 300 ml of the media composed of 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.05% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$, pH 7.4, taken into 3 liter Erlenmeyer flasks, acetanilide dissolved in alcohol was added to attain 0.05%. The cultured solution incubated at 37° for 48 hours, was acidified with dilute sulfuric acid and extracted with ethyl ether to isolate anthranilic acid. The ether extract was transferred to alkaline aqueous solution of sodium bicarbonate and again extracted with ether, after being made acidic. The dehydrated ether extract was then concentrated *in vacuo* and brownish yellow remains were once precipitated by the addition of cupric acetate solution. The sediment separated by decantation was resuspended in distilled water and treated with H_2S to remove cupric ion, and then the supernatant obtained, followed by the filteration in slightly alkaline state, was acidified and once more extracted with ethyl ether. The concentrated extract was chromatographed on column of alumina three-dimensionally, using the following solvent systems: (1) *n*-butanol-acetic acid-alcohol-water (8 : 1 : 4 : 4), (2) alcohol-water slightly alkalized with NH_4OH (1 : 4), (3) benzene-alcohol (8 : 1).

Fluorescent spot was taken out under ultraviolet light, eluted and further chromatographed with the concentrated eluate. The ether extract was concentrated *in vacuo* to form pale yellow crystals and recrystallized with methanol and water. The following is the result in elementary analysis. Found: C, 61.51; H, 5.20, Calcd. for $C_7H_7NO_2$; C, 61.31; H, 5.15% (m.p. 144° uncorr.).

Metabolism of Inhibitory Agents

From the experiments shown above, it has conclusively been resulted that anthranilic acid could be formed in the medium of the bacteria cultured in the presence of inhibitory agent.

There will be no more an evidence against it, unless such a proof to the contrary can be brought up as anthranilic acid formed is nothing but what is derived from the inhibiting agent itself. It has not, of course, been able to recognize the phenomenon hypothesized above in the present experiment as well as in the literature.

With the excepton of *p*-aminobenzoic acid, these substances used as an inhibiting agent can not be regarded as a metabolite in organisms. Even though they might be metabolizable, it would be of a slight modification as is usually observable in physiology of detoxication in animal body. For instance, *p*-aminophenol was found to be reversibly oxidized and reduced without any essential deterioration.

On the other hand, aniline or acetanilide appeared not to be metabolizable in the medium of the present bacteria, although they have been known in animal tissue to be converted to *p*- or *o*-aminophenol and to *p*-acetamidophenol, respectively, which may finally be excreted in conjugation with glucronic acid[13,14]. *o*-Phenylenediamine was recognized on paper chromatography as a brownish yellow spot, independently of the detection of anthranilic acid accumulated. On the other hand, it was noted in the case of *p*-aminobenzoic acid that with its appreciable consumption, anthranilic acid accumulation was brought about.

Pyocyanine Synthesis in Resting System

In the preceding work, the experiment was unsuccessful in the synthesis of pyocyanine from anthranilic acid by resting cells, and this reason has been assumed to be ascribable to the impairment of enzymic activity in resting cells or to the want of a moiety for this synthesis. In consideration of these possibilities, further experiments have been designed to find out the condition for pigmentation in resting system.

Requirement of amino acid. Experimental method was as follows: the resting cells harvested from the plate culture incubated at 37° for 24 hours according to the procedure mentioned previously,[15] were suspended at a level of 0.02 g of wet cells per ml of the solution composed of 0.001*M* $MgSO_4$, 0.001*M* anthranilic acid and 0.01*M* organic source to be tested, pH 7.6, and incubated at 37° for 12 hours.

It was found that glutamic acid or glycine was effective in the synthesis of pyocyanine as a source of the possible moiety and that phosphate was necessary to keep at an extremely low level besides requirement for $MgSO_4$, as will be

illustrated in the following section. Table 3 represents the effect of amino acid and other organic sources on the synthesis of pyocyanine. It was clearly observed that together with anthranilic acid, other organic source was required and that in the absence of anthranilic acid, pigmentation could never take place,

Table 3. Effect of amino acid and other organic sources on pyocyanine synthesis from anthranilic acid in resting system.

Organic sources	Pyocyanine	Organic sources	Pyocyanine
DL-Alanine	−	Pyruvic acid	−
L-Glutamic acid	++	α-Ketoglutaric acid	−
L-Aspartic acid	+	Acetic acid	−
Glycine	++	Fumaric acid	−
L-Phenylalanine	−	Malic acid	−
DL-Tyrosine	−	Succinic acid	−
DL-Threonine	−	Lactic acid	−
α-Aminobutyric acid	−	Citric acid	−

Reaction mixture: 0.001*M* $MgSO_4$, 0.001*M* anthranilic acid, 0.01*M* organic source and resting cells suspended at a level of 0.2 g wet cells per 10 ml of the solution. Incubation, at 37° for 12 hours.

suggesting that anthranilic acid would be an essential precursor of pyocyanine.

The aim of the experiment that to the concentration of 0.001*M* anthranilic acid amino acid is supplied at a tenfold level, is explained to be based on the consideration of a simultaneous energy source for synthesizing reaction. Therefore, even in the case in which glutamic acid is supplied at a level equivalent to that of anthranilic acid, the same result could be expected as far as the suitable carbon source, *e.g.* 0.01*M* succinate was provided.

On the other hand, it was noted that increasing effect on pyocyanine systhesis was revealed by the addition of methionine even in the resting system, although its synthesis was, at any rate, allowed to bring about in the case in which no methionine was added.

Effect of inorganic matters. Table 4 shows the effect of $MgSO_4$ and Fe. It has been led to approve a necessity of Mg ion, although the effect of Fe was

Table 4. Requirement of mineral matters for pyocyanine synthesis in resting system.

Concn. (m*M*, μ*M**)	2.0	1.0	0.5	0.25	0.1	0.05	0.025	0.010	0.005	Nil
$MgSO_4$	+	+	+	+	+	−	−	−	−	−
K_2HPO_4	−	−	−	+	+	+	+	+	+	+
$FeSO_4$	+	+	+	+	+	+	+	+	+	+

Substrate: 0.001*M* anthranilic acid, 0.01*M* succinate.

For the test of one component, concentration of others was kept constant: $MgSO_4$, 1.0m*M* ; K_2HPO_4, 0.1m*M* ; $FeSO_4$, no addition.

* Concentration of $FeSO_4$.

hardly recognized. As concerns the effect of PO_4 it was rather preferable not to use than to keep at the lower level. When phosphate was employed at a level of 0.002*M* at which pigmentation was usually revealed in growing system, pyocyanine formation could never be expected.

Unnecessity of adding Fe and PO_4 suggests that these ions are incorporated into the structure of the enzyme or coenzyme in pyocyanine synthesis system.

Other factors. The experiment on the effect of oxygen and pH, gave the results parallel to those of anthranilic acid metabolism mentioned precedingly[1] : under an anaerobic condition or at a lower pH level (below 7.0), pyocyanine could not be synthesized at all. In addition, for pyocyanine synthesis in the present system it was, on the whole, required to employ such a young cell as harvested within 24 hours' cultivation.

Relationship between the fluctuations of substrates and of pyocyanine. Fig. 5 shows the relation of the substrate disappeared to the pyocyanine appeared. Glutamic acid was determined by paper chromatographic and colorimetric techniques applied previously[16] to methionine determination, depending on the fact that the reaction products of these amino acids with ninhydrin reveal the same absorption peak at 560 mμ ($e=2900$ with glutamic acid). It was observed from the figure that pyocyanine formation was fairly parallel to the consumption of substrates, although the molar ratio between these fluctuating substances was not necessarily proportional.

The explanation for this illogicality will bring up the following discussion : anthranilic acid as well as glutamic acid would simultaneously be assigned a role as a precursor of the metabolites other than pyocyanine.

On the other hand, in parallel experiment the following test was tried to

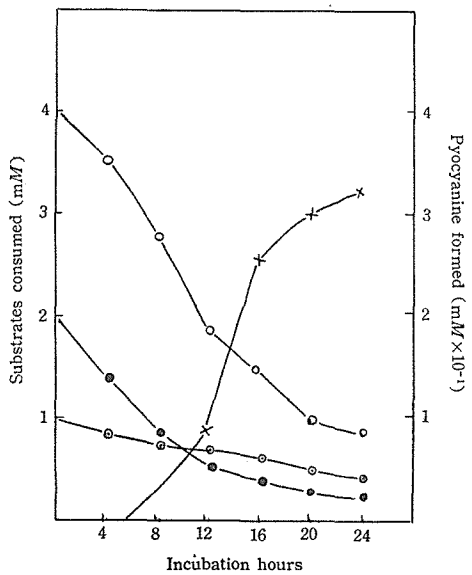

Fig. 5. Relationship between fluctuations of substrates and of pyocyanine. (●—●—●) Glutamic acid, (○—○—○) anthranilic acid, (◉—◉—◉) methionine, (×—×—×) pyocyanine.

exclude the possibility of dependence of pyocyanine synthesis on cell proliferation, for in the present experiment using carbon and nitrogen sources, the secondary growth might not be negated during the incubation process, although phosphate was scarcely or not employed. After the incubation for 12 hours when pyocyanine was synthesized, turbidity of the reaction mixture was measured to compare with that of no incubation. This result gave no evidence for the growth, indicating the equivalent or lower value of the turbidity in comparison with that before the incubation.

Pyocyanine synthesis from culture extract. From the above-mentioned experiment, the condition for the enzymatic reaction has been known about the synthesis of pyocyanine in resting system.

And so, the following experiment was designed: It has precedingly been pointed out that the accumulation product considered to be identical with the one derived from anthranilic acid, which has also been regarded as an intermediate in pyocyanine synthesis, was recognized in the culture medium containing excess of phosphate.

The reaction mixture of the above product obtained from the cultured solution by extracting with butanol, gave the result that the amount of pyocyanine synthesized was twice as much as that from anthranilic acid itself. However, in the experiment using the sample through the paper chromatographic procedure, pyocyanine was hardly synthesized probably on account of its oxidative degradation.

SUMMARY

1. It has been demonstrated by isolating anthranilic acid in crystalline form that anthranilic acid was accumulated in the culture medium of the bacteria incubated in the presence of inhibitory agent, and clarified that the inhibitors, aniline or acetanilide, *o*-phenylenediamine and *p*-aminobenzoic acid are a metabolite analogue of anthranilic acid.

2. Anthranilic acid has been shown to be formed through anabolic as well as catabolic pathway and it was postulated that this formation might occur to be, in part, derived directly from indole by way of the reverse reaction of its synthesis from anthranilic acid, depending on the oxidative splitting of its nucleus.

3. In the resting system, pyocyanine was synthesized from anthranilic acid in the presence of glutamic acid or glycine, and methionine revealed, even in this system, an increasing effect on pyocyanine synthesis.

4. Of the inorganic matters which have hitherto been put forward, Mg was found to be essential for the enzyme reaction in pyocyanine synthesis, while phosphate showed rather inhibiting effect on pigmentation. No recognition of the effect of Fe in this system was regarded to be ascribable to its incorporation into the enzyme structure.

5. The product detected in the culture medium of the bacteria which has been regarded to be derived from anthranilic acid was also found to be synthesized into pyocyanine under the same condition as in the case of anthranilic

acid.

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