ON THE BIOSYNTHESIS OF PYOCYANINE[1]

L. H. FRANK[2] AND R. D. DEMOSS

McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland, and Department of Bacteriology, University of Illinois, Urbana, Illinois

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The elucidation (Wrede and Strack, 1929) of the structure of pyocyanine, the characteristic blue pigment of *Pseudomonas aeruginosa*, represents the first reported instance of the occurrence of the phenazine nucleus as a natural product. Since the structure of pyocyanine was proved in 1929, a number of other substituted phenazine pigments have been shown to be produced by different *Pseudomonas* species. Kogl and Postowsky (1930) established the structure of the green pigment, chlororaphin, produced by *Pseudomonas chlororaphis*, as the amide of phenazine-1-carboxylic acid. Clemo and Daglish (1950) demonstrated that iodinin, the purple pigment of *Pseudomonas iodinum*, is the di-N-oxide of 1,5-dihydroxyphenazine. Recently, Haynes *et al.* (1956) and Kluyver (1956) found that the yellow pigment of a new pseudomonad, *Pseudomonas aureofaciens*, is phenazine-1-carboxylic acid.

The production of differently substituted phenazines by organisms of the Pseudomonas group constitutes an interesting aspect of comparative microbial biochemistry. From this point of view, it would be of value to elucidate the pathways involved in the synthesis of these pigments. The present report is concerned with the biosynthesis of pyocyanine by *P. aeruginosa*.

A number of workers have studied the nutritional requirements associated with pyocyanine production by growing cultures of *P. aeruginosa*. The most complete study appears to be that of Burton *et al.* (1947). From the results of this group it is clear that there is no unique nutritional environment responsible for the production of pyocyanine. Thus, a variety of amino acids, present singly, together with glycerol resulted in pigment production. Combinations of amino acids, notably alanine and leucine, were found to be more effective.

While the present investigation was in progress, Blackwood and Neish (1957) reported on the incorporation of C^{14} labeled substrates into pyocyanine during growth of *P. aeruginosa* on the glycerol-alanine-leucine medium developed by Burton *et al.* (1947). Of a variety of compounds tested, glycerol proved to be the best substrate for pyocyanine synthesis; amino acids, including alanine, were relatively poor substrates.

After the present work was completed, a paper by Grossowicz, *et al.* (1957) appeared which claimed pyocyanine synthesis by nonproliferating cell suspensions of *P. aeruginosa*. The results of these workers will be compared to those obtained by the present authors.

MATERIALS AND METHODS

Bacteriological. P. aeruginosa ATCC no. 9027 was obtained from the American Type Culture Collection. The organism was grown in a medium containing, per L: DL-alanine, 10 g; glycerol, 20 ml; K_2HPO_4, 0.139 g; $MgCl_2 \cdot 6H_2O$, 4.06 g; Na_2SO_4, 14.2 g; ferric citrate, 0.1 g. The final pH was adjusted to 7.0 to 7.2. Sterilization was accomplished by autoclaving at 120 C for 15 to 20 min.

P. aeruginosa was subcultured in this medium at 24-hr intervals, using a 5-ml inoculum per 100 ml media (500-ml Erlenmeyer flask). The cells used in most of the experiments were harvested from 400 ml cultures (2-L Erlenmeyer flask) in the defined medium as already described. These cultures had been initiated with 10 ml inocula from the standard 24-hr culture. All cultures were incubated at 30 C on a rotary shaker operating at 150 to 200 rpm.

In general, cells were washed twice with 250 to 300 ml of a salt solution (salts E) containing, per L: Na_2SO_4, 14.2 g; $MgCl_2 \cdot 6H_2O$, 4.06 g; K_2HPO_4, 0.087 g. Samples of a suspension of cells in salts E were added to the various incubation mixtures which occupied a total volume of

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[2] Predoctoral Fellow of the National Cancer Institute, 1955–1957. Present address: Benjamin Rose Hospital, Cleveland, Ohio.

20 ml (125-ml Erlenmeyer flask stoppered with cotton). Incubations were carried out at 30 C on the rotary shaker. Unless indicated otherwise, all incubation mixtures had a final salt composition corresponding to that of salts E. These conditions of incubation are referred to as standard conditions.

Analytical. Bacterial protein was determined with the biuret reagent described by Layne (1957), using crystalline bovine albumin as a standard. For the purpose of this assay, cells were precipitated with trichloracetic acid and dissolved in 1 M KOH by heating in a boiling water bath for 10 min.

The concentration of pyocyanine in cultures or in incubation mixtures was determined in the following manner. A 2-ml sample of the culture was added to 2 ml of 20 per cent trichloracetic acid in a glass centrifuge tube. The tube was heated in boiling water for 5 to 10 min, followed by cooling to room temperature and centrifugation. To 2 ml of the supernatant were added 4 ml of 2 M tris(hydroxymethyl) aminomethane in a colorimeter tube. The tube was shaken vigorously to insure the complete autoxidation of any reduced pyocyanine and the light absorption at 690 mμ measured in an Evelyn colorimeter or Beckman spectrophotometer. Pyocyanine concentration was calculated using an extinction coefficient of 4306×10^6 cm^2 per mole. This value had been determined previously using a crystalline sample of pyocyanine prepared from pigment of biosynthetic origin. Within experimental error, the same value was obtained by converting pyocyanine to 1-hydroxyphenazine (Wrede and Strack, 1929) with measurement of the latter compound accomplished spectrophotometrically using a pure sample of the compound as a standard.

The concentration of alanine in culture supernatants was determined by chromatographing a sample on Whatman no. 4 paper in a solvent system containing isopropanol-water, 4:1 (v/v). The alanine spot was located by spraying lightly with ninhydrin, cut out, and carried through the quantitative ninhydrin procedure of Rosen (1957). The latter method was also used to measure the concentration of alanine-C^{14} preparatory to plating (see below).

All specific activities were determined by plating a known quantity of the compound in question on stainless steel planchets of 7.5 cm^2 area, drying *in vacuo* over Drierite, and counting in the proportional region with a gas flow counter. Counting was done in a concentration range such that self-absorption was not manifest.

Pyocyanine and 1-hydroxyphenazine were purified for counting by extraction from the incubation mixture and subsequent paper chromatography.

Pyocyanine was extracted into chloroform from the incubation mixture supernatant and then re-extracted into 0.2 N HCl. To the deep red acid solution was added 0.4 M borate-NaOH buffer, pH 10, until the color changed to blue and the pyocyanine again extracted into chloroform. This cycle was repeated 2 or 3 times, resulting in a clear solution of pyocyanine in chloroform. The latter was evaporated to a small volume (*ca.* 1 ml) in a stream of cold air and then chromatographed on Whatman no. 4 in a solvent composed of *n*-butanol-acetic acid-water, 13:4:7 (v/v). Pyocyanine was eluted from the chromatogram with 0.2 N HCl and extracted into chloroform after treatment with borate buffer, as above. The chloroform solution was then rechromatographed in isopropanol-water, 4:1 (v/v). Again, the pigment was eluted with 0.2 N HCl and samples of the resulting solution used for the determination of concentration and for plating.

Materials. L-Alanine-U-C^{14} was purchased from Nuclear-Chicago Corp. DL-Alanine-1-C^{14} and DL-alanine-2-C^{14} were gifts from Dr. M. Gibbs. All other amino acids used in this investigation were products of Nutritional Biochemical Corp. 1-Hydroxyphenazine was obtained through the courtesy of Dr. D. Vivian, National Institutes of Health. 1-Hydroxyphenazine-C^{14} was prepared by alkaline degradation of pyocyanine-C^{14} (Wrede and Strack, 1929) which had been synthesized from L-alanine-U-C^{14} by *P. aeruginosa*.

RESULTS

The growth of *P. aeruginosa* in the defined medium described under Methods is illustrated in figure 1, together with the concomitant production of pyocyanine in the same culture.

Incubation of washed cells, harvested from cultures of 22 to 26 hr, resulted in pyocyanine synthesis. The incubation medium contained 0.05 M DL-alanine and salts, as described under Methods. Omission of either alanine, phosphate, magnesium, or sulfate greatly reduced or completely inhibited pigment formation.

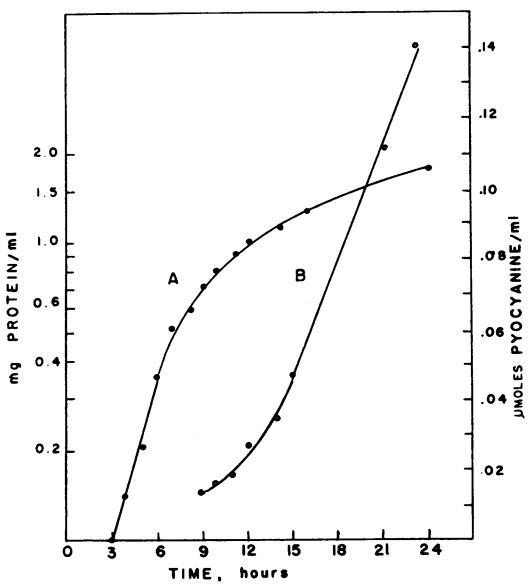

Figure 1. The time-course of growth and pyocyanine synthesis. One hundred ml media inoculated at 0 hr and incubated as described under Methods. *Curve A*, cell concentration measured directly as mg protein per ml, plotted on a logarithmic scale. *Curve B*, pyocyanine concentration plotted on an arithmetic scale.

The standard salt concentrations (salts E) used throughout this work were developed in a series of experiments in which the cells were washed with distilled water and resuspended in mixtures containing 0.05 M DL-alanine, graded concentrations of the salt in question, with the other salts held at the levels obtaining in salts E (figure 2). Pyocyanine was measured after 12 hr of incubation. Under these conditions, the concentration of $MgCl_2$ was not critical between 2 and 343 $\times$ 10^{-4} M whereas pyocyanine production was found to be near-optimal at 5 $\times$ 10^{-4} M phosphate. Higher phosphate concentrations, over a range of pH values were markedly inhibitory. Unfortunately, due to the low level of phosphate employed, the buffering capacity of the mixtures was low, allowing the pH to rise as high as 9 as a result of ammonia production from the substrate. Other buffer systems tested, in the presence of phosphate, were inhibitory. Consequently, the "standard conditions" of incubation are to be regarded as empirical rather than as an index of physiological requirements. In agreement with the results of Burton *et al.* (1948), obtained with growing cultures of *P. aeruginosa*, the extent of pyocyanine formation increased with increasing levels of sulfate to about 0.1 M (figure 2); higher concentrations of Na_2SO_4 were inhibitory.

The high level of sulfate (0.1 M) required for optimal pigment synthesis under the described conditions seems to represent a combination of sulfur-requirement and ionic strength effects. Thus, low levels of methionine could replace Na_2SO_4, but only in the presence of added salts. Similarly, lower concentrations of Na_2SO_4 would yield comparable quantities of pyocyanine, provided the medium was supplemented with another salt.

Addition of iron to the incubation mixture containing alanine as substrate did not stimulate pyocyanine synthesis. However, if the cells were grown in the synthetic medium with iron omitted, addition of ferric citrate or ferric chloride to the washed-cell incubation was found to stimulate pigment production (table 1). The difference would perhaps be larger if precautions were taken to free the medium of contaminating iron.

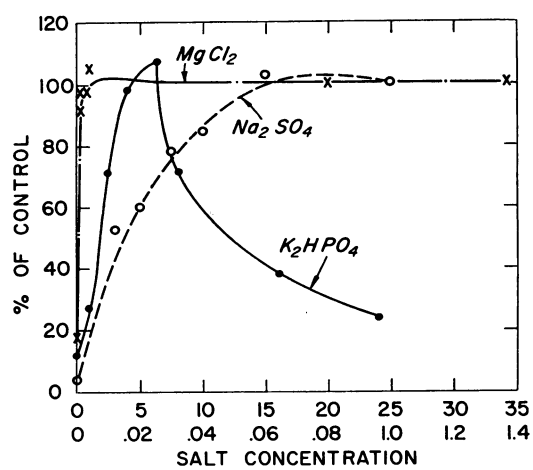

Figure 2. The effect of salt concentration on pyocyanine production. The amount of pyocyanine produced is expressed as a percentage of the quantity formed in control flasks incubated under the standard conditions described in the text. At each indicated concentration of a salt, the other two salts were present at the levels obtaining in salts E. In all cases, the substrate was DL-alanine, 0.05 M. The upper row of figures on the abscissa refers to the molar concentrations of $MgCl_2$ and K_2HPO_4 multiplied by 10^3 and 10^4, respectively, while the lower row of figures gives the molar concentration of Na_2SO_4.

TABLE 1

Effect of iron on pyocyanine formation

Ferric Citrate (0.1 mg per ml)		Pyocyanine Formed
Growth medium	Reaction mixture	
		μmole/ml
−	−	0.209
−	+	0.364
+	−	0.380
+	+	0.360

Washed cells were incubated under standard conditions for 20 hr. Initial cell concentration, 0.25 mg protein per ml.

TABLE 2

Incorporation of alanine into pyocyanine

Substrate		Pyocyanine	Pyocyanine/Alanine
	cpm/μmole	*cpm/μmole*	
L-Alanine-U-C^{14}.....	2,364	9,275	3.9
L-Alanine-U-C^{14}.....	2,535	11,667	4.6
DL-Alanine-1-C^{14}.....	2,720	5,211	1.9
DL-Alanine-2-C^{14}.....	452	2,389	5.3

Washed cells were incubated under standard conditions for 24 hr. Initial cell concentration, 0.1 mg protein per ml. Theoretical specific activity ratio of pyocyanine to uniformly labeled alanine, assuming alanine to be the sole source of pyocyanine carbon, is 4.33.

That essentially all the pigment produced during these washed-cell incubations arose from the added substrate, i. e., alanine, could be demonstrated by the use of alanine-U-C^{14} (table 2). The same conclusion is dictated by the the results of an experiment in which C^{14}-labeled cells were allowed to synthesize pyocyanine from nonisotopic alanine. Although the cellular carbon had a calculated[3] specific activity of 590 cpm per μmole carbon, the isolated pyocyanine contained only 16 cpm per μmole carbon.

The use of DL-alanine labeled with C^{14} in the 1- or 2-positions indicated that, of the 13 carbon atoms in the pyocyanine molecule, the equivalent of 2 and 5 atoms originated from the carboxyl and *alpha* carbons, respectively, of the alanine (table 2). In the absence of specific methods of degradation, the distribution of isotope among the pyocyanine carbon atoms cannot be deduced.

Addition of glycerol or pyruvate to the incubation mixtures containing alanine resulted in enhanced synthesis of pyocyanine. That these compounds were actually incorporated into the pigment molecule could be inferred from the dilution of isotope when glycerol or pyruvate was used in conjunction with alanine-U-C^{14} (table 3). The relative efficacy of alanine and glycerol in promoting pyocyanine synthesis does not appear to be an absolute one, but rather to depend upon the environmental conditions. Thus, at the usual phosphate concentration, appreciable amounts of alanine-C^{14} were incorpo-

TABLE 3

Effect of glycerol or pyruvate on incorporation of alanine into pyocyanine

Substrates	Pyocyanine
	cpm/μmole
L-Alanine-U-C^{14}.....	4,287
+ pyruvate.....	2,009
L-Alanine-U-C^{14}.....	13,087
+ glycerol.....	8,635

Washed cells incubated under standard conditions for 20 hr. Each substrate present at 0.05 M concentration. Initial cell concentration, 0.1 mg protein per ml.

TABLE 4

Effect of phosphate on pyocyanine formation from glycerol

Substrates	Pyocyanine	
	5×10^{-4} M PO_4	13×10^{-4} M PO_4
	cpm/μmole	*cpm/μmole*
L Alanine-U-C^{14}.....	13,087	—
L-Alanine-U-C^{14} + glycerol..	8,635	524

Washed cells incubated under standard conditions for 24 hr. Each substrate was present at 0.05 M concentration. Initial cell concentration, 0.1 mg protein per ml.

[3] The isotopically labeled cells were prepared by growth on alanine-U-C^{14} as the sole source of carbon. Under the conditions used, a fivefold increase in cell mass occurred. Thus, the specific activity of cellular carbon was of the order of four-fifths the specific activity of the substrate carbon, which was 733 cpm per μmole.

rated into the pigment in the presence of glycerol; however, at a higher (inhibitory) phosphate concentration, most of the pigment arose from glycerol (table 4). These results are to be compared with the finding of Blackwood and Neish, cited above. In the instance cited, phosphate (0.003 M) was present at a concentration which favors pyocyanine synthesis from glycerol rather than alanine.

The addition of 1-hydroxyphenazine to the standard system in the presence of alanine did not result in increased yields of pyocyanine. Moreover, the replacement of alanine by 1-hydroxyphenazine alone or in combination with methionine, betaine, sarcosine, creatine, or serine as possible methyl donors did not lead to pyocyanine formation. That exogenous 1-hydroxyphenazine was neither incorporated into pyocyanine nor exchanged with it was demonstrated by adding 1.5 μmoles of 1-hydroxyphenazine-C^{14} to the standard system containing alanine. Although 3.0 μmoles of pyocyanine were formed, it contained no C^{14}. The specific activity of the reisolated 1-hydroxyphenazine was unchanged.

Most of the common amino acids were tested for their ability to promote pyocyanine synthesis by replacing alanine with each amino acid singly (table 5). The following amino acids did not support pyocyanine synthesis or growth under these conditions: β-alanine, D- or DL-threonine, D- or DL-methionine, L-cysteine, glycine, sarcosine, betaine, DL-serine, L-lysine, D-aspartic acid, D- or DL-histidine. It is interesting to note (table 5) that D-glutamic acid was an effective substrate whereas L-glutamic acid was not. Perhaps correlated with this was the observed inactivity of α-ketoglutarate plus ammonia. Pyruvate or lactate plus ammonia constituted good substrates for pyocyanine synthesis.

In the comparison of the amino acids as substrates for pyocyanine synthesis, it is important to point out that all the compounds listed in table 5 were tested under the same conditions of cell concentration. The relative effectiveness of the amino acids, however, was later found to be a function of cell concentration. Thus, with alanine as substrate the amount of pyocyanine produced was an inverse function of the initial cell concentration, whereas with other amino acids, such as DL-isoleucine, the reverse was true. This complication, of necessity, leaves open the question of which substrate is the most efficient precursor of pyocyanine.

TABLE 5

Effect of amino acids on pyocyanine and protein synthesis

Substrate	Pyocyanine	Protein
	%	%
DL-Alanine	100	100
L-Alanine	102	100
D-Alanine	100	100
L-Glutamic acid	13	30
D-Glutamic acid	321	200
γ-Aminobutyric acid	113	120
L-Proline	139	150
Hydroxy-L-proline	65	80
DL-Ornithine	51	70
L-Leucine	13	27
D-Leucine	100	85
DL-Isoleucine	226	242
D-Isoleucine	0	30
L-Tryptophan	0	25
D-Tryptophan	0	11
DL-Phenylalanine	24	65
L-Tyrosine	23	50
L-Aspartic acid	17	25
DL-Valine	0	55
L-Arginine	34	80

Washed cells incubated under standard conditions for 24 hr. Each compound present at 0.05 M concentration, including tyrosine which was not completely in solution. The values are given as per cent of those obtained in the incubation mixtures containing DL-alanine. Initial cell concentration, 0.6 mg protein per ml.

The poor yields of pyocyanine (i. e., 300 to 500 μg from 9 mg alanine) obtained with amino acids suggested that, in the system under study, one or more competing reactions was occurring. In the case of alanine, at least, the small amount of pigment formed could not be attributed to incomplete utilization of the amino acid, since measurement of the reaction mixture supernatant revealed that all of the alanine eventually disappeared.

Of the various competing reactions to be considered, the most obvious one was growth of the washed cells during incubation. Indeed, with alanine as substrate, growth did occur (table 6). On investigation of the various amino acids as substrates for growth it was found, without exception, that only those amino acids that

TABLE 6

Growth of Pseudomonas aeruginosa during incubation under standard conditions

Growth Parameter	Initial	Final	Final/ Initial
Protein (turbidity), mg/ml	0.29	1.37	4.72
Protein (direct), mg/ml	0.29	1.35	4.66
Total cells/ml	1.27×10^9	5.20×10^9	4.09
Viable cells/ml	1.06×10^9	5.00×10^9	4.72

Washed cells incubated under standard conditions for 12 hr. Protein concentration was estimated from turbidity measurements at 540 mμ by means of an independently prepared standard curve, or according to a biuret method. Total cell concentration was determined by direct count in a Petroff-Hauser counting chamber. Viable cells were determined by colony counts after plating on nutrient agar.

TABLE 7

Effect of inhibitors on pyocyanine and protein synthesis

Inhibitor	Pyocyanine	Protein
	Inhibition	
	%	%
NaF, 10^{-2} M	0	0
KCN, 10^{-3} M	100	60
Na_2HAsO_4, 10^{-3} M	100	100
NaN_3, 10^{-3} M	67	0
NaN_3, 2×10^{-3} M	80	20
Chloramphenicol:		
1.5×10^{-5} M	60	32
3.0×10^{-5} M	74	54
6.0×10^{-5} M	95	75

Washed cells incubated under standard conditions for 12 hr. Initial cell concentration, 0.2 mg protein per ml.

supported growth would also support pigment synthesis.

The close relationship between growth and pigment production was manifest also in the presence of a number of metabolic inhibitors (table 7); those inhibitors that produced useful inhibition of growth also inhibited pyocyanine formation. It is of some interest that chloramphenicol, which is reported (Hahn *et al.*, 1955) to be a fairly specific inhibitor of protein synthesis, was an effective inhibitor of pyocyanine production.

DISCUSSION

The immediate objective of the present investigation, to develop a simplified system in which to study the biosynthesis of pyocyanine, was not realized. Undoubtedly, the primary obstacle was the failure to dissociate pyocyanine formation from growth. Because of this, the system studied possesses little advantage over the employment of growing cultures for the biosynthetic investigation.

It is of interest to consider the possible reasons for the simultaneous occurrence of pigment synthesis and growth under all the conditions tested. Probably the most obvious hypothesis is as follows. Although amino acids and metabolically related compounds support pyocyanine synthesis, their derivatives which are actually incorporated into the ring system of the pigment are quite different. Extensive modification of the amino acid substrates by the cell, while permitting pigment formation, invariably results in the production of compounds that are utilized for growth. The failure of some amino acids to support both growth and pigment synthesis would then be due to deficiencies in the enzyme content of the cell. It is not surprising that a variety of compounds serve as precursors of pyocyanine in view of the extreme versatility of pseudomonads in attacking substrates of diverse nature.

The close association between growth and pigment production effectively obscures any attempt to decide which of the substrates tested may be a specific precursor of pyocyanine. Thus, even if a given amino acid were incorporated intact into the ring system of the pigment, that same amino acid might also be a good substrate for growth and its efficiency as substrate for pyocyanine might not be observable.

The recent publication of Grossowicz *et al.* (1957), reporting the synthesis of pyocyanine by resting cell suspensions of *P. aeruginosa*, is pertinent since the observation, if correct, would resolve the question as to whether pyocyanine synthesis depends upon over-all cellular growth. However, the present authors question the validity of this claim. In general, the pattern of results obtained in the two laboratories is quite similar in that: (a) a number

of single amino acids serve as substrates for pyocyanine synthesis; (b) the time course of of pyocyanine synthesis exhibits a lag period followed by a linear increase in pigment; (c) the times scales involved are comparable; and (d) the yields of pyocyanine are small.

The evidence cited by Grossowicz, *et al.* for their claim of a nonproliferating system involves (a) colony counts and turbidity measurements and (b) the "proportionality" between cell concentration and pyocyanine synthesis. With regard to the first point, data are not presented nor is it clear whether several measurements were made or merely "initial" and "final." A possible explanation could be partial lysis of the cells after initial growth in the experiments of Grossowicz *et al.* In the present investigation, it was repeatedly established by kinetic measurements that during the course of incubation under standard conditions, protein concentration increased to a time coinciding with the complete disappearance of alanine, after which a constant level was maintained for only ½ to 1 hr due to lysis. The present authors reject the second line of evidence on the basis that in the present studies, although pyocyanine synthesis is a direct function of isoleucine concentration, cell growth occurred with this amino acid. It would seem unlikely that such similar results could be obtained in two systems, when only one of them was complicated by cellular growth.

The results of the experiments dealing with salt effects are similar to those observed by Burton *et al.* (1948) but clarify the situation somewhat with regard to the high level of sulfate required for pyocyanine synthesis. Whether the inability of methionine as sulfur source to support pigment synthesis in the absence of added salt reflects a specific influence on sulfur metabolism by this organism or merely an indirect environmental influence was not determined.

SUMMARY

The biosynthesis of the phenazine pigment, pyocyanine, by washed cell preparations of *Pseudomonas aeruginosa* was studied. Elaboration of the pigment was found to require magnesium, phosphate, and a sulfur source in addition to an amino acid substrate. Isotope experiments indicated that all of the pigment formed during incubation arose from the added amino acid substrate (alanine). Through the use of alanine-C^{14} and glycerol as joint substrates, it was shown that the relative effectiveness of the two compounds as precursor of pyocyanine was a function of environmental conditions. Synthesis of pyocyanine, under all the conditions investigated, was accompanied by protein synthesis.

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