

Studies on the Biosynthesis of Pyocyanine. (IV)

On the Effect of Methionine and Other Promoting Factor in Peptone

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In the present paper, methionine was proved to be one of the effective components of peptone, and was discussed concerning the mechanism of its effect on pyocyanine formation. On the other hand, from the fact that there exists the bacterial strain to be indifferent to methionine, another effective factor in peptone was pointed out, and some informations were presented on the characteristics of this effective constituent.

INTRODUCTION

It has been reported in the preceding paper¹⁾ that one of the effective constituents of peptone for pyocyanine formation was Fe, and that besides Fe other promoting factor should be considered to exist in peptone. In general, with the medium prepared from residual part of peptone treated by active charcoal or with the synthetic medium without peptone, pyocyanine formation cannot so satisfactorily be expected as with peptone medium, even if Fe ion may be supplemented. As was already reported, acid-hydrolysate of peptone could effectively be used for pyocyanine formation as well as peptone itself²⁾, so that the effective constituent other than Fe would be anticipated to be some amino acids or other acid-stable substance. From the paper partition chromatography of acid-hydrolysate of peptone, it was concluded that one of the effective components was methionine.

On the other hand, it has, in some cases, been observed that peptone hydrolysate was very much inferior on pyocyanine formation to peptone itself, especially when H_2SO_4 was used for its hydrolysis. This difference was found to be ascribable to bacterial mutation. On the other hand, effect of methionine on pigmentation was also found to be different according to the kind of bacterial strains: methionine must, in general, be one of the effective constituents of peptone, but it was quite useless for pyocyanine formation with some strains of the bacteria.

Therefore, it may be suggested that there exists another factor in peptone. But at any rate, methionine can be regarded as an essential metabolite in pyocyanine synthesis or bacterial growth system, since it is produced together with other amino acids in bacterial cells, and some strains reveal both growth and pyocyanine formation in the synthetic medium without methionine and other amino acids. In the present paper, methionine is shown to be one of the effective

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components of peptone and the mechanism of its action on pyocyanine synthesis is discussed.

Furthermore, it is pointed out that besides methionine other stimulating factor may exist in peptone, and some informations are presented about the characteristics of this substance.

MATERIALS AND METHODS

Bacterial Strain

Six strains of *Pseudomonas aeruginosa* were used, which had been isolated in this laboratory.

Two strains of them were observed to respond hardly to methionine, although a remarkable pigmentation was revealed in peptone medium.

Determination of Pyocyanine

Pyocyanine was ordinarily estimated by extraction treatment of cultured solution, but when Beckman photometer was used, especially in the measurement at 690 m μ , the extraction treatment might be omitted, as the direct method reported previously was applicable.⁹⁾ For the separation of bacterial cells, ZnSO₄ was found to be available instead of calcium phosphate gel, which was a modified method of Somogyi¹⁰⁾ for precipitation of protein. Since the cultured solution is usually alkaline, the bacterial cells and other protein substances were precipitated and clear filtrate could be obtained simply by the addition of ZnSO₄, and yet NH₄OH was added if necessary. In the very aged cultured solution, the determination of pyocyanine at 520 m μ with acid solution would not be successful, since wine-red pigment possibly derived from green fluorescent pigment is occasionally recognized.

Determination of Glycerol

In order to know the relation between the fluctuation of glycerol and the degree of bacterial growth, the determination of glycerol was carried out according to the periodate method of Varis *et al.*¹¹⁾

Determination of Methionine

Methionine was estimated by the following procedure : the reaction product of methionine with ninhydrin showed an absorption maximum at 560 m μ , and therefore, from the optical density standard curve for methionine determination was established (Figs. 1a and 1b). Five ml of cultured solution was treated with calcium phosphate gel to make the solution clear, and this solution was extracted with chloroform in order to remove pyocyanine formed. The colorless solution thus obtained was aspirated to evaporate chloroform, adjusted to pH 7.2, 2% ninhydrin solution was added and final volume was adjusted to 20 ml after being allowed to stand for 30 minutes at 40° so as to react completely with methionine. The measurement was carried out at 560 m μ by using Beckman spectrophotometer, model DU.

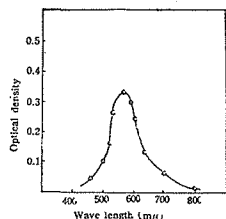


Fig. 1a. Absorption spectrum of the reaction product of methionine with ninhydrin. Methionine concentration is approximately $10^{-3}M$.

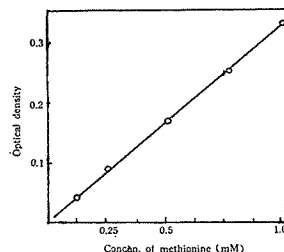


Fig. 1b. Standard curve expressing the relation between the amount of methionine and the value of optical density of its reaction product with ninhydrin. Optical density is of value at $560\text{ m}\mu$.

Even when the solution was composed of amino acids more than two kinds, the quantitative determination would easily be accomplished by dividing the total amount of amino acid shown with the solution into the ratio of the amino acid of each spot appeared on paper chromatograms.

Estimation of Bacterial Growth

Bacterial growth was expressed as the cell number or the turbidity of cell suspension in comparison with the standard curve reported in the preceding paper¹⁾. When bacterial growth is estimated by its turbidity, the dispersion of cell suspension must be uniform. However film of cells which obstructs the uniformity is usually formed by the present bacteria. Therefore the cultured solution followed by heating treatment was shaken so intensively in test tube with rubber stopper so as to make cell dispersion uniform, before the measurement of the turbidity. On the other hand, one should be careful for a decrease in turbidity after several days of incubation owing to the autolysis of bacterial cells (see Fig. 2).

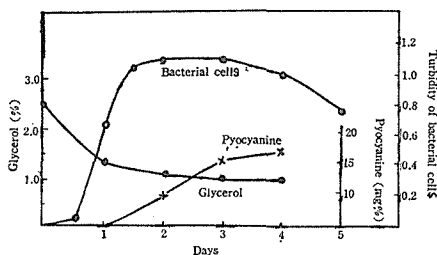


Fig. 2. Growth curve of the bacteria. Data show the result with the synthetic medium containing 0.5% glutamic acid and 0.1% NH_4NO_3 as source of nitrogen.

This objection may be avoided by keeping the cell suspension through heating treatment during the maximum state of the growth prior to the estimation.

EXPERIMENTAL AND DISCUSSION

Adsorption Treatment of Peptone

As mentioned before, the effective constituents of peptone were adsorbed

onto charcoal, so that the following procedure was chosen for their isolation : to 1 liter of 10% peptone solution adjusted to pH 4 to 5, 50g of active charcoal were added, allowed to stand for several hours at 45° and the charged adsorbent was eluted with ammoniacal 60% acetone, after being washed with worm water until no more ninhydrin reaction was observed in the washed solution.

Ammonia and acetone were evaporated in order to concentrate the eluted solution which was once filtered during the concentration, and further concentrated into syrup.

Paper Chromatography

On one-dimensional paper chromatogram of the eluate thus obtained, there appeared one clear and one faint small spots. The syrup-like substance was hydrolyzed with 6*N* HCl at 100° for 16 hours and the hydrolysate was repeatedly distilled with the excess of water to remove HCl, and then calamel-like substance was eliminated by treating with a small amount of charcoal.

The concentrated hydrolysate was developed by two-dimensional paper chromatography with the following solvent systems : (1) phenol-ethanol-water (2 : 1 : 1) and (2) *n*-butanol-acetic acid-water (4 : 1 : 2). For two-dimensional paper chro-

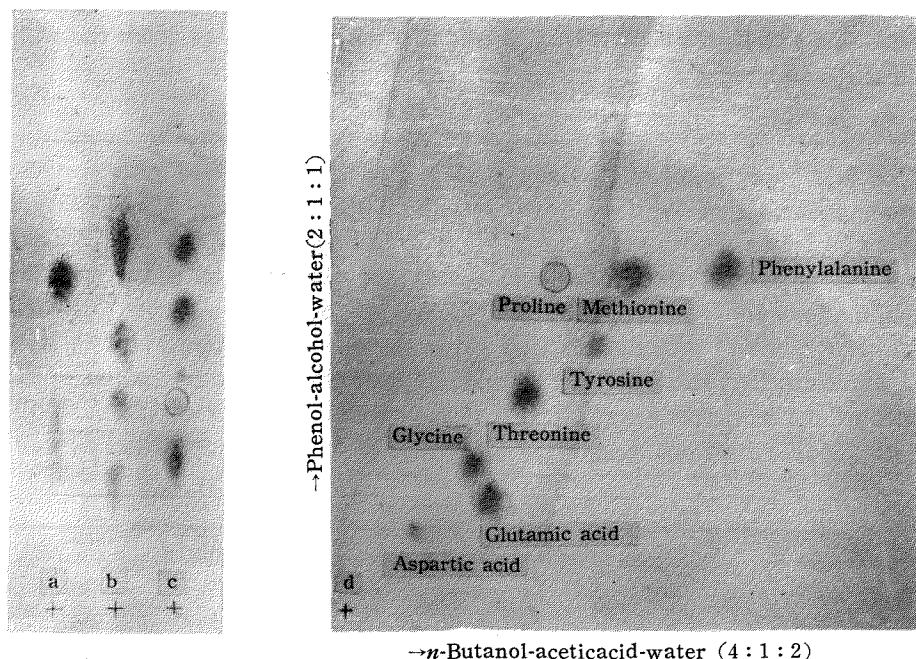


Fig. 3. Paper chromatography of acid-hydrolysate of peptone treated by charcoal adsorption.

- a, The eluate before hydrolysis, with the solvent system: *n*-butanol-acetic acid-water (4 : 1 : 2).
- b, The hydrolysate of the eluate with the solvent system: phenol-alcohol-water (2 : 1 : 1).
- c, The hydrolysate, with the same solvent system as in a.
- d, The two-dimensional chromatograms of the hydrolysate, with the solvent systems: phenol-alcohol-water (2 : 1 : 1), and *n*-butanol-acetic acid-water (4 : 1 : 2).

matography of amino acid the present solvent system (1) was found to be more excellent than the others hitherto presented for the separation of amino acid. Fig. 3 represents the chromatograms of acid-hydrolysate of pepton treated by charcoal adsorption. Six distinct ninhydrin positive spots were observed to be corresponding in Rf value to proline, methionine, phenylalanine, threonine, glycine and glutamic acid. Very faint spot of amino acids similar to aspartic acid and tyrosine was also detected. In order to examine whether the effective constituent of peptone will be relating to amino acid, the effect of each fraction on pyocyanine formation has been tested by eluting the spots corresponding to Fig. 3, c.

Table 1. Effect of each fraction of peptone hydrolysate.

Fractions	No. 1	No. 2	No. 3	No. 4	No. 5	Control
Pyocyanine formed (%)	0.0050	0.0072	0.0042	0.0038	0.0042	0.0036

Basal medium : 2.5% glycerol, 0.2 % urea, 0.05 % MgSO₄·7H₂O, 0.025 % K₂HPO₄ and 0.0005% Fe₂(SO₄)₃ ; pH 7.4.

As seen in Table 1, fraction No. 2 revealed the most remarkable effect on pigmentation and it was ascertained to coincide with methionine from the nature of the chromatography.

Effect of Methionine

The effect of methionine along with the other amino acid was tested on pyocyanine formation with synthetic medium which contained corresponding amount of various amino acids constituting 1g of casein⁶⁾ (Table 2). As might have been expected, the remarkable effect was observed with methionine on pyocyanine formation, although any noticeable effect on bacterial growth or carbon (glycerol or glucose) assimilation was never pointed out (Tables 3 and 4). It was noted therefore that the effect of methionine on pyocyanine formation is independent of the bacterial growth and that the mixture of each amino acid other than methionine does not reveal any significant effect on pigmentation as well as

Table 2. Amino acid component of casein.

DL-Alanine	0.037 (%)	L-Lysine	0.086 (%)
L-Arginine	0.041	L-Methionine	0.033
L-Aspartic acid	0.078	L-Phenylalanine	0.054
L-Cystine	0.005	Hydroxy-L-proline	0.117
L-Glutamic acid	0.233	DL-Serine	0.064
Glycine	0.021	DL-Threonine	0.048
L-Histidine	0.034	L-Tryptophan	0.014
L-Isoleucine	0.066	L-Tyrosine	0.033
L-Leucine	0.105	DL-Valine	0.070

bacterial growth, while peptone shows a remarkable effect in both systems. Ester *et al.*⁷⁾ have already reported the effect of amino acid such as DL-alanine

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Table 3. Effect of methionine and other amino acids on pyocyanine formation.

Medium	Pyocyanine formed (mg%)					Glycerol remained (%)					Bacterial cell number ($\times 10^8$ /ml)				
	Strains					Strains					Strains				
	B ₁₆	B _a	B _k	B _o	B ₁	B ₁₆	B _a	B _k	B _o	B ₁	B ₁₆	B _a	B _k	B _o	B ₁
A	4.4	7.3	2.5	2.5	5.0	1.51	1.49	1.48	1.42	1.14	11.5	11.0	9.8	9.0	10.0
AM	12.0	12.5	3.5	3.8	10.0	1.43	1.45	1.48	1.38	1.36	10.8	9.8	8.8	11.5	10.5

Basal medium is the same as in Table 1.

A : among all the amino acids shown in Table 2, methionine is omitted.

AM : all the amino acids including methionine are contained in the corresponding concentrations shown in Table 2.

Table 4. Effect of methionine formation.

Medium	*Pyocyanine formed (mg%)			Glycerol remained (%)			**Glucose remained (%)				*Bact. No. ($\times 10^8$ /ml)		
	Strains			Strains			Strains				Strains		
	B ₁₆	B _a	B ₁	B ₁₆	B _a	B ₁	B ₁₆	B _a	B _o	B ₁	B ₁₆	B _a	B ₁
U	4.0	5.2	4.4	1.17	1.00	1.13	0.97	—	0.80	—	10.6	11.0	9.6
UM	11.0	11.4	6.2	1.02	1.07	1.11	0.72	—	0.82	—	9.8	10.6	—
G	4.8	8.0	5.4	1.08	1.14	1.20	0.97	0.60	0.80	0.80	10.0	10.8	10.2
GM	11.2	12.0	8.0	1.00	1.25	1.19	0.84	0.90	0.87	0.90	11.0	11.8	11.2

U : medium composed of 2.5% glycerol, 0.2% urea, 0.05% MgSO₄·7H₂O, 0.025% K₂HPO₄ and 0.0005% Fe₂(SO₄)₃. UM : U+0.05% methionine. G : urea in medium U was replaced by 0.5% glutamic acid and 0.1% NH₄NO₃. GM : G+0.05% methionine.

*Data with glycerol medium.

**Glycerol in media was replaced by 2.5% glucose, and CaCO₃ was added ;—, no measurement.

or L-leucine, but it was not supported by the present author concerning the formation of pyocyanine. However, as regards the bacterial growth, the effect of amino acid could not necessarily be denied, because the lag period was found to be shortened by the administration of amino acids such as alanine especially β -alanine, glutamic and aspartic acids. These effects were also observed with the

Table 5. Effect of the concentration of methionine on pyocyanine formation.

Methionine added (mg%)		100.0	50.0	25.0	10.0	5.0	2.5	1.0	0.5	control
Pyocyanine found (mg%)	B ₁	12.6	12.4	10.6	11.8	8.2	6.3	4.2	5.5	4.4
	B ₁₆	14.2	9.2	14.0	10.6	8.8	5.2	4.7	4.8	4.8

B₁, B₁₆ : kinds of strains.

Basal medium is the same as in Table 1.

members of tricarboxylic acid cycle such as α -ketoglutarate, fumarate or malate, when one of them was added to the synthetic medium⁸⁾. As shown in Table 5, pyocyanine formation was increased when an increasing amount of methionine was administered, but a far smaller amount of methionine did not cause a noticeable acceleration. In order to eliminate the effect on bacterial growth, methio-

nine was added to the culture medium at a requisite stage of the growth and incubated for more than 12 hours, as seen in Table 6. And it was found to take fairly long time for the effect of methionine on pyocyanine formation. In this case, it is worth to note that D-form of methionine is also useful for pyocyanine formation in the same activity as in L-methionine. It is anticipated from the above result that methionine is rather a metabolite directly relating to

Table 6. Effect of methionine on pyocyanine formation by its addition at stationary growth phase.

Stage of addition (hours)	24	48	72	96	control
Pyocyanine found (%)	0.010	0.008	0.006	0.005	0.006

Strain used : B_a

Medium was the same as in Table 1.

an intermediate than a cofactor of the enzyme system in pyocyanine synthesis. The further experiments were carried out with various methyl donors in expectation of methionine which might play the role of transmethylation in course of pyocyanine synthesis. As shown in Table 7, it was found that methionine could be replaced by other substances generally recognized to be methyl donor such as choline and betaine, or acetyl choline in the same effect on pyocyanine formation,

Table 7. Effect of other substances to be replaced for methionine.

Strains	Substances				
	Methionine	Acetylcholine	Choline	Betain	Control
B ₁₆	0.0108	0.0142	0.0068	0.0110	0.0044
B _a	0.0140	0.0120	0.0090	0.0094	0.0060
B _k	0.0038	0.0040	0.0044	0.0050	0.0036
B _s	0.0042	0.0052	0.0038	0.0048	0.0040
B ₁	0.0070	0.0082	0.0070	0.0082	0.0042

Data show the amount of pyocyanine expressed in %.

Basal medium is the same as in Table 1.

Concentration of each substance used is 0.002M.

as was observed with methionine. From the above experiment, methionine is suggested to carry the methyl group to the intermediate in the formation of pyocyanine, although a decided evidence could not yet be manifested.

Metabolism of Methionine

Further studies were attempted to investigate methionine fluctuation in the culture solution. Although methionine has been shown to be deaminated by L- or DL-amino acid oxidase,^{9,10} it could not be utilized by the present bacteria as a source of nitrogen, so that a multiplication of bacterial cells did not take place in the absence of the other nitrogenous matter, including single amino acid (Table 8).

However, methionine in the medium was observed to be decreased during the incubation process (Fig. 4).

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Table 8. Assimirability of amino acids as source of nitrogen.

	Growth	Pyocyanine formation		Growth	Pyocyanine formation
DL-Alanine	+	+	L-Lysine	trace	--
L-Arginine	+	+	L-Methionine	--	--
L-Aspartic acid	+	--	L-Phenylalanine	--	--
L-Cystine	--	--	Oxy-L-proline	--	--
L-Glutamic acid	+	--	L-Serine	+	--
Glycine	+	+	DL-Threonine	trace	--
L-Histidine	+	+	DL-Tyrosine	trace	--
L-Isoleucine	+	--	L-Tryptophan	+	--
L-Lencine	trace	--	DL-Valine	trace	--

Basal medium: 2% glycerol, 0.05% $MgSO_4 \cdot 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Fe_2(SO_4)_3$, pH7.4. Each amino acid was tested at a concentration of 0.1% as sole source of nitrogen.

On the other hand, not only bacterial growth but also pyocyanine formation was observed in the synthetic medium containing methionine, even in the absence of sulfate which was an essential element for both the pigmentation and the bacterial growth, as had been reported previously.^{1,2)} It is suggested from the above result that sulfate would serve as a source of methionine in course of pyocyanine formation, and otherwise that methionine may be utilized for the synthesis of other sulfur compound. Methionine has been recognized to play the

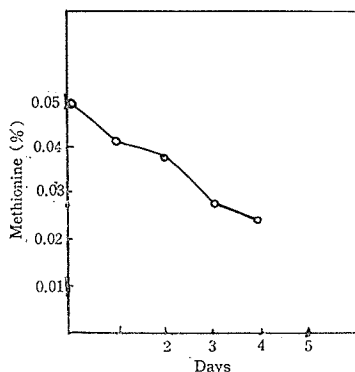


Fig. 4. Consumption of methionine in the cultural solution. To the same medium as shown in Table 1, 0.05% methionine was added.

role of methyl transfer in animal body, and the demethylation product may be expected to be homocysteine, judging from the fact that methionin is synthesized by methylation of homocysteine in the presence of choline or betaine.¹¹⁻¹⁴⁾

On the other hand, it has been shown that from mthionine, homoserine was derived when it was incubated with liver preparation, and that cystathionine which was derived from methionine as an intermediate in the formation of homo-serine and cysteine, was formed by the combination of homocysteine with serine.¹⁵⁾

In order to detect homocysteine or homoserine in an incubation mixture owing to transmethylation reaction, the following experiment was carried out: the

bacterial cells grown on the synthetic medium prepared with urea as a sole source of nitrogen, were harvested by centrifugation after being incubated for 36 hours when pyocyanine was appreciably formed and the cells were washed with 0.01 *M* phosphate buffer of pH 7.2. One g of the wet cells was suspended in 100 ml of 0.01 *M* phosphate buffer of pH 7.4, containing 0.05 g of $MgSO_4 \cdot 7H_2O$ and 0.1 g of Na-succinate. To 50 ml of this suspension, methionine was added to attain 0.002 *M* concentration and incubated at 37° for 16 hours with control experiment without methionine. The reaction mixtures were treated with calcium phosphate gel to make the solution clear, concentrated and then mounted on paper chromatography. The result is shown in Fig. 5. The paper chromatograms

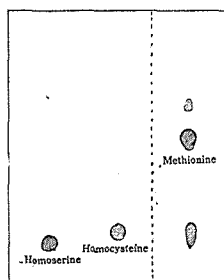


Fig. 5. Paper chromatography of a reaction product of methionine by resting cells compared with known amino acids (left), using the solvent system: *n*-butanol-acetic acid-water (5 : 1 : 2).

of the reaction mixture showed two faint spots besides the spot of methionine, whereas with the control experiment any spot did never appear, suggesting no autolysis of the bacterial cells. Among the three spots, the one was found to be corresponding rather to homocysteine than to homoserine, although the other spot was not yet identified. On the other hand, in the experiment with the growing state of the bacteria, these spots were hardly recognized. The reason of this phenomenon may be explained to be ascribable to the further metabolism of methionine in growing system. The experiment with the cell-free preparation of the bacteria which were harvested after the cultivation of 24 hours has also succeeded in obtaining the result similar to that of the experiment with resting cells mentioned above.

Although vitamin B_{12} was observed to be hardly effective in the metabolism of methionine, the effect of methionine on pigmentation is assumed to play the role of methyl carrier in course of pyocyanine biosynthesis. As regards the transmethylation, a noticeable fact has been found: in paper chromatographic study of the methionine metabolism, the spot corresponding to homocysteine was more markedly detected by the administration of anthranilic acid which might be assigned a role of methyl acceptor. The result of further studies on this problem will be presented later on.

Other Effective Component of Peptone

As was already illustrated, the strain unresponsive to methionine which revealed the remarkable pigmentation in peptone medium, was recognized among

the strains used in the present experiment. For the reason why no effect of methionine was observed with this strain, the following assumption will be proposed. Methionine would possibly be offered for the bacterial growth before the synthesis of pyocyanine, and therefore the requirement of methionine could be divided according to the kinds of bacterial strain, depending on their capacity for the synthesis of methionine. Similarly, the other stimulating factor for pigmentation which exists in peptone, may also be required by the bacteria according to their capacity for its synthesis in the synthetic medium without peptone. In other words, methionine may be no more than the effective substance which invests the bacteria with one of the conditions necessary for pyocyanine formation. It was found that in residual part of phosphotungstic acid-precipitation of peptone solution, the promoting effect on pyocyanine formation with major kinds of strain was remained, and that the same effect as with peptone itself was still observed to be maintained in the experiment with the methionine-unresponsive strain, after the deamination treatment of peptone by means of nitrous acid.

It is, on the other hand, interesting to point out that the effective substance similar to peptone is found in cow's milk serum or yeast extract. These effective substances are supposed to serve rather as a stimulating factor than as a precursor of pyocyanine. Although the effective substance recognized in peptone can not yet be obtained in pure state owing to its less solubility in organic solvent, it is presumed to be an aromatic compound, according to the phosphomolybdate test¹⁶⁾: when phosphomolybdic acid is added to the acidified solution of this effective substance and is made alkaline, it is immediately colored to blue as is recognizable with ordinary benzene compound.

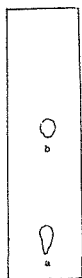


Fig. 6. Paper chromatography of the constituent of peptone being regarded as a stimulating factor for pyocyanine formation.

Solvent is the same as in Fig. 5.

- a, With the sample obtained by the adsorption treatment.
- b, Detected in the cultured solution of the bacteria grown on peptone medium.

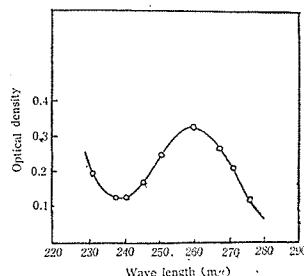


Fig. 7. Absorption curve of the component of peptone expected to be a stimulating factor for pyocyanine formation, obtained by the adsorption treatment and paper partition chromatography.

On the other hand, the following result has been offered as a further evidence: the aqueous eluate of one of the fluorescent spots (Fig. 6, a) obtained by paper chromatography according to the same procedure as was described before, revealed an increasing effect on pyocyanine formation. Furthermore, the photometric

experiment has shown that its absorption maximum exists at 260 m μ , as seen in Fig. 7. It is of interest to note that another fluorescent spot has appeared on paper chromatograms from the butanol extract of the cultured solution of the bacteria grown on peptone medium, while it was not observed with synthetic medium without peptone (Fig. 6, b).

SUMMARY

1. It has been shown that one effective constituent of peptone was identified with methionine, and that the other amino acid components did not reveal any effect on pyocyanine formation.

2. It was assumed that methionine would play the role of methyl carrier in course of pyocyanine synthesis, since the same effect was observed with known methyl donor such as choline and betain, or acetylcholine, and the product derived from methionine was expected to be identical with homocysteine.

3. From the characteristics of the bacterial strain unresponsive to methionine, the other stimulating factor was suggested to exist in peptone and some informations on this substance were presented.

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