STUDY OF BACTERIAL FLUORESCENCE IN VARIOUS MEDIA

I. INORGANIC SUBSTANCES NECESSARY FOR BACTERIAL FLUORESCENCE

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INTRODUCTION AND HISTORICAL

While working with fluorescing organisms it was found that certain lots of media failed to show the production of fluorescence when used for the growth of *B. fluorescens-liquefaciens*.[1] However, media of the same composition, when made with different lots of material, gave a good production of pigment. In looking up the literature on this subject it was found that conflicting statements appeared as to the constituents necessary for the production of fluorescence.

One of the first synthetic media used for the production of pigment with fluorescing organisms was proposed by Hueppe (1880). It contained a solution of ammonium tartrate, neutral potassium phosphate, magnesium sulphate, and calcium chloride.

Gessard (1892) studied the production of fluorescence with *B. pyocyaneus* in a medium very similar to that used by Hueppe. He found that phosphates were essential for pigment production, and that $\frac{1}{8000}$ part of phosphate could be detected in a medium by the production of fluorescence. Nägeli (1895) stated that calcium could be substituted for magnesium and that fluoresence could still be obtained. Thumm (1895), while working with *B. pyocyaneus*, studied fluorescence in a medium similar to that used by Gessard. For the organic constituent, he tried a number of

[1] This organism is now called *Pseudomonas fluorescens* by Bergey.

organic compounds and concluded that, in addition to a source of organic nitrogen, a phosphate and magnesium sulphate must be present. He disagreed with Nägeli's conclusion that calcium could be used in lieu of magnesium.

Lepierre (1895) studied a fluorescent bacillus which he isolated from water. He found that the only common organic acid salts, which gave fluorescence when used with the proper inorganic constituents, were citric, succinic, oxy-glutamic, and glutaric acids. He concluded that fluorescence depends, first, upon the dibasicity of the acid; second, upon the presence of at least two CH_2 groups in the molecule.

Jordan (1899) studied the production of fluorescence with six species of fluorescing bacteria. He used ammonium salts of a number of organic acids with magnesium sulphate and sodium phosphate. He found that the dibasicity of the acid and the presence of CH_2 groups were not essential as stated by Lepierre. Jordan concluded that a sulphate and a phosphate salt were the only inorganic constituents needed, and that, regardless of the base, equally good fluorescence was given. In his words: "The nature of the base associated with the phosphorous and sulphur appears to be a matter of complete indifference. Sodium, potassium, and magnesium salts give similar results in whatever way they are combined. Even if ammonium phosphate and ammonium sulphate be used together, fluorescence appears, but it is somewhat less intense than in the presence of one of the bases mentioned above."

Jirou (1901) found that the simplest medium for production of fluorescence consisted of a mineral salt containing nitrogen, a hydrocarbon, and an alkaline or alkaline-earth phosphate. Ammonium citrate will suffice, he stated, if one adds ammonium carbonate and a phosphate.

Sullivan (1905) agreed with Jordan. He concluded "that the fluorescent pigment could be formed whenever, in addition to asparagine, there was present in the solution both phosphates and sulphates, irrespective of the base." He also agreed, in the main with Lepierre that the production of pigment was favored by the carboxyl and methylene groups.

Tanner (1918) studied one hundred strains of fluorescent bacteria isolated from water in Uschinsky's medium, Sullivan's medium, and Frankel's medium. In addition to other ingredients, the two media first mentioned contain magnesium sulphate and a phosphate. Frankel's medium contains sodium chloride, monocalcium phosphate, ammonium lactate, and asparagine. According to Tanner all three of these media gave a good pigment production. It will be noted that Frankel's medium contains neither sulphates nor magnesium. One, therefore, might conclude that these elements were not essential for the production of fluorescence. However, some of the chemicals may have been impure.

As will be noted by the foregoing brief survey of the literature all investigators do not agree on the constituents especially the inorganic salts, which are necessary for the production of fluorescence. All of them, however, believe that some nitrogen containing organic compound, such as asparagine or ammonium succinate, is necessary as the organic constituent. Therefore, the organic compounds necessary for fluorescence will be considered settled: asparagine will be taken as the organic constituent in this research, and the constituents under investigation will be the inorganic salts.

METHODS AND MATERIALS

In all, ten different cultures of fluorescing organisms were used. Four of these were from standard collections and six were isolated from water supplies. All of these cultures were identical with, or closely allied to, *Pseudomonas fluorescens* (Flügge).

Great care was used in the selection of glassware and chemicals, owing to the fact that the least amount of impurity might vitiate the results. The glassware was thoroughly cleansed, rinsed in tap water, then with alcohol, and finally three times with distilled water. The chemicals used were the purest to be obtained on the market. In most cases analyzed chemicals were used. These were, however, tested for impurities. Over fifty samples were analyzed, and it was found that many of the samples contained far too great an amount of impurities, so that several recrystallizations were necessary in many cases.

All of the media were made up very carefully with water, redistilled from pyrex glass. A liter of this water gave no weighable residue. About 7 cc. of the nutrient solution were placed in each test tube and sterilized in steam for thirty minutes on three successive days.

Inoculations were made from forty-eight-hour broth cultures. In cases where the various sulphates were being tested for their effect on fluorescence, the broth contained no magnesium salts for fear that a trace of magnesium might be carried over into the medium tested. Likewise, in cases where some other salt was being tested for a given impurity, this ingredient was eliminated from the broth in which the stock cultures were grown.

EXPERIMENTAL

A number of preliminary experiments indicated that very good fluorescence was produced when asparagine, magnesium sulphate, and dipotassium hydrogen phosphate were used as a basis for the medium. Experiments were then conducted to see if this medium could be simplified. Media were made containing the following ingredients in varying amounts: (1) asparagine, (2) asparagine and a potassium phosphate, (3) asparagine and magnesium sulphate, (4) asparagine and magnesium chloride, (5) asparagine and the various alkali sulphates. None of the above media gave any fluorescence except in a few isolated tubes.

As noted in the historical review, Jordan and Sullivan state that sulphates and phosphates, regardless of the base, are the only inorganic elements necessary for the production of fluorescence. Therefore, this phase was next investigated. Media were prepared containing 0.2 per cent asparagine, with varying amounts of dipotassium phosphate and sulphates of sodium, potassium, and ammonium; and also sulphuric acid. Control media were also prepared from each of the above media with the addition of 0.01 per cent anhydrous magnesium chloride. Very little fluorescence was produced in any of the media without magnesium as may be seen by the results of one of the experiments given in table 1. Table 2 represents the same media plus 0.01 per cent magnesium chloride. The quantity of fluorescence is indicated by plus signs.

Four plus signs indicate the maximum. A negative mark indicates no fluorescence. Final readings were made at the end of four days. In all of the experiments where no fluorescence was given, a satisfactory growth was, nevertheless, produced. The lack of fluorescence, therefore, can not be charged to lack of growth.

The experiments with the other sulphates gave similar results to those recorded in tables 1 and 2. These results seem to indi-

TABLE 1

Growth of fluorescing organisms in media containing asparagine, K_2SO_4, and K_2HPO_4

K_2SO_4	K_2HPO_4			
	0.001 per cent	0.01 per cent	0.1 per cent	0.2 per cent
per cent				
0.0001	−	−	−	−
0.001	−	−	+	−
0.01	−	−	−	−
0.1	−	+	−	+
0.2	+	++	+	+

TABLE 2

Growth of fluorescing organisms in media when 0.01 per cent $MgCl_2$ was added

K_2SO_4	K_2HPO_4			
	0.001 per cent	0.01 per cent	0.1 per cent	0.2 per cent
per cent				
0.0001	+	+	+	−
0.001	+	+++	++++	++
0.01	++	++++	++++	+++
0.1	++	++++	++++	+++
0.2	+	++	++	−

cate that fluorescence will not be produced equally well by any sulphate, save that of magnesium. In some cases, the media containing the larger amounts of the sulphates gave a slight production of pigment. It was thought that these might contain a small amount of magnesium as an impurity. However, when 5 grams were analyzed no magnesium was detected.

A large number of C.P. analyzed sulphates were obtained. Five samples of each of the sulphates of sodium, potassium, and

ammonium were selected. Analysis of each sample was made for magnesium, using 100 grams. The samples of sodium sulphate contained magnesium sulphate varying from 0.003 to 0.0184 per cent, the potassium sulphates from 0.0039 to 0.0432 per cent, and the ammonium sulphates from none to 0.0014 per cent.

Analyses were also made on five samples each of the phosphates of sodium, potassium, and ammonium. Two samples of disodium phosphate, one sample of dipotassium phosphate, and four samples of diammonium phosphate were found to be free from magnesium and sulphates.

Because of the expense of asparagine only 10 grams were used for the analysis. Six samples out of eleven were found to be free from magnesium, sulphates, and phosphates. On account of the small amount of asparagine used for the analysis, it was decided to use a biological method of analysis for impurities. Media were prepared using each sample of asparagine with different combinations of the inorganic constituents. The pH of all of the media used in this research was between 6.8 and 7.2. The following were prepared with 0.2 per cent asparagine:

1. Asparagine and 0.01 per cent phosphate.
2. Asparagine, 0.01 per cent phosphate, and 0.01 per cent Na_2SO_4.
3. Asparagine, 0.01 per cent phosphate, and 0.01 per cent $MgCl_2$.
4. Asparagine and 0.01 per cent $MgSO_4$.
5. Asparagine, 0.01 per cent phosphate, and 0.01 per cent $MgSO_4$.

The research to this point seems to indicate that the inorganic constituents necessary for fluorescence are magnesium, sulphate, and phosphates. Assuming this to be true, then fluorescence in the above media would indicate certain impurities. Positive results in number 1 would show the presence of sulphates and magnesium; in number 2, magnesium; in number 3, sulphates; in number 4, phosphates. Number 5, of course, was used as a control. The results for one of the five organisms used are listed in table 3. The figures in parentheses indicate the number of hours necessary for the appearance of the maximum fluorescence

The results by the biological method did not show perfect agreement with the chemical method. Sulphates were shown by the

biological method, in more samples, than by the chemical test. The reverse was true with magnesium. There were six of the eleven samples free from impurities when tested chemically. Five of these six were also free from impurities when tested biologically.

The purest samples of asparagine and dipotassium phosphate were selected as the samples to be used in all future media. Experiments had shown that there was little difference in the fluorescence produced with the sodium and potassium phosphates. The ammonium phosphates were not quite as satisfactory.

TABLE 3

Fluorescence with media made from different samples of asparagine (0.2 per cent), organism C

SAMPLE ASPARAGINE NUMBER	0.01 PER CENT K_2HPO_4	0.01 PER CENT K_2HPO_4 AND 0.01 PER CENT Na_2SO_4	0.01 PER CENT K_2HPO_4 AND 0.01 PER CENT $MgCl_2$	0.01 PER CENT $MgSO_4$	0.01 PER CENT K_2HPO_4, 0.01 PER CENT H_2SO_4 AND 0.01 PER CENT $MgCl_2$
1	−	−	−	−	++++(36)
2	−	++(84)	−	−	++++(36)
3	−	++(84)	−	−	++++(48)
4	−	+++(60)	−	−	++++(36)
5	+(60)	++(60)	+(72)	−	++++(36)
6	−	−	−	−	++++(36)
7	+(72)	+(72)	++(48)	−	+++(48)
8	−	++(72)	−	−	++++(36)
9	−	−	−	−	++++(36)
10	−	−	−	−	++++(36)
11	−	−	−	−	++++(36)

All of the sulphates, previously analyzed, were used for the preparation of media. Even if the sample did show considerable magnesium, it was used to show the effects of impurities. The media contained 0.2 per cent asparagine, 0.01 per cent dipotassium phosphate, and the sulphate in varying amounts. Similar media were made as above except that magnesium chloride was added; 0.0001, 0.001, and 0.01 per cent were used. With the media made from the purest sulphates, little fluorescence was found. Table 4 gives the results with the purest sulphate used, and table 5 gives the results with one of the C.P. analyzed sulphates which showed

the largest amount of magnesium present as impurity. Of course, all the media to which magnesium chloride was added gave excellent fluorescence.

In studying the media made from the different sulphates, it was found that the greater fluorescence was produced by the sample containing the most magnesium as impurity. It is possible that a sample of sulphate may contain enough magnesium to produce fluorescence and still show no magnesium on analysis, owing to

TABLE 4

Fluorescence in media containing 0.2 per cent asparagine, 0.01 per cent K_2HPO_4, and $(NH_4)_2SO_4$ no. 1

$(NH_4)_2SO_4$	ORGANISM A	ORGANISM C	ORGANISM E	ORGANISM F	ORGANISM G	ORGANISM H
per cent						
0.00001	−	−	−	−	−	−
0.0001	−	−	−	−	−	−
0.001	−	−	−	−	−	−
0.01	−	−	−	−	+(60)	−
0.1	+(48)	−	−	+(72)	−(60)	+(60)

TABLE 5

Fluorescence in media containing 0.2 per cent asparagine, 0.01 per cent K_2HPO_4, and K_2SO_4 no. 3

K_2SO_4	ORGANISM A	ORGANISM C	ORGANISM E	ORGANISM F	ORGANISM G	ORGANISM H
per cent						
0.00001		−	−	−	−	−
0.0001	+(60)	−	−	+(60)		+(72)
0.001	+(60)	+(72)	+(60)	+(60)	+(48)	+(72)
0.01	++(60)	+(60)	+(72)	+(72)	+(72)	+(48)
0.1	++(48)	++(72)	+(84)	+(84)	+(48)	+(48)

the solubility of the magnesium ammonium phosphate during the analysis.

As the result of over eleven hundred tests with the sulphates, it may be seen that magnesium is essential for the production of pigment. The media, containing the sulphates of the alkalies, gave little or no fluorescence, but in every case where magnesium chloride was added a good fluorescence was produced.

In order to prove definitely that the alkali sulphates will not suffice to produce fluorescence, it was decided to prepare a sulphate absolutely free from magnesium. This was done by selecting the purest ammonium hydroxide and sulphuric acid, and distilling each separately from an all quartz apparatus. The products were received in platinum dishes. The two were mixed in the proper proportions to form ammonium sulphate. A medium was made, using this pure sulphate with a pure sample of dipotassium phosphate and asparagine. The medium was first put in soft glass tubes, but a slight fluorescence, in some cases, was produced. Later quartz tubes and then pyrex tubes were used. Part of the results are recorded in table 6.

TABLE 6

Fluorescence in media made from pure $(NH_4)_2SO_4$ in different kinds of tubes (0.2 per cent asparagine, 0.01 per cent K_2HPO_4)

$(NH_4)_2SO_4$	SOFT GLASS No. 1	SOFT GLASS No. 2	QUARTZ GLASS	PYREX GLASS	MEDIA PLUS 0.01 PER CENT $MgCl_2$
per cent					
0.00001	−	−	−	−	++(60)
0.0001	−	+(84)	−	−	+++(60)
0.001	+(72)	−	−	−	++++(48)
0.01	−	−	−	−	++++(48)
0.1	+(60)	+(72)	−	−	+++(48)

The results recorded in table 6 show that a medium made with ammonium sulphate in place of magnesium sulphate will not produce fluorescence. A slight fluorescence was produced when soft glass test tubes were used. It may be that a small amount of magnesium was dissolved from the glass.

The previous experiments have shown that magnesium must be present in a medium in order to produce fluorescence. The next question to be taken up was whether the sulphate was necessary. A number of C.P. analyzed samples of magnesium chloride and magnesium ammonium chloride were selected. These were analyzed for sulphates. Three out of seven samples of magnesium chloride gave no sulphates, and one sample out of three lots of magnesium ammonium chloride was free from sulphate.

The above samples, together with some of the impure samples, were used in making up the test media. These media contained 0.2 per cent asparagine, varying amounts of potassium phosphate from 0.001 to 0.1 per cent, and ten different amounts (from 0.00001 to 0.5 per cent) of the magnesium chloride or magnesium ammonium chloride. This made fifty different media for each chloride used. The media, containing the chlorides free from sulphates, gave very little fluorescence, and such as did appear was probably due to impurities which could not be detected on analysis. In the media made with one sample of magnesium chloride pigment production was given in only three tubes out of the fifty tested. The samples of chlorides which contained the sulphate as impurity gave much more fluorescence especially when the larger amounts of magnesium chloride were used. To the fifty different media made from each chloride, 0.01 per cent sodium sulphate was later added. Each of these solutions was tested with the same organisms as above. In each medium, where the magnesium was present in amounts greater than 0.001 per cent, a good fluorescence was produced. This set of experiments shows clearly that the sulphate is necessary for pigment production.

Up to this point magnesium and sulphate have been shown to be indispensible for the production of pigment. The only other ingredient of the medium under question is the phosphate. Several sets of media were made with asparagine and magnesium sulphate, but no fluorescence was observed. When a phosphate was added, a good fluoresence was produced. Preliminary experiments had already shown that fluoresence was produced when either sodium, potassium, lithium, or ammonium phosphate was used in media with asparagine and magnesium sulphate. The intensity was not quite so great with the ammonium salt. A medium was then prepared with magnesium phosphate, asparagine, and magnesium sulphate. A fair fluorescence was produced with this combination. No attempt was made to analyze the chemicals for alkali metals.

It may be concluded, therefore, that in addition to an organic constituent such as asparagine, the medium must contain a phosphate, magnesium, and sulphate. This conclusion is not in

accord with the reports of the two most recent workers in this field. Both Jordan and Sullivan found that magnesium was not necessary for the production of fluorescence. It is practically impossible to obtain alkali sulphates free from magnesium. Also, there may be enough magnesium present to produce fluorescence and still not be detected on an analysis of a small amount of the sample. In addition, it is apparent that a little magnesium may be dissolved from the test tubes.

In order to determine the amounts of the different constituents which, when present in a medium, will produce the best fluores-

TABLE 7

Organism A in media with 0.2 per cent asparagine; $MgSO_4$ and K_2HPO_4

$MgSO_4$	K_2HPO_4						
	0.0001 per cent	0.001 per cent	0.005 per cent	0.01 per cent	0.05 per cent	0.1 per cent	0.5 per cent
per cent							
0.00001	−	−	−	−	−	−	−
0.00005	−	−	−		−	+(84)	−
0.0001	−	−	+(72)	+(72)	+(84)	++(60)	−
0.0005	−	++(72)	+(72)	+(60)	+(72)	+(72)	−
0.001	−	+(72)	++(60)	+++(60)	+++(60)	++(72)	+(72)
0.005	−	++(60)	++(60)	++(48)	+++(60)	++(60)	+(72)
0.01	−	++(60)	+++(60)	++++(48)	++++(48)	+++(60)	++(60)
0.025	−	++(60)	+++(48)	+++(48)	+++(48)	++++(48)	++(60)
0.05	−	++(60)	+++(48)	++++(48)	++++(36)	++++(48)	+(72)
0.1	−	++(60)	+++(60)	++++(48)	+++(48)	+++(60)	+(72)
0.2	−	++(60)	+++(60)	+++(48)	+++(48)	++(60)	+(72)
0.5	−	++(60)	++(60)	++(48)	+++(60)	+(60)	+(72)

cence, a series of experiments was run. Media were, therefore, made with varying amounts and combinations of the three ingredients. Table 7 shows the results with 0.2 per cent asparagine with one of the organisms. Media containing 0.1, 0.3, 0.5, and 0.7 per cent asparagine were also used.

The fluorescence in the lower percentages of asparagine was about equally good. The medium containing 0.3 per cent was probably a little better. The pigment production was very good when the phosphate content was between 0.005 and 0.1 per cent,

and when the magnesium sulphate was between 0.001 and 0.2 per cent. The best and quickest pigment production was in the media containing 0.3 per cent asparagine, 0.05 per cent dipotassium phosphate, and 0.05 per cent magnesium sulphate. Considering all of the tests similar to the one given in table 7, very little or no fluorescence was produced when the phosphate content was less than 0.0005 per cent or when the magnesium was less than 0.00005 per cent. There was, of course, some variance with different organisms.

In light of the above the following medium is given as the most satisfactory for the production of fluorescence:

Magnesium sulphate, anhydrous	0.5 gram
Dipotassium phosphate, anhydrous	0.5 gram
Asparagine	3.0 grams
Distilled water	1000 cc.

CONCLUSION

1. A comprehensive study of the inorganic constituents necessary for the production of bacterial fluorescence has been reported. Over 4500 inoculations have been made.

2. The presence of magnesium, phosphate, and sulphate has been found to be essential for pigment production.

3. Highly purified chemicals may contain enough impurities to cause the production of fluorescence.

4. Media in some kinds of ordinary soft glass test tubes may, apparently, dissolve enough magnesium to permit of the production of some fluorescence. The use of pyrex or quartz test tubes eliminated this source of magnesium as shown by the absence of pigment formation in magnesium-free media when used in such tubes.

5. The most satisfactory medium for the production of bacterial fluorescence has been suggested.

6. The production of fluorescence may be used in place of chemical tests as a very delicate method for the detection of sulphates, phosphates, and magnesium.

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