BACILLUS PYOCYANEUS AND ITS PIGMENTS.

By EDWIN O. JORDAN, PH. D.

(From the Bacteriological Laboratory of the University of Chicago.)

The blue or blue-green stains that sometimes appear upon surgical dressings long ago attracted the attention of observers and, even before the cause of the phenomenon had been discovered, Fordos* carried out some important investigations upon the nature of the coloring substance. In 1882 Gessard† proved that the color is produced by a bacillus (*B. pyocyaneus*) which he was able to isolate in pure culture, and whose morphological and physiological characters he carefully described. Since Gessard's discovery, *B. pyocyaneus* has been frequently subjected to the crucible of experiment and its biological peculiarities have been scrutinized with a degree of minuteness not yet exercised in the study of most microorganisms.

Attempts to determine the nature of the pigment, and the conditions under which the chromogenic property is manifested have not, however, led to concordant results. At one extreme we find an investigator‡ who maintains that only one pigment is formed by the different "races" or "varieties," and that this pigment is identical with the ordinary blue-green fluorescent substance produced by the growth of a number of common water bacteria, and who also is of opinion that the several "races" differ chiefly in their power to produce ammonia, a substance that, like other alkalies, modifies the color of the fluorescent pigment; at the other extreme we find observers§ who claim that one and the same variety of *B. pyocyaneus* is able to produce as many as four distinct pigments, black, blue, green and

* *Compt. rend. Acad. des sciences*, 1860, li, p. 215.

† *La pyocyanine*, Thèse. Paris, 1882.

‡ Thumm, Beiträge zur Biologie der fluorescierenden Bakterien, *Arb. a. d. Bact. Inst. d. techn. Hochschule zu Karlsruhe*, 1895, i, 291.

§ Charrin and de Nittis, *Compt. rend. Soc. de biol.*, 1898, 10. s., v, 721.

yellow, at the same time and in the same culture medium; while between these extreme positions are found some investigators who hold to the existence of different races or varieties of *B. pyocyaneus*, each race being characterized by the ability to produce a different pigment or pigments; there are also some who maintain that by varying the nutrient substratum one and the same race may be compelled to form differently colored metabolic products.

In the existing chaotic condition of bacterial classification and description, it behooves all investigators to move circumspectly in the matter of identifying the "species" or "races" with which they are working, and in the present instance I have thought it desirable to describe with some fulness the cultures that I have employed.*

I have made use in all of seven cultures. All these have certain characteristics in common. The bacteria are small bacilli with rounded ends, and for the most part average from 0.3μ - 0.5μ in breadth by 1μ - 2μ in length, but there is considerable variation upon different media, and these dimensions are frequently exceeded in one and the same culture. They are sometimes grouped in short chains, but are usually single. All are actively motile. Spores have never been observed. The bacilli stain readily with the ordinary aniline dyes, but lose the stain when treated by Gram's method. Growth under the mica plate, when it occurs at all, is very scanty, and none of the cultures produces pigment in the absence of oxygen. All grow more luxuriantly at 37.5° C. than at the room temperature. Gelatin is liquefied by all. The origin of the various cultures was as follows: Four, designated respectively as "*B. pyocyaneus* α , Gessard," "*B. pyocyaneus* β , Ernst," "*B. pyocyaneus* pericarditidis" and "*B. pyocyaneus* γ , Freudenreich," were sent to me from Kral's collection; one ("*B. pyocyaneus*, Mich.")† was sent to me through the kindness of Dr. Novy of Ann Arbor; one ("*B. pyocyaneus*, Albany") was obtained at an autopsy "from a focus of broncho-pneumonia" (Jan., 1899) and was sent to me through the courtesy of Dr. George Blumer

* I am indebted to Mr. H. E. Davies and Mr. E. E. Irons for assistance in the study of these cultures.

† Dr. Novy informs me that the parent culture was brought by him from the Berlin Hygienic Institute in 1888.

of the Bender Hygienic Laboratory; and one ("B. pyocyaneus, Rush") was furnished me through the kindness of Mr. Danielson of the Rush Medical College and was originally obtained (March, 1898) from the body of a guinea-pig that had died after inoculation with a fragment of diphtheritic membrane.

These cultures, while agreeing in certain points, showed among themselves differences which, though in some respects trivial, have proved fairly constant and characteristic. All the cultures, except B. pyocyaneus, Albany, have been in my possession for at least a year. Save where otherwise indicated, I have employed the methods recommended by the Bacteriological Committee.*

As regards morphological characters, I have been able to detect few uniform or significant differences. Variations in dimensions and grouping similar in character and extent to those commonly displayed among the varieties of the colon bacillus have, however, been observed. For example, B. pyocyaneus, Albany, when grown on potato, develops larger forms and shows a greater tendency to form chains than the other varieties. B. pyocyaneus γ displays a tendency both in broth and on potato to form short chains, and takes the stain more evenly than in the other cultures. B. pyocyaneus β is, on the average, not so large as the other bacilli.

I have observed no noteworthy differences in respect to behavior toward stains. All the cultures stain somewhat irregularly when treated with Löffler's methylene-blue, a point I have not seen mentioned by other observers. None of the seven cultures hold the stain when treated by Gram's method; on this head my observations accord completely with those of Růžicka.†

Gelatin.—In the growth upon gelatin plates I have been able to discover no salient and constant differences in either the macroscopic or microscopic appearances of the colonies, although differences in the rapidity of growth and of liquefaction can be noticed. Divergences in growth upon gelatin are best seen in stab-cultures. The most rapid liquefaction occurs with B. pyocyaneus α , but B. pyocya-

* *Journ. Amer. Pub. Health Assoc.*, Jan., 1898.

† *Archiv f. Hyg.*, 1899, xxxiv, p. 153.

neus β , pericarditidis, and Albany are not far behind. *B. pyocyaneus*, Rush, grows at first somewhat more slowly, but in about 7 days overtakes the others and in 15 days outstrips them all. *B. pyocyaneus*, Mich., always lags behind the five already mentioned and the rear is brought up by *B. pyocyaneus* γ , which liquefies the gelatin most tardily. In all the cultures except *B. pyocyaneus* γ the liquefaction is at first superficial, a shallow, saucer-shaped depression forming slowly and then gradually extending to the sides of the tube. The liquefied gelatin, which is more or less colored, is sharply separated by a horizontal line from the unaltered medium below; some growth takes place along the inoculation line, but at first little or no liquefaction. Later, however, considerable liquefaction occurs along the inoculation line in *B. pyocyaneus*, Rush, Albany and β . The growth of *B. pyocyaneus* γ is of the stocking-shaped variety from the start, and even after 15 days' liquefaction has not reached the walls of the test-tube. In all a pellicle is formed on the surface of the liquefied gelatin; this is most marked in the case of *B. pyocyaneus*, Mich.

Agar.—Upon sloped agar there are few important divergences. The features most worthy of note are shown by *B. pyocyaneus*, Rush; this culture forms a very thin, delicate film which rapidly covers the whole surface and has a pronounced metallic lustre. In the other cultures the surface growth, which is projecting, is of a dull yellowish-white.

Broth.—In ordinary peptonized meat broth of the standard reaction, kept at 37.5° C., *B. pyocyaneus*, Rush, is the first to produce a pigment. The broth is rendered turbid by all the species and a heavy flocculent sediment is formed; a surface pellicle is produced by all, but is more tenacious in the cultures of *B. pyocyaneus*, Mich., than in the others.

Potato.—Interesting differences appear in the growth upon potato. The growth is luxuriant, dry, projecting and of a chocolate brown color in four cases, viz.: *B. pyocyaneus*, Mich., γ , β , and pericarditidis. The growth produced by *B. pyocyaneus* α is moist and glistening, but in other respects resembles that of the four cultures already mentioned. The potato itself is not discolored by these five species.

B. pyocyaneus, Rush, however, colors the potato a beautiful deep blue and *B. pyocyaneus*, Albany, imparts to it a greenish hue, the color in the latter case developing less rapidly and never becoming so intense as in the former. The growth of *B. pyocyaneus*, Rush, is more spreading and less projecting than the others.

The "chameleon phenomenon," as it was termed by P. Ernst,* is manifested by *B. pyocyaneus*, Rush, but by none of the others. The chameleon phenomenon, it will be remembered, consists in the change of color observed when the growth upon potato is touched with a platinum needle. It depends doubtless on the well-known fact that a substance is produced by the growth of the bacillus which by contact with the air is oxidized to the blue pigment known as pyocyanin. The shifting play of tints seen when a colony is touched is due to the conflicting action of the atmospheric oxygen and of the reducing substances doubtless present among the metabolic products of the bacillus. The exposure of the pyocyanigenic substance to the air affords an opportunity for the development of the blue color. I may here anticipate the remainder of this paper so far as to state that my observations indicate that the reason for the appearance of the chameleon phenomenon in some races of *B. pyocyaneus* and not in others lies in the varying degree in which pyocyanin is produced. Those races that produce pyocyanin vigorously and abundantly manifest the "chameleon phenomenon;" others, in which the pyocyanigenic function is weaker or altogether absent, are unable to display this peculiarity.

Indol.—Only two of the cultures possess the power of forming indol in any quantity in Smith broth; these are *B. pyocyaneus*, Rush, and *B. pyocyaneus*, Mich., the former showing itself much the more vigorous in this particular. *B. pyocyaneus*, Albany, produced a very slight amount of indol and the other four never gave a positive reaction. An attempt was made to accentuate or develop the indol-forming power by passing the organism through a succession of peptone cultures according to the method suggested by Peckham,† but

* *Zeitschr. f. Hyg.*, ii, p. 369.

† *Journal of Experimental Medicine*, 1897, ii, p. 549.

without avail so far as the awakening of any latent indol-forming power was concerned. A few such transfers, however, exalted perceptibly the indolfacient power of *B. pyocyaneus*, Rush, and to a lesser degree that of *B. pyocyaneus*, Mich. Freshly isolated cultures of *B. pyocyaneus* are, in general, recorded as yielding a positive indol reaction.*

Milk.—All the cultures curdled litmus milk at 37.5° in about 2 days. *B. pyocyaneus* γ , however, was usually 24-36 hours behind the other cultures. The color of the litmus was discharged, but on testing the reaction of the milk at the expiration of 2 days, and again after 10 days, it was found alkaline in all cases. When the cultures were allowed to stand the casein was slowly digested. Control tubes showed no change. At the room temperature the action upon milk was similar, but less rapid than in the thermostat, curdling not beginning until after the lapse of 5 or 6 days.

Reduction of Nitrates.—In nitrate broth *B. pyocyaneus*, Rush, reduced nitrates to nitrites quickly and completely; *B. pyocyaneus* α showed a much slighter reducing power, and the other five cultures gave a negative reaction. Sewerin† observed active reduction of nitrates in a culture of *B. pyocyaneus* recently isolated by himself from horse-dung. Dyar‡ observed reduction of nitrates in a culture of "*B. pyocyaneus*" from the laboratory collection and found also that a culture of "*B. fl. liquefaciens*" reduced nitrates well, but not completely; while another culture found in the exudate from a sick lepidopterous larva resembled the latter in all respects except in not reducing nitrates. Thumm§ and Růžicka,|| two of the investigators who have recently carried out extended comparative studies upon this group of organisms, make no statements regarding reduction of nitrates.

Production of Gas.—None of the cultures produced gas in 2 per cent glucose broth in the fermentation tube.

* Cf. e. g. Růžicka, *Arch. f. Hyg.*, 1899, xxxiv, p. 162, and Lartigau, *Journal of Experimental Medicine*, 1898, iii, p. 604.

† *Centralbl. f. Bakt.*, II. Abth., 1897, iii, p. 504.

‡ *Annals of N. Y. Acad. of Sciences*, 1895, viii, p. 322.

§ *Op. cit.*

|| *Op. cit.*

Pigment Production.—It is in connection with the production of pigment that the most interesting, and, in some respects, the most significant differences among my seven cultures are to be noted. One culture (*B. pyocyaneus*, Rush) produced only one pigment, the typical, unmistakable pyocyanin; the other six, with the exception of *B. pyocyaneus* γ , produced both pyocyanin and fluorescent pigment; the variety labelled *B. pyocyaneus* γ , when it first came into my possession, produced only the fluorescent pigment, and during the year in which I have had it under observation it has lost the power of forming any pigment at all, and none of the efforts I have made to restore this power by growing the organism in media known to be favorable to the fluorescogenic property has yet proved successful.

I have made a rather detailed study of the conditions under which the pigments are produced.

Fluorescent Pigment.—The general conditions that permit the formation of the blue-green fluorescent pigment are exactly similar to those that I have elsewhere described* as necessary to the manifestation of this property by other bacteria of the same group. The presence of both phosphate and sulfate is essential; the associated cation is a matter of indifference—sodium, potassium or magnesium being equally available. Asparagin, ammonium succinate, ammonium lactate and ammonium citrate all proved suitable for the development of the fluorescent pigment.† In ammonium urate solution (amm. urate, 0.05 per cent) a small amount of pigment was produced by the cultures designated as *B. pyocyaneus* α and *B. pyocyaneus*, Mich., but the others gave only the merest trace of color. In ammonium acetate solution *B. pyocyaneus*, Albany, *B. pyocyaneus*, Mich., *B. pyocyaneus* α , *B. pyocyaneus* β and *B. pyocyaneus* pericard. produced pigment abundantly. Solutions of ammonium tartrate, oxalate and formate proved entirely unfavorable for the production of the fluorescent pigment, none of the species employed being able to form any pigment whatsoever in media con-

* *Botanical Gazette*, xxvii, Jan., 1899, p. 19.

† The standard solution used contained in each case 0.5 per cent of the ammonium salt and 0.1 per cent each of neutral sodium phosphate and magnesium sulfate.

structed from these salts. In the ammonium tartrate solution, abundant growth occurred in the culture of *B. pyocyaneus* pericard. and *B. pyocyaneus*, Albany, and a somewhat less abundant growth in the culture of *B. pyocyaneus*, Mich. In no case, however, was any pigment formed. To put the whole matter concisely, *the study of five varieties of B. pyocyaneus which produce fluorescent pigment in suitable media has entirely confirmed the conclusions arrived at in my previous work upon the production of fluorescent pigment by bacteria.**

Pyocyanin.—In spite of the inability of Thumm† during his extended study of pigment production to discover any evidence for the existence of pyocyanin, there is no doubt whatever that a blue pigment with definite optical and chemical characters is formed by some bacteria belonging to the same group of organisms that Thumm studied. The cause of Thumm's failure will be made clear presently. All the cultures that I have worked with, excepting the undoubtedly degenerate *B. pyocyaneus* γ , have formed pyocyanin, although in varying degrees and under different conditions. The blue pigment termed pyocyanin can be readily distinguished from the blue-green fluorescent pigment. It is soluble in chloroform while the fluorescent pigment is not; viewed by artificial light, pyocyanin is as brilliant and distinct as by daylight, while the fluorescent pigment loses its vivid emerald green tint altogether and appears a dim muddy yellow. Either of these tests for the detection of pyocyanin in the presence of the fluorescent pigment can be applied very simply. A small amount of chloroform shaken up with a fluid culture becomes, within a few seconds, a deep blue if pyocyanin be present. If cultures when viewed by daylight appear to contain nothing but the fluorescent pigment—as under certain conditions was the case with *B. pyocyaneus* α and *B. pyocyaneus*, Albany—pyocyanin can be easily detected by viewing the cultures under gaslight, when any pyocyanin present will be plainly seen owing to the blotting out of the fluorescent substance that in daylight effectually screens the other pigment.

* *Botanical Gazette*, Jan., 1899.

† *Op. cit.*, p. 367.

Another well-known difference between the pigments may also be mentioned here. Pyocyanin turns red when treated with acid, while the fluorescent substance becomes colorless, both pigments regaining their original hue when the solution is again rendered alkaline. In all the cultures that I have worked with, pyocyanin is produced more slowly than the fluorescent pigment. *B. pyocyaneus*, Rush, develops pyocyanin at the room temperature only after three to four days' growth, while the races that produce the fluorescent pigment at all vigorously show color 24-48 hours earlier.

One of the races that I have used for experiment, *B. pyocyaneus*, Rush, does not produce the fluorescent pigment, and for this reason has been particularly serviceable for the study of the conditions under which pyocyanin is produced. In ammonium succinate or asparagin solutions, prepared according to the formulæ given above, pyocyanin is produced abundantly, and I have found that after ten transfers in ammonium succinate, made at intervals of 6-8 days, the power of this race to form pyocyanin persists unimpaired, and at the end is manifested as energetically as at the beginning. This race also produces pyocyanin in lactate, acetate, and citrate solutions. A very faint blue tinge, due to pyocyanin, appears in the urate, but in the solutions of tartrate, oxalate and formate no pigment at all is formed, although a very good growth occurs in both tartrate and oxalate media. Pyocyanin is also formed by this race in beef broth and upon ordinary nutrient gelatin and agar.

Upon testing the behavior of the other varieties in the several media interesting differences came to light. *B. pyocyaneus*, Mich., and *B. pyocyaneus*, Albany, produced both pyocyanin and fluorescent pigment in succinate, lactate, acetate and citrate solutions, while *B. pyocyaneus* α , *B. pyocyaneus* β and *B. pyocyaneus* pericard. produced only the fluorescent pigment in these media, and did not develop any pyocyanin even after 30-40 days' growth. In the urate solution a small amount of fluorescent pigment, but no pyocyanin, was formed by *B. pyocyaneus*, Mich., and *B. pyocyaneus* α ; *B. pyocyaneus* β did not form any pigment although rendering the solution turbid. Neither pyocyanin nor fluorescent pigment was formed by these four races in tartrate, citrate or formate media.

In standard beef broth *B. pyocyaneus*, Mich., and *B. pyocyaneus*, Albany; produced pyocyanin scantily, and the other three races formed only a small amount of fluorescent pigment. In broth from which the muscle sugar has been removed by Smith's method of inoculation with *B. coli* the fluorescent pigment is produced much more abundantly, but I have not observed so great a difference in the formation of pyocyanin. *B. pyocyaneus*, Rush, produces some pyocyanin in broth to which 2 per cent of glucose has been added, but the pigment appears more slowly than in Smith broth and the culture assumes a yellow tinge. *B. pyocyaneus*, Mich., also produced some pyocyanin in a solution composed of 0.2 per cent asparagin; 0.2 per cent glucose; and 1 per cent potassium sulfate. Nicolle and Zia Bey* note that their cultures produced the blue pigment in "milieux sucrés."

In nutrient gelatin all five cultures produced both pyocyanin and the fluorescent pigment; this is the only medium I have yet found in which *B. pyocyaneus* α , *B. pyocyaneus* β and *B. pyocyaneus* pericard. form pyocyanin.

Further experiments have been made with the three races capable of forming pyocyanin in non-proteid media with a view of determining the chemical elements essential to this activity.† The conditions are distinctly different from those governing the production of the fluorescent substance. Neither sulfate nor phosphate is essential. In a nutrient solution composed of asparagin, 0.2 per cent; magnesium sulfate, 0.1 per cent; sodium chloride, 0.5 per cent, *B. pyocyaneus*, Rush, produced pigment almost as abundantly and rapidly as in a standard sulfate-phosphate solution, and the pyocyanigenic power was not perceptibly weakened during four transfers in the same medium. The same thing proved true of *B. pyocyaneus*, Mich. If only one salt, as sulfate alone or phosphate alone, was used with the asparagin some pyocyanin was formed, but the amount in such cases was much less than under conditions more favorable to the multi-

* *Ann. de l'Inst. Pasteur*, 1896, x, p. 670.

† The cultures were kept at the room temperature unless otherwise indicated.

plication of the organism, although even in a pure 1 per cent asparagin solution some pyocyanin is formed by the three races named. It is entirely clear, therefore, that the pyocyanigenic property is in a sense more intimately bound up with the life processes of these organisms than is the fluorescigenic, since it is less dependent upon the presence of definite chemical compounds in the culture medium. Gessard* has also noticed the production of pyocyanin in a medium to which no phosphate was added, but is inclined to attribute it to the presence of small traces of phosphate carried over in the first sowing. This is of course possible, but the amount of phosphate in such a solution must be exceedingly small and the pyocyanigenic power of the organism certainly shows no such abject dependence upon the presence of this salt as is manifested in the case of the fluorescent pigment. In a medium containing 0.2 per cent asparagin, 0.1 per cent magnesium sulfate, and 0.001 per cent of neutral sodium phosphate *B. pyocyaneus*, Mich., produces both pyocyanin and fluorescent pigment. If the phosphate be altogether omitted pyocyanin alone is formed, and continues to be formed for at least four generations of successive transfers, which is as far as I have followed it in this species. The medium is prepared from recrystallized C. P. salts as free from phosphate as possible, and does not respond to the delicate phosphomolybdate test. Thumm† refers to Gessard's statement regarding the appearance of a blue color in the absence of phosphate and asserts that he has observed the same phenomenon in cultures of all the fluorescent bacteria that he has studied and explains the effect in this way: "Diese Färbungen sind jedoch nie auf Pyocyaninbildung sondern einfach auf Lichtbrechungserscheinungen zurückzuführen, indem jede leicht getrübe Flüssigkeit einen blauen Schimmer zeigt." Such an explanation in respect to the effects that I have observed is totally inadmissible, since I have demonstrated the presence of pyocyanin by all the applicable tests. Thumm's failure to obtain any pyocyanin here and in his other experiments with non-proteid media undoubtedly lay in the fact that the races with which he worked, whatever might have

* *Ann. de l'Inst. Pasteur*, 1892, vi, p. 810.

† *Op. cit.*, p. 367.

been their earlier history, at the time of his investigation were unable to produce pyocyanin in media constructed from ammonium salts. Three of the races in my possession show this same incapacity and the fact should be noted that they, like the races employed by Thumm, had been for some time under artificial cultivation and were perhaps obtained from the same laboratory. One of Thumm's* races seems to have behaved exactly like my own culture of "B. pyocyaneus γ " in losing its power to produce even the fluorescent pigment after it came into his possession.

Gessard† is inclined to lay great stress upon the production of pyocyanin in very simple media. "La fonction essentielle du bacille pyocyanique est donc la fonction pyocyanogène. . . . C'est à bon droit qu'elle entre dans son nom spécifique. C'est celle dont ne le séparent pas même ces conditions précaires d'existence, cette véritable misère physiologique qui aboutit à la mort, après un petit nombre de générations dans le milieu dépourvu de phosphate. L'autre fonction est surajoutée; elle n'est pas essentielle." I see no reason for establishing so sharp a distinction between the pyocyanigenic and the fluorescigenic function. It can hardly be doubted that cultures like B. pyocyaneus α and B. pyocyaneus β , which at present produce fluorescent pigment but no pyocyanin in ammonium succinate solutions, are merely modified descendants of races which when first isolated were more vigorous producers of pyocyanin. In fact there can be no objection to the view that the cultures of "B. pyocyaneus" that are not uncommonly found in laboratory collections and that secrete only the fluorescent pigment are nothing but degenerate offspring, so to speak, of a parent stock that originally produced both pigments. Such evidence as we possess is distinctly in favor of the view that the pyocyanigenic property is sometimes spontaneously lost while the fluorescigenic persists. The former "function" can hardly be looked upon, then, as more "essential" than the latter.

Cultural Modifications.—Gessard‡ was among the first to call atten-

* *Op. cit.*, p. 341.

† *Ann. de l'Inst. Pasteur*, vi, pp. 811, 812.

‡ *Ann. de l'Inst. Pasteur*, 1891, v, p. 63.

tion to the possibility of manufacturing new races of *B. pyocyaneus* by subjecting cultures to different conditions. According to this author the "typical" race, yielding both pyocyanin and fluorescent pigment, could be converted into a race possessed only of the fluorescogenic property either by the action of heat (five minutes' exposure to 57° C.) or by passage through the body of a rabbit; a pyocyanigenic race, on the other hand, could be created by growth of the typical race for a series of generations upon egg-albumen; while by the action of heat, by passage through the body of a rabbit or by spontaneous degeneration the pyocyanigenic race could be changed into a race incapable of producing any pigment; by the action of heat, too, the fluorescogenic race could be converted into a non-pigmented variety.

Acting upon these statements I have attempted to abolish or modify the pyocyanigenic power of *B. pyocyaneus*, Rush, by several methods. One of the first to be carried out was the passage through successive cultures in media particularly favorable to the production of fluorescent pigment, with the aim of developing some latent fluorescogenic power, but, as I have stated above, a series of ten generations in a standard succinate-sulfate-phosphate solution did not perceptibly affect the amount of pyocyanin produced and there was not the slightest approximation towards a fluorescogenic variety; on the other hand, *B. pyocyaneus* α , when grown in the same sulfate-phosphate medium, continued to produce only the fluorescent pigment after a parallel series of transfers. In ammonium tartrate solution no pigment is produced by either variety during the course of five generations, but on transfer to ammonium succinate the pigment characteristic of each reappears in the first generation.

Following the method suggested by Gessard, I subjected a fluid culture of *B. pyocyaneus*, Rush, to a temperature of 57° C. for 5 minutes, but this procedure failed to destroy the pyocyanigenic power, although development was slightly retarded in the subculture. Nicolle and Zia Bey* obtained a result similar to my own when they applied the heat method to some recently isolated cultures of

* *Ann. de l'Inst. Pasteur*, x, p. 669.

B. pyocyaneus, but Jakowski* has corroborated Gessard's statement, that the pyocyanigenic power is abolished in a culture warmed for 5 minutes at 57° C. Different races possibly differ in this regard. Neither Jakowski nor Nicolle and Zia Bey on the other hand observed any loss of pyocyanigenic power when the organism was passed through the body of rabbits. My own experiments with animals were made with mice and guinea-pigs, and are hence not strictly comparable with Gessard's, but in these animals at least no alteration of the chromogenic function could be observed in cultures isolated after death from the heart's blood, the kidneys, spleen or liver. Kukula, as quoted by Růžicka,† observed sometimes exaltation, sometimes attenuation of the chromogenic power when *B. pyocyaneus* was passed through the animal body.

A particular interest attaches to modification experiments in view of Růžicka's recent comparative studies‡ upon cultures of "*B. pyocyaneus*" and "*B. fluorescens liquefaciens*." The close relationship existing between cultures of organisms bearing these names has long been recognized. Intermediate forms are common, and no hard and fast line can be drawn between the "varieties" of the two "species." The ability to grow and produce pigment at 37° C. is perhaps as distinctive of *B. pyocyaneus* as any single physiological character, but the well-known ability of bacteria to adapt themselves to varying temperatures and the actual existence of a whole series of "liquefying fluorescent bacteria" that vary in behavior to temperature under natural conditions do not permit us to lay much stress upon this point of difference. Růžicka attaches some importance to a freer growth of *B. pyocyaneus* beneath the surface of gelatin or agar media, but as I have stated elsewhere in this paper, I have not found a uniform behavior in this regard among the various pyocyanin-producing cultures in my possession. As respects indol-formation, nitrate reduction, action upon milk and growth in the ordinary nutrient media, I share Růžicka's opinion that almost all characters and combinations of characters can be found among members of this group of organ-

* *Zeitschr. f. Hyg.*, xv, p. 474.

† *Arch. f. Hyg.*, 1899, xxxiv, p. 173.

‡ *Op. cit.*, p. 149.

isms. One culture will form indol, grow at 37° C. and reduce nitrate; another will not form indol but will agree with the first in all other characters, and so on through a long series in which all possible combinations of physiological capacities seem to be represented among the cells of one or another culture.

Růžicka does not, however, appear to grasp the full significance of the difference between "*B. pyocyaneus*" and "*B. fluorescens liquefaciens*" in the matter of pigment production. Gessard's experiments consisted simply in removing the power to form one or the other pigment from an organism originally capable of forming both pigments. I have not been able to find in the literature a single satisfactory instance of the acquisition of pyocyanigenic power by an organism primarily devoid of this property. Kruse and Pasquale* mention the discovery of an organism resembling *B. pyocyaneus* except in ability to produce pigment, but do not affirm that it acquired chromogenic power on cultivation. Remlinger† records the occurrence of a non-chromogenic variety in splenic pulp and states that color appeared after the sixth or seventh sowing in broth, but I have not been able to satisfy myself that this statement necessarily implies acquisition of pyocyanigenic power. Růžicka‡ describes an experiment upon the transformation of one variety into another in the course of which he noticed the formation of a "*gesättigt blaugrünen Farbstoff*" in a typical culture of *B. fluorescens liquefaciens*, but his description is not sufficiently detailed to carry conviction upon the point at issue.

I have made a number of experiments designed to augment the pyocyanigenic power of my cultures and to evoke the power in cultures in which it was latent, but thus far with only indifferent success. The pyocyanigenic power of *B. pyocyaneus*, Rush, was very slightly but perceptibly increased by passage through a series of cultures in ordinary peptone broth and the pigment was produced more rapidly and abundantly at the end of eight transfers than at the beginning. Similar attempts to increase the amount of pyocyanin formed by *B.*

* *Zeitschr. f. Hyg.*, xvi, p. 63.

† *Arch. de. méd. exp.*, 1898, x, p. 167.

‡ *Op. cit.*, p. 173.

pyocyaneus, Albany, and *B. pyocyaneus*, Mich., did not prove successful, and no pyocyanin at all was produced by *B. pyocyaneus* pericard. during the course of fifteen generations. A series of sowings in nutrient gelatin did not exalt in any degree the pyocyanigenic power in the three organisms that manifested it only in this medium. In this respect there is an agreement with what is noticed concerning the indolfacient power of the organisms.

There is, as might be expected, a difference in chromogenic power among the cells of one and the same culture. *B. pyocyaneus*, Mich., for example, was plated from a pure culture obtained from a single colony in the usual way. From nine separate colonies selected at random from the gelatin plate nine separate tubes of standard ammonium succinate solution were inoculated. Fluorescent pigment made its appearance in one tube on the fourth day after inoculation, in five more on the fifth day, in two more not until the eleventh day and in the ninth tube no pigment appeared until the fifteenth day. After twenty-four days the cultures were tested for pyocyanin and eight tubes showed the presence of this pigment accompanying the fluorescent. The ninth tube (one of those in which fluorescent pigment appeared on the fifth day) contained no pyocyanin, and further experiment showed that the descendants of this cell were devoid of pyocyanigenic power.

Other Pigments.—The production of other pigments besides the blue and the blue-green has been mentioned by some authors. Charrin and de Nittis* observed the simultaneous formation of black, blue, green and yellow pigments in one and the same culture. The yellow or yellow-brown pigment is, as I have shown elsewhere, an oxidation product of the green fluorescent pigment and is commonly seen in old cultures and among races beginning to lose fluorescigenic power. The black pigment has been recently attributed by Gessard† to the oxidation of tyrosin. I have observed the formation of the black pigment in old agar and gelatin cultures of *B. pyocyaneus*, Rush, *B. pyocyaneus*, Mich., and *B. pyocyaneus*, Albany; in other words, in the races that produce pyocyanin most vigorously. *B. pyocyaneus*,

* *Compt. rend. Soc. de biol.*, 1898, p. 721.

† *Compt. rend. Soc. de biol.*, 1898, p. 1033.

Rush, which is by far the best producer of pyocyanin that has come into my hands, develops the black pigment most profusely. To test the relation of this pigment to tyrosin, I inoculated a solution of 0.05 per cent tyrosin (Merck), 0.1 per cent neutral sodium phosphate and 0.1 per cent magnesium sulfate with the several cultures. Growth occurred with all, but not quite so abundantly as in the ammonium succinate medium. *B. pyocyaneus*, Rush, formed pyocyanin quite abundantly but rather slowly, the first appearance of the pigment being noticed in 12 days; *B. pyocyaneus*, Mich., and *B. pyocyaneus*, Albany, formed both pyocyanin and fluorescent pigment, but in scanty amounts. The chromogenic behavior of these organisms in a tyrosin medium does not, therefore, differ materially from their behavior in an ammonium succinate solution. The culture of *B. pyocyaneus*, Rush, in tyrosin, *which was kept in the dark*, contained only pyocyanin and had no trace of black pigment at the end of 113 days. The black pigment is, in fact, due to the oxidation of the pyocyanin. Exposure of a pyocyanin solution (an old ammonium succinate culture of *B. pyocyaneus*, Rush) to the action of strong north light causes a change, first to a bottle-green hue, then to a brownish-black; the same change goes on in the dark in some media, but very much more slowly than in tubes exposed to daylight. This change is one of oxidation, as is shown by the fact that a dilute solution of potassium permanganate added gradually to a pyocyanin solution produces exactly the same conversion of the blue into the bottle-green and then into the black pigment, as is wrought more slowly by the action of light and air.* I have elsewhere shown† that the conversion of the fluorescent pigment into the yellow-brown depends upon a similar oxidizing process. The formation of four pigments, then, can be easily explained on the basis of the oxidation of the blue and the fluorescent pigments into black and yellow respectively. The almost uniform occurrence of a yellow-brown tint in old stock cultures of *B. pyocyaneus* that have lost their ability to form either pyocyanin or fluorescent pigment may be most plausibly referred, perhaps, to an accentuation of oxi-

* Charrin and de Nittis, *loc. cit.*, state that the black pigment is produced at the surface of the agar.

† *Botanical Gazette*, 1899, xxvii, p. 19.

dizing processes, and the real explanation of the loss of pyocyanigenic and fluorescogenic power may perhaps lie in the impetus given to metabolic processes of oxidation by the conditions of life in our artificial peptone media. It must not be forgotten that both pyocyanin and the fluorescent pigment are themselves oxidation products of colorless substances.

Natural Varieties.—The confusion into which the study of *B. pyocyaneus* and its varieties has fallen is in part due to the rapid degeneration of many stock cultures under conditions of artificial cultivation and the consequent disappearance of all chromogenic power, and in part also to the occurrence of natural varieties characterized by considerable physiological divergence. The most common natural variety, to judge from an examination of the literature, as well as from the study of a number of freshly isolated cultures that I have obtained from different sources, is the one that produces both pyocyanin and the fluorescent pigment. This is the variety that was originally described and studied by Gessard, and I would suggest that the name *B. pyocyaneus*, var. α be given to freshly isolated cultures possessed of this double chromogenic power. The culture with which Ernst worked* and to which he gave the designation of " α ," was, so far as I can determine from his description, a culture wholly devoid of pyocyanigenic power and one that produced, while in his hands, only the fluorescent pigment.† The most natural inference is that in respect to pyocyanin production this culture was degenerate. Many cultures seem overtaken by this fate. It is perhaps fair to assume that the large number of cultures found in laboratory collections bearing the name of *B. pyocyaneus*, but without any power to produce pyocyanin, are degraded scions of a more vigorous parent stock.‡ It is worthy of note that the pyocyanigenic property is apparently the first to be lost. I have never observed a case where the fluorescogenic

* *Zeitschr. f. Hyg.*, ii, p. 369.

† Cf. e. g. the statement on p. 373, *op. cit.*, that the color of cultures of the *a*-bacillus disappears when viewed by gaslight.

‡ The failure to preserve unimpaired the original pyocyanigenic power is clearly shown in the usual inability of old cultures to manifest the "chameleon phenomenon," while most freshly isolated cultures display this power (cf., for example, Schürmayer (cited below) and Lartigau (*loc. cit.*)).

power was lost by spontaneous degeneration while the pyocyanigenic persisted, nor have I found mention of such a case in the literature.

It is a matter of common experience that there is great diversity among cultures of *B. pyocyaneus* as regards rapidity of degeneration. Some lose the power to produce either pyocyanin or fluorescent pigment within a short time after isolation; others weaken quickly in pyocyanigenic power, but retain the fluorescigenic much longer; others again, but these are the rarer cases, show almost undiminished capacity for the formation of pyocyanin and fluorescent pigment through a long series of generations. *B. pyocyaneus*, Mich., is the most notable instance of the last group with which I am acquainted and is a marvelously stable variety; it has shown no attenuation in chromogenic power during the three years and a half I have had it under observation.

In the relative proportion of pyocyanin and fluorescent pigment there is likewise considerable variation among freshly isolated cultures. New varieties have even been founded by some authors upon the differing nuances of color due to varying admixtures of the two pigments, but the advisability of such distinctions is questionable. It must be acknowledged that there are found races of the variety that I have termed var. α , some of which are more or less sharply distinguished from one another by intensity and persistency of pigment production as well as by such physiological traits as the formation of indol, reduction of nitrates and growth on potato, but it must be left to future bacteriological investigation to interpret the significance of these deviations and to determine to what extent subvarieties and races shall be established.

The culture I have designated as *B. pyocyaneus*, Rush, produces only pyocyanin under ordinary conditions of cultivation, and has resisted all my attempts to communicate to it any fluorescigenic power. I would suggest that the name *B. pyocyaneus*, var. β , be employed to distinguish this non-fluorescigenic variety. I am unable to determine whether the variety studied by Ernst,* and designated by him as the " β variety," produced only pyocyanin, but the coloring of his

* *Zeitschr. f. Hyg.*, ii, p. 369.

figures (*op. cit.*, p. 372), and the peculiar liquefaction of gelatin there shown, together with the statement regarding tardy appearance of the color, suggest that this may have been the case. Schürmayer* and Jakowski† both worked with cultures of the pyocyanigenic-fluorescigenic variety, which I have called the “ α ” variety. Nicolle and Zia Bey‡ also appear to have been dealing with the α -variety, although they state that their four cultures were “much more pyocyanigenic than the type.” I have not been able, in fact, to find in the literature any unimpeachable statement of the existence of a purely pyocyanigenic variety such as I have worked with, although it is not difficult to discover instances where the pyocyanigenic power of a culture has been recognized to be much higher than is usually the case.

The occurrence of a fluorescigenic variety seems to be more common. The variety I have termed the α -variety frequently degenerates spontaneously into this. The chief difference between this variety and the organism called *B. fluorescens liquefaciens* seems to lie in the difference of temperature optimum and perhaps in behavior in the animal body (Růžicka). I have not been able to confirm Růžicka's observations respecting a constant difference in gelatin stab-cultures. It is a tempting hypothesis that *B. fluorescens liquefaciens* is a degenerate or modified form of *B. pyocyaneus*, but more convincing evidence than we now possess is needed to establish this point.

The natural occurrence of a non-chromogenic variety has been affirmed by Remlinger§ and others. Kruse and Pasquale|| have recorded the finding of a non-chromogenic variety exactly like the ordinary *B. pyocyaneus*, but without power to produce pigment. I have not been able to obtain a natural variety of this kind. It will be generally recognized that the method of repeated transfer of such a variety from one tube of fluid culture to another (Remlinger) is open to objection unless frequent control platings are carried out. My own attempts to restore chromogenic power to spontaneously or artificially etiolated cultures have failed.

* *Zeitschr. f. Hyg.*, xx, p. 281.

† *Zeitschr. f. Hyg.*, xv, p. 474.

‡ *Ann. de l'Inst. Pasteur*, p. 669.

§ *Op. cit.*

|| *Op. cit.*

CONCLUSIONS.

The principal conclusions that seem to me justified are as follows:

1. The fluorescent pigment formed by some varieties of *B. pyocyaneus* is produced under conditions identical with those governing the production of the pigment by other "fluorescent bacteria."*

2. The production of pyocyanin is not dependent upon the presence of either phosphate or sulfate in the culture medium. It is formed in non-proteid as well as in proteid media, but is not a necessary accompaniment of the metabolic activities of the organism (*e. g.* tartrate solution).

3. The power of producing pyocyanin under conditions of artificial cultivation is lost sooner than the fluorescogenic power.

4. There are greater natural and acquired differences in pyocyanigenic power than in fluorescogenic.

5. The fluorescent pigment may be oxidized slowly by the action of light and air as well as by reagents into a yellow pigment, and pyocyanin may be similarly oxidized into a black pigment.

6. A convenient separation of *B. pyocyaneus* into four varieties would be the following: var. α , pyocyanigenic and fluorescogenic (most common); var. β , pyocyanigenic only (rare); var. γ , fluorescogenic only (not uncommon, closely related to "*B. fluorescens liquefaciens*"); var. δ , non-chromogenic.

7. Except for the occasional loss of one or another function the different varieties are not so plastic as sometimes assumed, and cannot be readily converted into one another by subjection to varying conditions of life.

8. The signification and correlation of the almost countless physiological variations among the members of this group in respect to growth in gelatin, behavior to temperature, indol production, etc., remain to be determined. It is not yet clear that the variations in chromogenic power can be in any way correlated with the presence or absence of other physiological functions.

* Cf. *Botanical Gazette*, xxvii, p. 19.