THE PIGMENT PRODUCTION OF ACTINOMYCES COELICOLOR AND A. VIOLACEUS-RUBER[1]

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INTRODUCTION

Some species of *Actinomyces* produce very striking pigments which have aroused the interest of many investigators. As a result a great deal of work has been done on the genus, but, unfortunately, failure to understand the nature of these pigments has led to considerable confusion on this subject. It has been long recognized that these pigments have indicator properties, and their similarity to litmus has been mentioned in the literature; they may therefore be one color on one medium and an entirely different color on a second medium. In spite of this fact, authors of species frequently fail to describe the medium on which they have grown their cultures; so that it becomes very difficult to tell whether varying descriptions apply to different organisms or all to the same one.

Some students of the genus even take the point of view that all cultures producing a litmus-like (*i.e.*, blue-red) pigment form a single species. The present series of experiments were planned to investigate this point, the study beginning with two pigment-producing strains, isolated from soil, which superficial examination indicated to be quite different, although both produced pigments that were blue under alkaline conditions and red when acid. A comparison of these with other cultures and with descriptions in the literature yielded some interesting results.

REVIEW OF LITERATURE

Some of the most detailed investigations on this subject were made by Müller (1908), one of the earliest workers in this field. He described in detail an organism producing a blue pigment on potato which he called *Streptothrix coelicolor*. This organism is now described in Bergey's Manual as *Actinomyces coelicolor*. Müller, however, did not stop with a description of the organism. He also extracted the pigment by washing it out of potato with water and studied its properties. He was unable to crystallize it, but he observed the effect of various reagents, including acid and alkalies, on the solution and found it to turn red in acid, green in alkali.

Waksman (1919) described an organism which he called *Actinomyces violaceus-ruber*. He stated that this organism produced a soluble red-blue pigment which had indicator properties. His description was not as detailed as that of Müller,

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but his organism seemed to be very similar to Müller's, if not identical with it. Waksman himself later came to the conclusion that the two organisms were probably identical, and in the 5th edition of Bergey *Actinomyces violaceus-ruber* was, at his suggestion, considered synonymous with *Actinomyces coelicolor*. The available evidence, however, did not seem sufficient to settle this point satisfactorily either way. It is interesting to note in this connection that Kriss (1937) discussed in detail the variability of *Actinomyces coelicolor*. Then, in an entirely different section of his article, he referred to a culture of *Actinomyces violaceus-ruber* obtained from Waksman, giving the impression (although he did not actually say so) that he considered the two to be different organisms.

Conn (1921) was perhaps the first to call attention to the importance of the composition of the medium in studying pigment production by *Actinomycetes*. He observed that the same species would produce a different color on different media, and suggested that it might be due to the final hydrogen ion concentration. This work was confirmed by Tompel (1931) who found variation in color on different media but constancy under constant conditions. Further work along this line was done by Plotho (1940) who observed that the color produced by the various pigment-producing species of *Actinomyces* was generally darker in an alkaline medium than in an acid one. Furthermore, when the pigment was made acid, it was less soluble and precipitated on heating.

The chemical nature of these pigments has been discussed by Erikson, Oxford, and Robinson (1938), Frampton and Taylor (1938) and Kriss (1936), but without reaching very definite conclusions. The chief point at issue has been whether or not the pigments are anthocyanins like certain red-blue pigments of higher plants.

The attempts of the early workers to classify the *Actinomycetes* according to pigment production were all unsatisfactory. More recently Waksman (1940) has developed a method of classification by which the family is divided into five sub-groups according to the arrangement of the spore-producing bodies on dried agar plates. His method, however, is still not entirely satisfactory because the form of the spore-producing bodies is so variable and so hard to determine that individual investigators frequently disagree on the condition present in any one species. Kriss (1937) believes that the Actinomycetes can be classified if the classification is based upon many characteristics, if the morphological and physiological characteristics are broadly used, and if variability because of the outside environment is taken into consideration. He does not consider descriptions and classifications of any value which are based on single characteristics.

EXPERIMENTAL

Purpose

In view of the existing confusion, further work with this group of organisms seemed desirable. Accordingly, two pigment-producing strains of *Actinomyces* isolated from soil were selected for study. One of these produced a blue pigment on the synthetic agar used, the other a violet pigment. A comparison of the

cultural characteristics and of the chemical properties of the pigments of the two strains was undertaken with two objects in mind: (1) to determine whether or not the pigments produced by these two strains were identical, and (2) to determine whether either of them agreed with the description of Müller's *Actinomyces coelicolor* or with that of Waksman's *Actinomyces violaceus-ruber*.

Superficial appearance of organisms

For routine study the cultures were grown on a synthetic agar containing 0.085 per cent asparagin, 1 per cent glycerol and 0.1 per cent K_2HPO_4. This is a modification of the medium described by Conn (1921) as giving fairly characteristic and vigorous growth with the majority of strains of *Actinomyces* used in his work. The two organisms used in this study were designated as V-1 and B-3. When grown on this medium, V-1 produced a leathery violet-colored surface growth with a white aerial mycelium and colored the medium a somewhat lighter violet. On the other hand, B-3 produced a rather moist blue surface growth with an abundance of white aerial mycelium and colored the medium a very deep blue. Thus, these two cultures were quite different in appearance and their pigments did not bear any superficial resemblance to one another. Furthermore, neither of them bore any marked resemblance to the culture obtained from Waksman as typical of his *Actinomyces violaceus-ruber*. On the same medium the last mentioned organism produced a reddish-violet surface growth with very scant white aerial mycelium, sometimes lacking entirely. The medium was colored a faint red or violet.

Cultural study

When a more detailed cultural study was undertaken, one of the first things noted was that the B-3 culture was contaminated, and probably had been from the beginning. The contaminating rod was not only easily overlooked, but proved rather difficult to eliminate. However, a pure culture was finally obtained, and it was interesting to note that this purified B-3, instead of producing a deep blue pigment, produced a violet pigment even lighter than that produced by V-1. This led to the suspicion that the two organisms might be identical after all. When NaOH was added to the medium upon which either culture had been grown, a deep blue color was produced similar to that observed in the contaminated culture of B-3. This would make it seem probable that the bacterial contaminant interfered in some way with the acid production by the *Actinomyces*, thus changing the pH and causing the pigment to appear in its alkaline phase instead of showing the intermediate violet hue.

Previous work (Conn and Conn, 1941) had shown that, when glucose was added to the glycerol-asparagin medium described above, the pigment produced by these cultures was an insoluble red instead of a soluble violet. Increasing amounts of glucose were used and the final hydrogen-ion concentrations of the cultures were determined. The results showed that increasing the amount of glucose caused an increase in the final hydrogen-ion concentration and a corre-

TABLE 1

Cultural studies

CULTURAL CHARACTER	V-1	B-3 (PURIFIED)	STREPTOTHRIX COELICOLOR (MÜLLER'S DATA)	ACTINOMYCES VIOLACEUS-RUBER (WAKSMAN)	
				Observed	Waksman's data
Standard agar at 25°	Light blue White aer. myc.	Scant blue White aer. myc.	Good growth No aer. myc.	Medium blue White aer. myc.	Blue White aer. myc.
Standard agar at 37°	Very scant blue White aer. myc.	No pigment White aer. myc.	No pigment	No pigment White aer. myc.	Not reported
Gelatin at 18°	Liquefaction starting in 7 days	Liquefaction starting in 7 days	Liquefaction starting after 4–6 days	Very slight liquefaction in 14 days	Liquefaction slow to medium
Milk at 25°	No coagulation	No coagulation	No coagulation	No coagulation	Not reported
Milk at 37°	Rennet curd and proteolysis on 3rd day	Rennet curd and proteolysis on 3rd day	Rennet curd in 3 to 4 4 days	Rennet curd and proteolysis on 5th day	No coagulation; hydrolysis and alkaline reaction in 15 days
Nitrate reduction	Positive	Positive	Positive	Positive	Not reported
Hemolysis of blood agar at 37°	Hemolysis on 4th day	Hemolysis on 4th day	Hemolyzed very rapidly	No hemolysis in 9 days	Strong hemolysis
Potato plug at 25°	Deep blue—7 days	Deep blue—7 days	Deep blue	Colorless	Blue—4–5 days
Fermentations	Glucose + Sucrose − Lactose + Mannitol −	Glucose + Sucrose + Lactose + Mannitol +	No acid from carbohydrates	Glucose + Sucrose − Lactose + Mannitol +	Not reported

sponding change in color from the soluble violet (alkaline phase) to the insoluble red (acid phase of the pigment).

After the B-3 culture was purified, cultural studies were made of these two experimental organisms and also of the culture of *Actinomyces violaceus-ruber* obtained from Waksman. The results of these studies are listed in table 1 together with the characteristics observed by Waksman (1919) for his organism and those given by Müller (1908) in his description of *Streptothrix coelicolor*.

From observation of table 1 it can be seen that the organisms V-1 and B-3 agree absolutely in all cultural characteristics tested except in the matter of acid production from carbohydrates and mannitol. B-3 produced acid from all carbohydrates used, but V-1 produced no acid from sucrose and mannitol. Furthermore, in all respects except this same matter of acid production the two experimental organisms agree with the description given by Müller for *Streptothrix coelicolor*. In regard to *Actinomyces violaceus-ruber* it will be noted that the characteristics observed for the organism do not agree with those reported in the literature in regard to coagulation of milk, hemolysis of blood agar, and production of pigment on potato plugs. This would make it appear probable that the strain of *Actinomyces violaceus-ruber* carried by Waksman in his stock cultures had become somewhat weakened by repeated transfers since it was originally described. Even the original description, however, disagrees with the other cultures studied since Waksman states that it shows good growth and abundant pigment production on standard agar, slow liquefaction of gelatin, and no coagulation of milk at 37°.

The matter of acid production from carbohydrates is rather hard to determine for these organisms without making time-consuming electrometric measurements. The indicator media ordinarily used for these tests are not satisfactory since the color of the indicator may well be masked by the pigment produced by the organism. Müller reported that there was no acid produced from carbohydrates when added in 1 per cent amounts to litmus ascites agar. This may mean that the color change of the litmus was masked by pigment production, or it may be that the medium used was not sufficiently favorable to permit acid production by the organism. In the present work, the carbohydrates tested were added to the synthetic agar described above in 2 per cent amounts and the production of acid was judged by observation of the indicator properties of the pigment. If the pigment produced was an insoluble red or light violet, acid was assumed to be present. On the other hand, if the pigment was a soluble blue-violet, it was concluded that no acid was produced. This method would seem to be fairly reliable. Nevertheless, the differences in acid production between V-1 and B-3 might well be due only to more active growth on the part of B-3.

If acid production from carbohydrates is disregarded, V-1 and B-3 appear to be identical with Müller's *Streptothrix coelicolor*, but not with Waksman's *Actinomyces violaceus-ruber*.

Extraction of pigments

In order to study the chemical properties of the pigments it was necessary first to extract them from the medium on which the organism had been grown. Nu-

merous laboratory solvents[2] were tried in an attempt to remove the pigment from an agar medium, but dioxane and NaOH were the only ones found to be effective. Both of these methods proved to be extremely slow, and required large amounts of solvent in proportion to the amounts of pigment obtained.

While searching for a better method, the discovery was made that the blue color produced on potato could be washed out readily with distilled water. Accordingly, the cultures were incubated on mashed potato for about four weeks at 25°C. At the end of this time the potato was colored a deep blue. The pigment was then extracted with distilled water, filtered through a Berkefeld filter, and sterilized. This method had several disadvantages: first, the extraction is very slow owing to the time required for filtering; secondly, the product obtained is not very pure; and third, it tends to decompose on standing, possibly because of the presence of organic matter from the potato. The pigment solution used by Müller in his studies of *Streptothrix coelicolor* was obtained by this method, although he admits that the solution was not pure.

Because of these disadvantages, further efforts were made to extract the pigment from agar cultures. The following method was finally developed: The culture was grown on glycerol-asparagin agar in large flasks, and incubated at 25°C., for two to four weeks, until the agar was colored a deep blue. The pigment was then extracted with very dilute NaOH and filtered. Fairly concentrated H_2SO_4 (about 10 *N*) was added to the deep blue solution resulting, causing it to turn red and precipitate. The precipitate was separated by centrifuging and dried.

The above method was satisfactory, but an even better method proved to be that described by Conn and Bottcher (1942), in which the organism was grown on cotton saturated with the nutrient solution used in making the agar. The pigment could be easily and quickly extracted from the cotton by squeezing it. The solution was then precipitated and centrifuged as before. The advantages of this method are that the organism grows faster on cotton than it does on agar, and the extraction is easier and quicker.

This method of extracting the pigments yielded a product that was realized to be far from pure. After various unsuccessful attempts to find a reagent which would precipitate them in crystalline form and therefore permit some hopes of actual purification, the effort to purify them was temporarily abandoned. The products obtained by precipitation from the extract of a culture growing on a synthetic medium were much purer than the extracts themselves (in that the substances soluble in dilute H_2SO_4 had been largely eliminated) and were very much purer than the extracts of organic media (*e.g.*, potato). In fact it was found that these precipitates were sufficiently pure to yield more valuable data concerning the solubilities of the pigments than could be obtained from the cultures before such treatment; and solutions made from them could be compared with one another by spectrophotometric analysis and thus supply some information as to the identity of the pigments present. Attention is specially called to

[2] See list in table 2. The following additional solvents were included: Dilute HCl, CCl_4, Ch_2Cl_2, pyridine.

this method of studying pigment-producing Actinomycetes. A distinct advantage of the spectrophotometer in studying such colored substances is that it furnishes much information concerning their nature without actual isolation of the pigments themselves.

Solubilities of pigments

The pigments of the two experimental organisms agreed with those of both Müller's (1908) organism and that of Waksman (1919) in that all were blue or green in alkali and red in acid. To determine whether or not the pigments are actually identical, a further study was made of their solubilities, using the two experimental organisms and the culture obtained from Waksman. For this purpose the pigments were extracted as described above, and small amounts of the washed and dried extract were added to the different reagents. The results are recorded in table 2. Also, for comparison are recorded as much about the solubilities of Müller's pigment as could be determined from his description. His results, however, are not strictly comparable since he used an extract obtained by washing the pigment out of potato with water and evaporating. By his own admission this extract was not pure.

From a study of the table, it can be seen that the pigment produced by the V-1 organism behaves in very much the same way as that produced by B-3. The only conspicuous differences are that the V-1 pigment is more soluble in amyl alcohol and the B-3 pigment is more soluble in phenol. Then the pigment from B-3 is very slightly soluble in benzene and xylene whereas that from V-1 is insoluble. The pigment from Waksman's organism on the other hand behaves very differently. It is generally less soluble in all reagents, being readily soluble only in NaOH. In acid solution it is insoluble; in alkali it is soluble but turns a light blue instead of the deep blue or green produced by the other pigments. In Waksman's description of the organism, he does not discuss these properties of the pigment so that it is impossible to tell whether or not the pigment has changed in nature since the culture was first isolated. From the data in the table, certain conclusions may be drawn. First, the pigments produced by V-1 and B-3 are probably identical, since it is hard to imagine that two different pigments would be so much alike in their solubilities. Second, the pigment isolated from Waksman's culture of *Actinomyces violaceus-ruber* is quite different from either of these. Third, judging from the reactions in acid and alkali, the pigment isolated from the experimental organisms resembles the pigment from Müller's organism much more closely than that from Waksman's.

Certain investigations were made to obtain some idea as to what might be the chemical nature of the pigment. The results were inconclusive, however, and as they add nothing to what is already in the literature, they are not reported here.

Spectrophotometric determinations

To obtain further evidence as to whether or not the pigments produced by these various cultures are identical, spectrophotometric studies of the pigments

TABLE 2

Solubility of pigments

SOLVENT	MÜLLER'S DATA CONCERNING STREPTOTHRIX COELICOLOR	PIGMENTS EXTRACTED FROM		
		V-1	B-3	Waksman's *Actinomyces violaceus-ruber*
Water	soluble—blue	v. sl. soluble—faint pink	sl. soluble—pink	insoluble
N/10 H_2SO_4	soluble—red	v. sl. soluble—faint pink	sl. soluble—pink	insoluble
10*N* H_2SO_4	soluble—red	soluble—red	soluble—red	insoluble
N/10 NaOH	soluble—green	very soluble—dark blue	very soluble—dark blue	soluble—light cloudy blue
N/1 NaOH	soluble—green	very soluble—dark blue green after 24 hrs.	very soluble—dark blue green after 24 hrs.	soluble—pale blue
Ethyl alcohol (absolute)	insoluble	soluble—red	soluble—red	v. sl. soluble—faint pink
Methyl alcohol		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Amyl alcohol		soluble—red	sl. soluble—pink	v. sl. soluble—faint pink
Benzene		insoluble	v. sl. soluble—faint pink	insoluble
Xylene	insoluble	insoluble	v. sl. soluble—faint pink	insoluble
Phenol		sl. soluble—pink	soluble—red	insoluble
Dioxane		very soluble—deep red	very soluble—deep red	partially soluble—red
Carbon disulfide		insoluble	insoluble	insoluble
Ethyl ether	insoluble	sl. soluble—pink	v. sl. soluble—faint pink	v. sl. soluble—faint pink
Acetone		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Chloroform	insoluble	insoluble	insoluble	insoluble
Petroleum ether		insoluble	insoluble	insoluble
Formaldehyde		very soluble—deep red	very soluble—deep red	sl. soluble—pink
Ethylene chloride		insoluble	insoluble	insoluble
Acetaldehyde		soluble—red	soluble—red	v. sl. soluble—faint pink

were made. The first studies were made on the pigments extracted from the potato cultures. These results were not satisfactory, however, because the solutions were so unstable that curves made two hours apart from the same solution were not alike. Subsequent studies were made using the aqueous extracts, both partially purified (by precipitation with H_2SO_4) and unpurified.

Figure 1 was made using the results obtained from the partially purified NaOH extract of B-3. Curve 1 was prepared from the unbuffered solution which had a pH of 10.73. The solution used for Curve 2 was buffered to pH 6.3 and that for

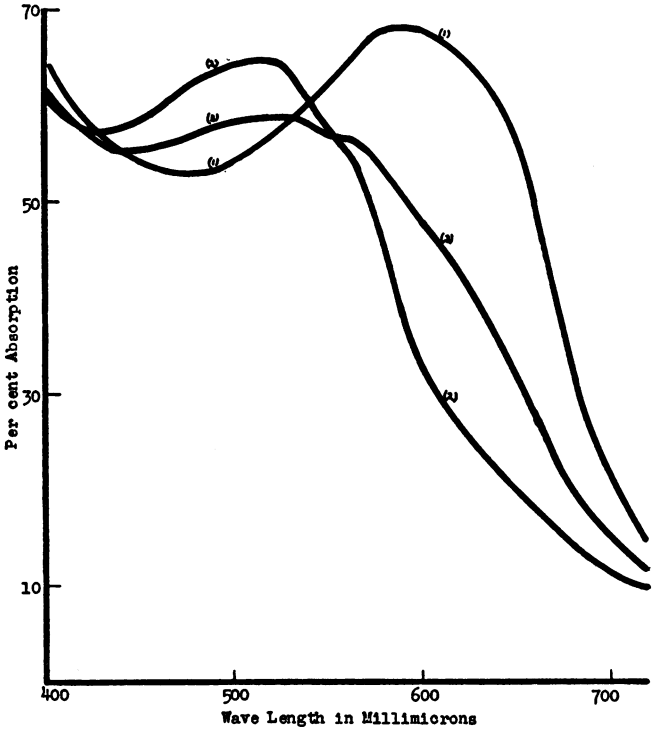

FIG. 1. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM B-3 AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

Curve 1, unbuffered; pH 10.73. Curve 2, buffered to pH 6.3. Curve 3, buffered to pH 7.9.

Curve 3 to pH 7.9. These curves also show the shift in wave length of the maxima from the acid to the alkaline solutions, with the maximum at 520 mμ at pH 6.3; 530 mμ at pH 7.9, with a secondary maximum at 560; and 590 mμ at pH 10.73. Figure 2 shows similar curves made from the purified NaOH extract of V-1. These curves are not absolutely identical with those of figure 1, but they have their maxima at absolutely the same wave lengths, even to the secondary maximum at 560 observed at pH 8.0.

The curves in figure 3 were made from aqueous unbuffered and unpurified solutions of the pigments of B-3 and V-1 that had been poured off from the base

of the flasks in which the cultures had been grown. These curves do not show a great deal in themselves, but are included for comparison with the data given by Müller (1908) concerning the spectrum of a similar extract of his organism. He states that the absorption was strongest between the D line (589.5 mμ) and the beginning of the pure green part of the spectrum (about 550 mμ) becoming gradually less on each side of this region until the C line (656.3 mμ) and the E line (526.9 mμ) were reached. Since his determinations were made from an unpurified solution, the results should be more nearly comparable with the curves shown in figure 3 than those of figure 1 and figure 2. The region where he observed the

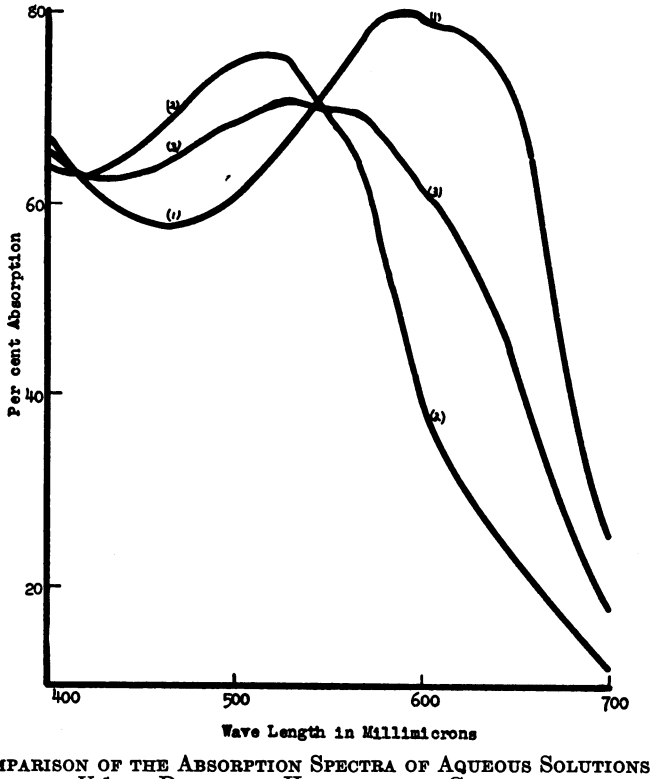

FIG. 2. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM V-1 AT DIFFERENT HYDROGEN-ION CONCENTRATIONS
Curve 1, unbuffered; pH 10.58. Curve 2, buffered to pH 6.3. Curve 3, buffered to pH 8.0.

most absorption is indicated by the block drawn at the base of figure 3. The limits of absorption are indicated by the lines drawn at 525 and 660 mμ.

Figure 4 shows curves made from the purified NaOH extract of Waksman's culture of *Actinomyces violaceus-ruber*. The solution used in Curve 1 was buffered to pH 6.3, that in Curve 2 to pH 7.2, Curve 3 to pH 7.7, and Curve 4 to pH 9.2. These curves are quite different in appearance from those shown in figure 1 and figure 2. The absorption maxima occur at approximately the same wave lengths, but are much less pronounced. The most alkaline solution

(Curve 4) has its maximum around 580 mμ which is about the same as the maxima for the pigments of V-1 and B-3. However, the more acid solutions have their maxima a little further to the left than do the corresponding ones for the other pigments. This might indicate that this pigment would change from blue to red at a higher pH than the other pigments. This observation is in accord with Waksman's statement (1919) that the pigment changes color at pH 7.6.

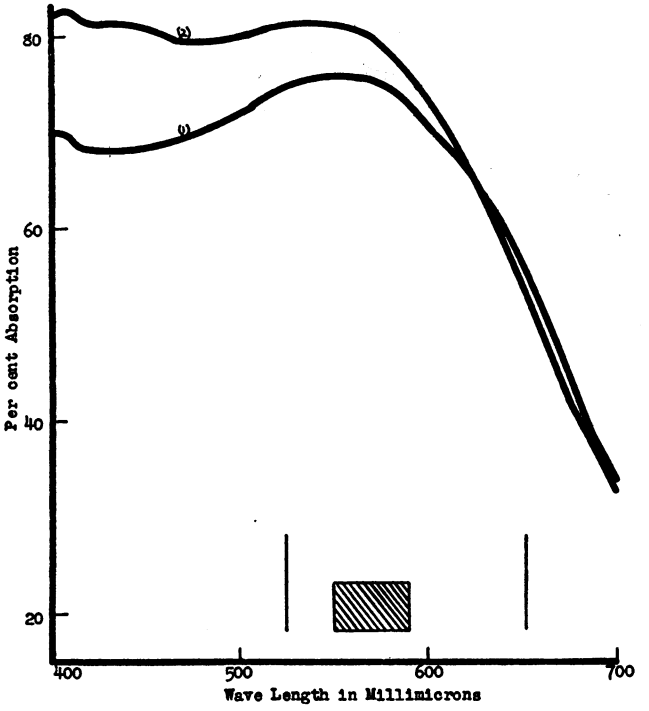

FIG. 3. A COMPARISON OF THE ABSORPTION SPECTRA OF UNPURIFIED AQUEOUS SOLUTIONS B-3 AND V-1 PIGMENTS WITH THE ABSORPTION DATA GIVEN BY MÜLLER FOR STREPTOTHRIX COELICOLOR

Curve 1, B-3 pigment at pH 7.35. Curve 2, V-1 pigment at pH 7.15.
The block at the bottom of the figure indicates the region where Müller observed the most absorption. The limits of absorption are indicated by the lines on either side.

A study of these curves shows beyond a doubt that there is no difference spectrophotometrically between the pigments produced by the organisms V-1 and B-3. In regard to the pigment from Waksman's *Actinomyces violaceus-ruber*, one of two things is indicated. Either this pigment is a quite different substance from that produced by the other organisms, or else the substance is a mixture of several compounds including a small amount of the same pigment which is found in the other cultures. This would be in agreement with Waksman's conclusion that the coloring matter produced by *Actinomyces violaceus-ruber* is a mixture of several pigments. No definite conclusions can be drawn con-

cerning Müller's organism from the absorption data which he gives, especially since he does not mention the pH of his solution; but the maximum which he gives is closer to that observed for the pigments of V-1 and B-3 than is that of Waksman's organism. The only curve for *Actinomyces violaceus-ruber* which shows a maximum between 550 and 590 mμ is the one made at pH 9.2, and it does not seem probable that Müller's solution was as alkaline as that.

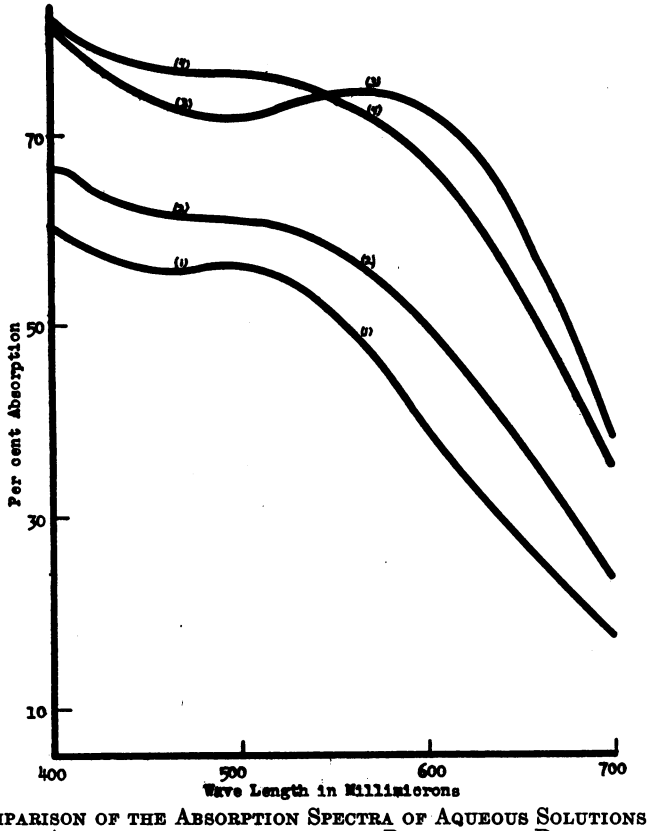

FIG. 4. A COMPARISON OF THE ABSORPTION SPECTRA OF AQUEOUS SOLUTIONS OF PIGMENT FROM ACTINOMYCES VIOLACEUS-RUBER BUFFERED TO DIFFERENT HYDROGEN-ION CONCENTRATIONS

Curve 1, buffered to pH 6.3. Curve 2, buffered to pH 7.2. Curve 3, buffered to pH 7.7. Curve 4, buffered to pH 9.2.

Comparison with litmus

Beijerinck (1914) mentions *Actinomyces coelicolor* as producing a pigment similar to that of Schröter and Cohn's "litmus micrococcus," *Micrococcus cyaneus* Cohn, and suggests that one of these organisms may have some connection with the commercial preparation of litmus. Beijerinck makes no mention of having made spectrophotometric studies. It seemed worth while, therefore, to make a comparison of litmus with the pigment of *Actinomyces coelicolor* by means of the spectrophotometric methods now available. For this purpose a solution was

made of a commercial sample of litmus purchased several years ago; also a solution of azolitmin, believed to have been imported before the war, was obtained from a commercial house in this country.

The absorption spectra of the pigments from culture B-3, of litmus, and of azolitmin were obtained at three different H-ion concentrations by mixing them with phosphate buffer solutions having the reactions of pH 6.2 and 8.0 respectively, as well as with a strongly alkaline solution (about pH 11–12). The absorption spectra in the alkaline solution are given in figure 5. The similarity

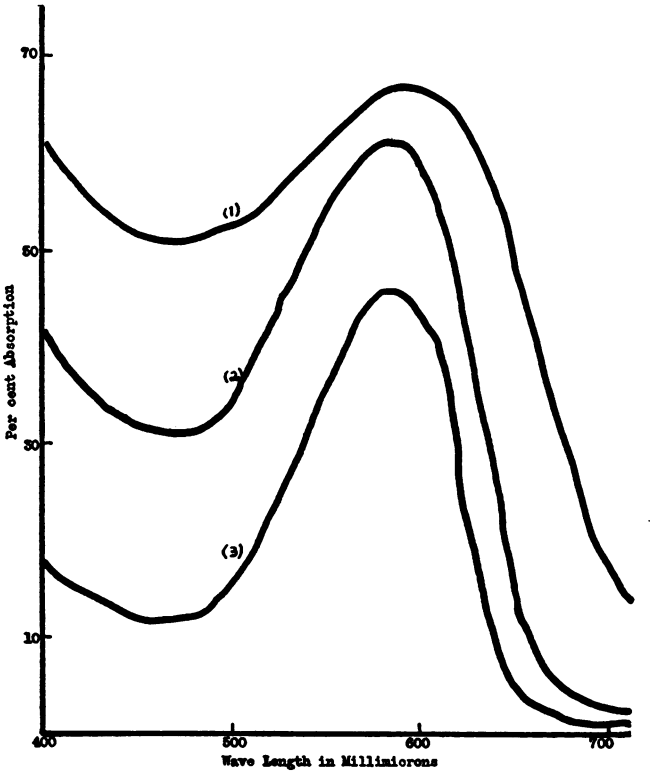

FIG. 5. A COMPARISON OF THE ABSORPTION SPECTRUM OF PIGMENT FROM B-3 WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN ALKALINE AQUEOUS SOLUTION (pH 11 TO 12)
Curve 1, actinomyces pigment. Curve 2, azolitmin. Curve 3, litmus.

of these curves is very striking both in their general shape and in the position of the maximum (close to 580 mμ).

Under acid conditions (pH 6.2), as will be seen from figure 6, a quite different type of curve was obtained with its maximum at about 500 mμ. (This is merely another way of stating that the solutions were then red instead of blue.) At this reaction, however, the curve for litmus proved distinctly different from those for azolitmin and for the pigment, in that it had a secondary maximum at about 580 mμ. Now azolitmin is generally regarded as the primary active constituent of litmus, although other colored compounds are known to be present. The

curve for litmus at pH 6.2 indicates that even under conditions as acid as this some substance is present which absorbs light of about 580 mμ wave length, although the blue color is not evident to the eye.

At a reaction of pH 8.0 (fig. 7) the differences between the *Actinomyces* pigment and either of the other colored substances became more evident. The pigment showed its maximum at about 530 mμ while that of litmus and of azolitmin was around 580 mμ. This indicates that the color of this pigment at this reaction

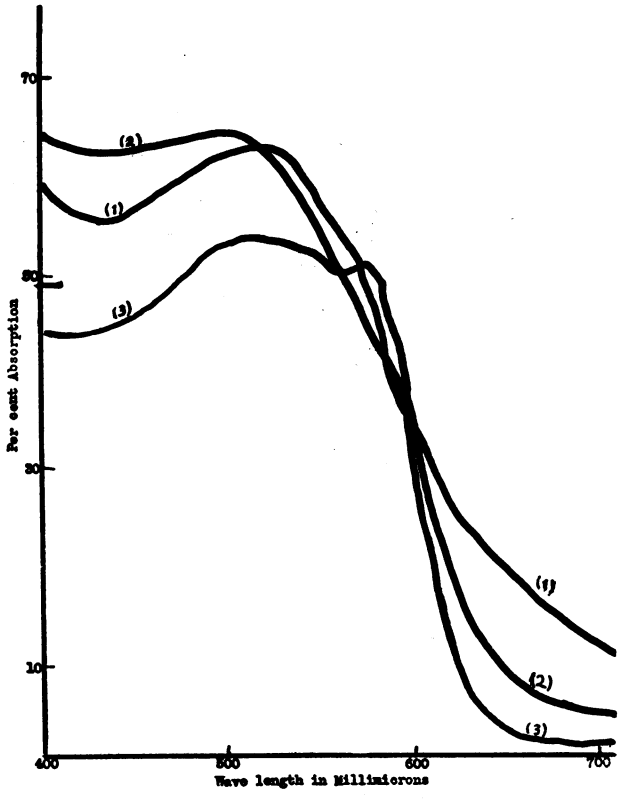

FIG. 6. A COMPARISON OF THE ABSORPTION SPECTRUM OF THE PIGMENT FROM B-3 WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN AQUEOUS SOLUTION BUFFERED TO pH 6.2

Curve 1, actinomyces pigment. Curve 2, azolitmin. Curve 3, litmus.

is somewhat redder than azolitmin (which is purplish in hue) and very much redder than litmus (which is blue). In other words the pH-value (dissociation constant) for the *Actinomyces* pigment is higher than that of azolitmin or litmus; in fact it is too high to allow it to be used as a substitute for litmus in certain indicator media (e.g., milk) used by the bacteriologist. This would indicate that although the pigment produced by this organism is very similar to azolitmin it is not identical with it, and it would hardly be suitable to nick-name this culture "the litmus *Actinomyces*".

By comparing these curves with those for the pigment from *Actinomyces violaceus-ruber* at various reactions (fig. 4) it will be seen that the latter pigment is even less like azolitmin.

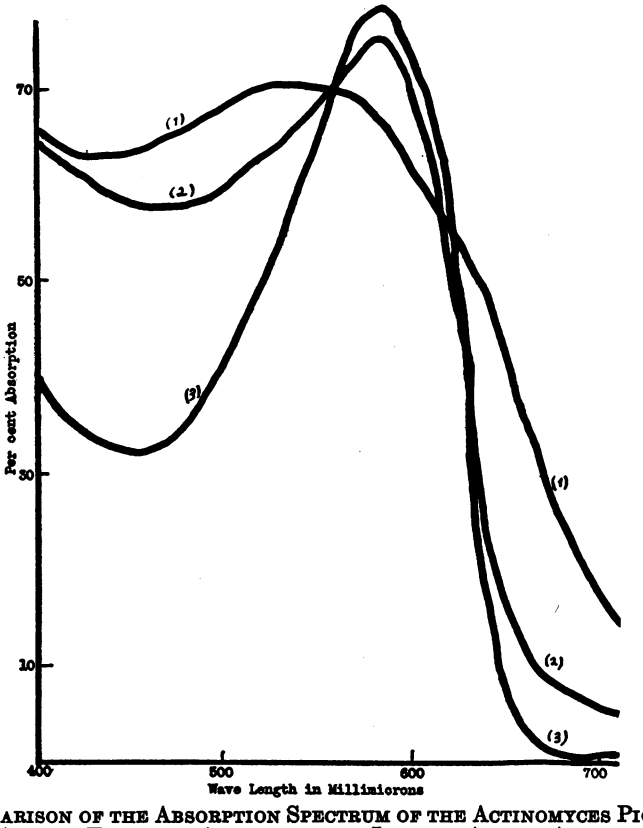

FIG. 7. A COMPARISON OF THE ABSORPTION SPECTRUM OF THE ACTINOMYCES PIGMENT (FROM B-3) WITH THOSE OF AZOLITMIN AND LITMUS; ALL IN AQUEOUS SOLUTION BUFFERED TO pH 8.0

Curve 1, actinomyces pigment. Curve 2, azolitmin. Curve 3, litmus.

DISCUSSION

These experiments have not completely clarified the confusion existing in the literature in regard to these pigments, but they have shown several reasons for this confusion. The difference in appearance of the B-3 culture before and after purification is interesting to note in this connection. If a contaminant, as hard to detect and to remove as this one was, can cause two almost identical cultures to look and to act so differently, it is no wonder that investigators have observed the same organism to produce different colors at different times even when grown under identical conditions. Further confusion has probably been caused by the indicator properties of the pigments. Many investigators have reported results on a "synthetic medium" without giving the exact composition of the medium. It can be readily seen how a slight variation in the composition of the

medium or in the vigor of the culture might alter the final hydrogen ion concentration of the organism, and thus cause the pigment to appear a different color. The temperature of incubation also has some effect. The organisms studied here will produce pigment well at 25° and 30°C., but not at 37°, even though they will grow well at the higher temperature.

This work has shown very clearly the similarity between the organisms designated as V-1 and B-3, which were thought at first to be different species. Culturally they are almost identical, and their pigments, studied both chemically and spectrophotometrically, appear to be the same compounds. During the entire course of investigation, no evidence was found to indicate that any of the organisms (except possibly Waksman's *Actinomyces violaceus-ruber*) produced more than one pigment.

In comparing these two cultures with the organisms described in the literature, it is evident that, in their cultural characters, they agree very closely with Müller's description of *Streptothrix coelicolor*, but are somewhat different from Waksman's *Actinomyces violaceus-ruber*. This distinction is further supported by chemical and spectrophotometric studies of their respective pigments. In view of this evidence, it appears probable that there are at least two species of *Actinomyces* which produce a pigment changing from blue to red that acts as a hydrogen-ion indicator. Because of the superficial resemblance of their pigments they appear to be quite closely related, but a more careful examination of their cultural characteristics and of the chemistry of their pigments indicates them to be distinct species. This contention is further supported by the work of Kriss (1937). His *Actinomyces coelicolor* is apparently the same organism as Müller's *Streptothrix coelicolor* and the two cultures studied here. In another part of his paper, however, he makes a reference to a culture of *Actinomyces violaceus-ruber* obtained from Waksman, and implies that he considers the latter a different organism.

This work shows that the members of the genus *Actinomyces* cannot be satisfactorily classified from superficial appearance alone. It is necessary, in accordance with the conclusions of Kriss (1937) to make careful cultural studies under controlled conditions. Furthermore, it would be advisable to supplement these studies with simple chemical tests of their pigments, especially as to their solubilities and their reactions with acids and alkalies, while spectrophotometric studies of partially purified preparations of these pigments are regarded as of special value.

CONCLUSIONS

From the above experiments, the following conclusions may be drawn:

1. Chemical and spectrophotometric studies show that identical pigments are produced by two strains of *Actinomyces* isolated from soil and thought at first to be separate species because of differences in color observed on superficial examination. This pigment is very similar to (but not identical with) azolitmin, the best known constituent of litmus.

2. The two strains are so much alike in their cultural characters that they may be considered the same species.

3. This species is the same one described by Müller as *Streptothrix coelicolor* and by Kriss as *Actinomyces coelicolor*. It is listed in the 5th edition of Bergey's Manual as *Actinomyces coelicolor*.

4. Waksman's *Actinomyces violaceus-ruber* is quite different culturally and in the chemistry of its pigment. Therefore, the name should probably be retained as that of a species distinct from *Actinomyces coelicolor*.

5. The method of comparing such cultures by spectrophotometric study of partially purified pigments proves sufficiently promising so that it is recommended for application to other pigment-producing Actinomycetes.

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