VALUE OF PIGMENTATION IN CLASSIFYING ACTINOMYCETES

A Preliminary Note[1]

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HISTORICAL

The genus *Actinomyces* is generally regarded as lying between the true bacteria and higher fungi, and contains a few parasitic species but many saprophytic forms. The latter occur mostly in the soil and many of them produce striking pigments. It is quite natural that efforts have been made to utilize these pigments in the identification of species; but the present unsatisfactory nature of the classification of this group suggests that—striking though these pigments are—they have not proved of great diagnostic value.

The first attempt to use pigmentation in classifying the organisms resulted in the recognition of two groups which were at that time considered species and were named by Gasperini *Actinomyces albus* and *A. chromogenus* respectively. The former was supposed to produce no pigmentation and the latter to cause a browning of the medium in which it was grown. The early work on which this classification was based was done with the use of media containing peptone or gelatin. It has since been shown that if the soil *Actinomycetes* are grown on synthetic media this particular brown pigment is never produced, but instead much brighter colors occur and species can be obtained showing nearly all the primary colors of the rainbow. On such media as

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this, many of the strains originally considered part of the species *Actinomyces albus* show striking pigmentation.

The first student of the group to describe the pigmentation on synthetic media and to use it in his classification was Krainsky (1914) who made a very important contribution in this line, although it is difficult today to recognize many of his species. A further effort to classify this group of organisms partially by the use of pigmentation was made by Waksman (1919), while one of the writers (Conn, 1921) pointed out the great amount of variation in color which could occur with the same species on two lots of media having almost identically the same composition. The latter paper concluded that the composition of such media must be controlled more carefully than had been done by any author who had attempted to classify these organisms in the past. Tempel (1931) agreed with previous writers as to this variation, but claimed that under constant conditions, constancy of color could be maintained.

More recently a small amount of work has been published concerning the nature of the pigments produced by these organisms. Thus Kriss (1936a) claimed to have isolated pigments from red, orange, and violet strains of Actinomycetes; and in a second paper (1936b) gave evidence (including absorption spectra) to indicate that at least one of these pigments was an anthocyanin. The presence of anthocyanins in flowering plants (especially grapes) is well established; but Erikson, Oxford and Robinson (1938) disagree with Kriss as to their occurring in Actinomycetes. The latter authors show that the blue pigments of the latter do not turn red in alkaline media, but retain their blue color even when boiled in a solution of NaOH; the suggestion is made that such a pigment may be a polyhydroxyphenazin. Plotho (1940) states that the color of Actinomycetes is regularly darker in alkaline media than in acid media; some strains have fat-soluble pigments while other pigments are insoluble in fats. He shows that such pigments are less soluble when acidified, and precipitate on heating. He calls attention to a yellow pigment which turns violet in alkaline solution and is not soluble in chloroform or ether.

These papers show that the color bears a certain relation to

H-ion concentration. They do not, however, give much indication as to the chemical nature of the pigments, nor do they answer the question as to whether such pigments can be used in classifying the organisms.

Waksman (1940) has, moreover, published a partial classification of this group of organisms, which is based primarily upon morphological characteristics such as the shape of the structures on which the conidia are borne, without paying much attention to pigmentation. Although no such statement occurs in the published paper, Dr. Waksman has privately expressed the opinion that pigmentation is too variable a characteristic to use in classifying these organisms, and that for this reason morphological differences are especially important.

EXPERIMENTAL

Meanwhile, the writers have tried a different approach to the subject of pigmentation in relation to classification; and hope that this characteristic may prove to have more diagnostic value than is generally supposed. The idea that the color produced by one of these cultures may be linked to H-ion concentration is not new, as it has been discussed informally by students of the group for a considerable length of time; in fact, it follows as a sort of corollary from the above-mentioned observations of Erikson, Oxford and Robinson, and of Plotho. No claim is made, therefore, for any originality in the idea; nevertheless there does not seem to be any reference in the literature to show definitely that color of Actinomyces cultures varies with final H-ion concentration, until the writers (Conn and Conn, 1940a, 1940b) called attention to the fact. It seems, however, that this latter work shows conclusively enough that some of these organisms produce pigments which act as H-ion indicators, and that the color which they show depends upon the reaction produced in the medium. In general, it can be said that these indicators are deepest in hue and most soluble at an alkaline reaction while their acid phase is of lighter hue and either insoluble or almost so.

Inasmuch as the final H-ion concentration of any culture depends not only upon the composition of the medium, but upon

the rate with which the culture produces acid from one source and alkalinity from another, it is clear that the color produced by any particular organism may vary greatly. It is quite easy to see why students of the group have come to distrust pigmentation as a means of characterizing species. It has, however, occurred to the writers that this feature may have more diagnostic value than has been supposed in the past provided the emphasis is laid on the nature of the pigment produced rather than on the color manifested.

To illustrate these points, certain data obtained with four cultures are given in Table 1. The first three of the cultures in this table were isolated in this laboratory from soil and have not yet been definitely identified. The fourth culture was obtained from Dr. Waksman of the New Jersey Agricultural Experiment Station, as representing the species to which he formerly gave the name *Actinomyces violaceus-ruber* but which he has subsequently regarded as synonymous with the previously named *Actinomyces coelicolor* (Müller) Lieske. These four cultures are selected for discussion because all of them produce a pigment which under certain circumstances is red. The data obtained in the table were secured by growing the cultures in a synthetic medium containing 0.085 per cent asparagin, 1 per cent glycerol and 0.1 per cent K_2HPO_4, but with varying amounts of glucose. The glucose varied in the ten different lots of media employed from 0.1 up to 5.0 per cent.

The result of this variation in the amount of glucose was a corresponding variation in the final H-ion concentration. Determination of pH-values of each culture after two weeks incubation was made with the quinhydrone electrode, using the technic previously described (Jean E. Conn, 1939); in this technic the agar is macerated with the quinhydrone, placed in a small piece of a drinking straw and attached to the proper terminus of the electrical system. Although the accuracy of determinations made by this technic is not guaranteed, they gave comparative values of sufficient significance for the purpose of this investigation.

Inspection of the data in table 1 shows that there is one point

in the series where there is a distinct change in color with each organism: from violet to red with B1; from blue to red with B3; and from bright red to light pink with R1 and *Actinomyces coelicolor*. With the first two cultures, which showed the most conspicuous change in color, the change occurred between two tubes

TABLE 1

Relation of final reaction to color of culture

	COLOR AND REACTION OF CULTURES ON ASPARAGIN-GLYCEROL AGAR CONTAINING GLUCOSE IN THE QUANTITIES INDICATED:									
	0.1 per cent	0.2 per cent	0.3 per cent	0.4 per cent	0.5 per cent	1.0 per cent	2.0 per cent	3.0 per cent	4.0 per cent	5.0 per cent
R1 (2 wks. old):										
Color:										
Growth	Light red		Bright red				Pink			
Medium	Red						Light pink			
Final pH	7.07	7.27	7.32	7.05	7.10	6.78	4.36	4.25	4.25	4.01
B1* (2 wks. old):										
Color:										
Growth	Violet						Red			
Medium	Violet					Slightly violet	Colorless			
Final pH	5.10	5.35	5.17	4.85	4.58	4.85	4.85	4.65	4.68	4.57
B3 (2 wks. old):										
Color:										
Growth	Blue					Red blue	Red			
Medium	Blue					Part blue	Colorless			
Final pH	6.99	7.44	7.56	6.96	7.38	6.80	5.02	5.44	5.39	4.72
A. coelicolor† (N. J. 3355) (3 wks. old):										
Color:										
Growth	Red		Slightly pink							
Medium	Light red		Colorless							
Final pH	5.0	4.95	4.75	4.65	5.17	4.60	4.55	4.57	4.60	4.50

* B1 on standard agar: Growth, blue; medium, deep blue; final pH, 8.31.

† An earlier series from this same culture showed the blue phase of the pigment in the tubes containing 0.1 to 0.5 per cent glucose, and the red (unsoluble) phase only with over 1 per cent glucose; final pH not determined.

having final pH-values of about 7 on the one hand and about 6 on the other. These two cultures apparently produce no acid from glycerol, and the reaction accordingly is about neutral when small quantities of glucose (1 per cent or less) are added, but becomes acid with larger quantities. The other two cultures (B1 and *Actinomyces coelicolor*), apparently produce acid from

glycerol as well as glucose, so the range of reaction is only from about pH 5.0 to about 4.6. With these two organisms, no blue or violet color is observed; the color change is a mere fading of the red and is not so definitely linked with a change in pH. It should be added that in an earlier series with *Actinomyces coelicolor* (whose results it has not yet proved possible to duplicate), a set almost exactly like those with B3 were obtained, changing from blue to red in color. Unfortunately the pH-values of this set were not determined; it is quite conceivable that in this instance the organism may have failed to produce acid from glycerol and that the final reaction in the absence of glucose may in that case have been around neutrality. Although this is pure speculation, it seemed quite possible after the characteristics of the pigment produced by this culture were studied.

Subsequent study, in fact, has shown that the entire color-change of the pigment is brought out by the data in the table only in the case of B3. The first suggestion that the data given in table 1 do not tell the whole story came from the observation that when grown on standard agar the color of B1 became deep blue. This seemed at first to indicate the production of a still different pigment. It was, however, discovered that the final reaction on this agar was pH 8.3; and that if a strong alkali were added to one of the violet cultures on the synthetic medium, the same deep blue color could be produced. It seems that this organism produces a pigment which acts as a H-ion indicator covering the range from pH 4.8 to 8, changing from red through violet to blue. A report on this pigment has already been made by the authors (Conn and Conn, 1940a).

With R1 it was also found that the pigment changed color through a H-ion range longer than that covered by the series of cultures reported in the table. This pigment can be extracted in alcohol, and appears normally as an orange-red solution in this reagent; if treated with NaOH, the solution turns violet, with H_2SO_4 it turns yellow. A previous report on this pigment (Conn and Conn, 1940b) shows it to be an indicator with a slightly different pH-range, yellow at pH 5 and changing through red at neutrality to violet at pH 8.5.

The four cultures here discussed can under certain circumstances exhibit very similar shades of red; the differences between them, however, are well brought out by a consideration of the characteristics of their respective pigments, as listed below:

R1. Pigment soluble in alcohol at neutrality, yielding an orange-red solution. Turns violet in dilute alkali and yellow in dilute acid; range of color change about pH 5.0 to 8.5. Acid phase almost insoluble, alkaline phase readily soluble in water. Slightly soluble in chloroform at neutrality, yielding a yellow solution.

B1. Pigment soluble in alcohol at neutrality, yielding a violet solution. Turns blue in dilute alkali, and red in dilute acid; range of color change, about pH 4.8 to 8.0. Alkaline phase soluble in water, but precipitates in red form (color acid?) when acidified. Dissolves in chloroform, at neutrality, yielding a bright red solution.

B3. Pigment insoluble in alcohol, chloroform, or any of the ordinary fat solvents. Insoluble in water at neutrality; in dilute NaOH it yields a deep blue solution, but precipitates in red form when neutralized by addition of acid.

Actinomyces coelicolor (Waksman, No. 3355). Pigment in its acid phase (red) insoluble in water, alcohol, chloroform or ether. Turns blue with addition of NaOH and in this phase is soluble in water or alcohol, but insoluble in chloroform or ether.

It is quite apparent that these pigments differ from one another. Nevertheless they have one or two characteristics in common: They are more soluble in alkali than in neutral solutions; their acid phase is almost insoluble in water; they act as H-ion indicators, their alkaline phase being deeper in hue than their acid phase. Presumably they are acid dyes with insoluble color acids but highly soluble sodium salts; but their actual chemical nature has not yet been determined. The change in color and solubility can cause great differences in the appearance of a culture according to its final H-ion concentration; if it remains alkaline in reaction the deep hue of the alkaline phase (blue or violet) colors the entire medium as well as the mass of growth; if it becomes acid, the lighter hued acid phase (red or orange) seems to pre-

cipitate in the mass of growth and the medium remains uncolored. It is apparent that these pigments differ distinctly from those of fruits (anthocyanins, etc.) in that the latter are most soluble in their acid phase.

The pigments produced by B3 and *Actinomyces coelicolor* are very similar. The only distinct difference so far observed between them is that the alkaline phase of the latter is soluble in alcohol while that of the former is insoluble. Nevertheless, cultures of the two organisms are very different in appearance on glycerol asparagin agar; the former is always blue in the absence of glucose, while the latter is usually red. As the red color of the latter usually coincides with a low pH-value, it is concluded that it is due to a difference in final H-ion concentration; presumably this species produces acid from glycerol, while B3 evidently does not.

So far as the writers have yet been able to determine, the production of one of these pigments is constant for any of the species yet investigated, even though color-production, as evident to the eye, is distinctly inconstant. If this is the case, pigment production can be used in the characterization of *Actinomyces* species. This is true, even though other pigments, of non-specific nature, may be produced in some instances. A little more study is necessary before it is possible to be sure how constant the characteristic may be. For the present it seems best not to name the cultures described in this paper; they differ in pigment production from any that the writers have obtained from other laboratories as named species, but whether they are separate species depends on whether the characteristic here under investigation is actually as constant as it now seems to be.

CONCLUSIONS

It is well established that the *colors* produced by species of *Actinomyces* although striking, show such variability that they cannot be used successfully in classification. This opinion is entirely confirmed by the present work.

Apparently, however, *pigment* production is fairly constant for any strain. The variability in *color* is due to the fact that

these pigments act as H-ion indicators, and that the appearance of any culture may vary greatly according to whether the pigment is present in its alkaline or acid phase or partly in each form. As the final H-ion concentration of such an organism on synthetic media is variable, accordingly the color varies.

There is good evidence that if instead of recording the color of a culture, one makes a few simple tests as to its solubility in certain reagents and the color assumed by its solutions at differing pH-values, one can use pigmentation as a feature sufficiently constant to be of diagnostic value.

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