THE ISOLATION, PURIFICATION, AND PROPERTIES OF LITMOCIDIN

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It was shown by Gause (1946) that *Proactinomyces cyaneus-antibioticus* produces an antibiotic substance of a new type, designated as litmocidin. The present article describes the isolation and purification of litmocidin and outlines some of its properties.

ISOLATION OF LITMOCIDIN

Extraction of the active principle from agar culture. Observations have shown that watery extracts of agar cultures of *P. cyaneus-antibioticus* contain a blue pigment and are very active in suppressing the growth of staphylococci. This pigment possesses indicator properties: it is red at an acid reaction, violet at neutrality, and blue at an alkaline reaction. It was also found that this pigment cannot be extracted from agar cultures by ethanol, ether, or acetone at a neutral reaction. Extracts made with these organic solvents after evaporation yield a colorless residue, which possesses no antibiotic activity. However, when organic solvents are acidified to a pH of 3.0, the pigment readily passes from agar into the solvent in the following order: ethanol, acetone, ether, amyl acetate. The evaporation of organic solvents leaves a red residue, which possesses strong antibacterial action. It was hence concluded that antibacterial activity is associated with the pigment, which is soluble in organic solvents at an acid reaction.

Isolation of the active principle from watery extracts. The antibacterial pigment readily passes from watery extracts of agar cultures into ether at a pH of 3 to 4. At a neutral reaction it is insoluble in ether, and hence differs in this respect from actinomycin studied by Waksman and Woodruff (see Waksman, 1945) and from mycetin (Fainschmidt and Koreniako, 1944). However, when a watery extract is acidified to a pH of 3 to 4 an abundant black sediment is immediately formed. It was found that this sediment consists of inactive protein and active pigment adsorbed upon it. These two components are easily separated in the following way: The sediment is filtered, washed, and dissolved in alkaline water. When the water is acidified, the sediment comes out again, leaving some of the pigment in the watery solution. This sedimentation was repeated 12 times and finally yielded pure protein without antibacterial action.

It was further recorded that the watery extract of an agar culture contains two pigments. One of these (litmocidin) is extracted by ether at an acid reaction, changes from red to blue with a rise in pH, and possesses antibiotic action. Another pigment, present in small amount, is insoluble in ether at an acid reaction, changes from orange to green with a rise in pH, and is inactive against bacteria.

Preparation of dry litmocidin. It was found practicable to prepare litmocidin in the following way: To a watery extract of an agar culture 1 per cent of charcoal is added, and the mixture is acidified to pH 3.5. In this procedure the pigment is completely adsorbed on the charcoal, and the liquid becomes colorless. The charcoal is filtered, and further repeatedly extracted by acetone at an acid reaction. The acetone is evaporated *in vacuo* to dryness, and the residue is dissolved in a small amount of ethanol. To four parts of ethanol one part of water is added, and the pigment is immediately sedimented. The sediment is centrifuged, dried *in vacuo*, and kept in powdered form.

Purification of litmocidin. Dry litmocidin can be further purified in the following way: It is dissolved in ethanol, and a few drops of muriatic acid are added to it. The ethanol solution is then mixed with ether, and water is added to this mixture until a watery layer clearly separates. Litmocidin passes into the ether, and impurities are left in the watery phase. This procedure is repeated several times. Finally, the ether is evaporated, the dry residue is dissolved in ethanol, and pure litmocidin is sedimented by water as described above.

PROPERTIES OF LITMOCIDIN

Acid and alkaline forms of litmocidin. The preparation of the acid (red) form of litmocidin has already been described. It is slightly soluble in water at acid reactions and soluble in ethanol and acetone. At a neutral reaction its solubility in water is greatly increased. When the acid (red) form of litmocidin is dissolved in ethanol and neutralized by alkali, and the ethanol evaporated *in vacuo*, the blue (alkaline) form of the pigment is obtained. This form is readily soluble in water. Both forms inhibit the growth of *Staphylococcus aureus* in nutrient broth in a dilution of 1:4,000,000.

Stability of litmocidin. Dry acid litmocidin is entirely stable. Boiling of 0.01 per cent watery solutions of litmocidin for 30 minutes under different conditions shows that it is stable at acid reactions, but at alkaline reactions the activity and coloration rapidly disappear.

It is possible to undertake a differential destruction of the coloration and the activity of litmocidin. Strong alkalies at room temperature destroy the activity but do not affect the color of litmocidin. On the other hand, the heating of litmocidin with 20 per cent HCl for 10 hours at 80 to 90 C affects the coloration but does not interfere with the antibiotic action of litmocidin.

Relation of litmocidin to anthocyans. Litmocidin has much in common with the anthocyanin pigments of plants, and a comparison of some of their properties is presented below.

Properties of anthocyans (Onslow, 1925)	*Properties of litmocidin*
1. Green or blue at alkaline reactions.	1. Blue at alkaline reactions.
2. Red at acid reactions.	2. Red at acid reactions.
3. Green or blue sediment with lead acetate.	3. Blue sediment with lead acetate.

Properties of anthocyans	*Properties of litmocidin*
4. Anthocyanidines (pigments of anthocyans) are completely extracted from water by amyl alcohol at an acid reaction.	4. Litmocidin is completely extracted from water by amyl alcohol at acid reaction. When reaction is made alkaline, it passes into water again and acquires a blue color.
5. Acid solutions of anthocyans become colorless when treated with zinc dust (i.e., by nascent hydrogen). On exposure to air the color returns.	5. Acid solutions of litmocidin are decolorized by zinc dust. On exposure to air the color returns.
6. Decolorized by addition of sodium bisulfite. The color returns on addition of strong acid.	6. Decolorized by bisulfite, but the color is not restored by strong acid.

It is clear that the pigment properties of litmocidin have much in common with the anthocyanidines. But whereas the anthocyanidine pigments of plants are combined with carbohydrates, it was found that litmocidin does not contain any carbohydrate in its molecule.

Repeated purification of litmocidin does not increase further its antibacterial action. Hence it is clear that we are dealing here with an individual body. Litmocidin also possesses a constant melting point. It melts (with decomposition) at 144 to 146 C.

SUMMARY

Litmocidin, a new antibiotic substance produced by *Proactinomyces cyaneus-antibioticus*, has been isolated and purified. It possesses a constant melting point (144 to 146 C). Litmocidin is a pigment, and it has much in common with the anthocyanin pigments of plants. Litmocidin can be obtained in two forms: acid (red) and alkaline (blue), possessing different solubilities in water. Both forms inhibit the growth of *Staphylococcus aureus* in a dilution of 1:4,000,000.

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