PIGMENT PRODUCTION BY NEUROSPORA CRASSA IN THE PRESENCE OF PARA-AMINOBENZOIC ACID

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Recent investigations of altered sulfonamide responses in Neurospora crassa (Emerson and Cushing, 1946) have disclosed that high concentrations of \( p \)-aminobenzoic acid (PABA) depressed and even inhibited the growth rate of various strains studied. The present paper reports observations upon a pigment produced by Neurospora crassa when growing in “minimal” medium (Beadle and Tatum, 1945) supplemented with \( 10^{-2} \) moles of PABA. Specific interest, beyond a general relation to studies of PABA metabolism, may be attached to these observations because of work showing that a strain of Mycobacterium tuberculosis (Mayer, 1944) also produces a pigment when growing in the presence of this factor. In addition, Mayer notes that Escherichia coli and Aspergillus niger produce pigments under similar conditions, and Spink and Vivino (1943) have found that various sulfonamides induce the formation of a pigment by resistant stains of staphylococci, which they believe is derived from the excess PABA produced by these strains. These various pigments, though seemingly related because of their specific association with PABA, differ among themselves and from the one described here.

The fact that the toxicity of PABA in high concentrations extends to such diverse organisms as dogs (Scott and Robbins, 1942), and to microorganisms (Cavill and Vincent, 1945) other than Neurospora lends additional interest to a consideration of the colored compounds produced in its presence. In this regard, Cavill and Vincent also note that the molds Aspergillus, Penicillium, and Byssoschlamys form an orange-yellow pigmentation in the mycelial felt and in the surrounding medium when growing in high PABA concentrations, but do not report further on this coloration.

The present experiments, unless otherwise noted, were conducted with several strains of wild type Neurospora crassa grown at 30° C on liquid medium (20 ml in 125-ml Erlenmeyer flasks). When this medium consisted of “minimal” supplemented with \( 10^{-2} \) moles PABA (pH adjusted to 5.5 at the start of the experiment and changing to 4.2 to 3 as growth progressed), the growth rate of Neurospora was depressed, as noted earlier. As growth proceeded, a green pigment gradually appeared in the vicinity of the mycelium and accumulated until, by 78 hours, the culture fluid was greenish yellow. By the end of 6 days full growth of the mycelium had been reached and abundant conidia produced. At this

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time the medium was conspicuously orange red in color. Separation of the mycelial mat by filtration showed this color to be due to a material that had accumulated in the hyphal strands, the medium itself remaining greenish yellow. Flasks containing the PABA medium, but not inoculated, remained colorless, showing that the pigments are a product of Neurospora metabolism. Concentrations of $10^{-1}$ moles of PABA completely inhibited growth, but did develop a greenish tinge spontaneously. This product, however, was found to have quite different properties from those described for the material produced by the mold. Growth in concentrations of $PABA = 10^{-2}$ moles or lower produced no pigmentation.

In addition to wild type strains from several sources, grown at 35 C as well as at 30 C and on solid as well as liquid media, the following mutant strains also produced the colors described: $p$-aminobenzoic 15835a (from the California Institute of Technology) and the sulfanilamide-resistant (C-40) and sulfanilamide-requiring (E-15172A) strains described elsewhere (Emerson and Cushing, 1946; Emerson, 1947). In addition, an albino strain (12-2) obtained in this laboratory also reacted as did the wild type with respect to the production of pigment, although it retained its albino characteristics.

An attempt was made to obtain genetic alterations in the response of Neurospora through adaptation. To this end, four parallel series of serial transfers of cultures growing on $10^{-2}$ moles of PABA were started, but at the end of 4 months representing 8 such transfers no detectable deviations from the wild type behavior in pigment production or growth rate were found.

That pigment production is specifically associated with PABA is shown by the fact that like concentrations of several amino acids and vitamins (M. Schwartz, unpublished data) added individually to minimal medium had no effect, even on the growth rate, upon wild type Neurospora. In addition, neither benzoic nor anthranilic acids stimulated pigment production; for these either inhibited growth at $10^{-2}$ moles or gave colorless growth at lower concentrations.

The effects of PABA were not altered in media containing, in addition to $10^{-2}$ moles of this factor, mixtures of the vitamins or the amino acids normally used in this laboratory for testing for biochemical mutations in Neurospora and present in the concentrations used for these tests (McElroy, Cushing, and Miller, 1947).

The pigment was also produced when glucose was substituted for sucrose. This is different from the reactions of Mycobacterium when glucose is substituted for glycerol (Mayer, 1944).

The relationship between the yellow-green pigment occurring in the medium and the orange-red pigment in the hyphae has not been established, and it has been convenient to consider the two occurrences separately. The results reported below are concerned only with the pigment found in the filtrate. This material has not yet been obtained in crystalline form, the best preparations being amorphous orange-red powders.

Extraction of the colored material is best done with the concentrated filtrate
at pH 4 and peroxide-free ether; lesser yields are obtained at pH 1 and pH 11. The substance is readily soluble in the lower alcohols, and may be extracted from the concentrated filtrate with n-butyl alcohol. Hydrocarbons, e.g., hexane, benzene, toluene, as well as chloroform and carbon tetrachloride, do not extract the pigment from aqueous solution. However, high specific solubility is shown by the dried material in that, whereas it is insoluble in carbon tetrachloride and the hydrocarbon solvents, it is soluble in chloroform.

The amorphous powder mentioned above was obtained from toluene solution as follows: Excess toluene was added to the ether extract of the pigment, and the ether was removed by distillation, during which process some insoluble material formed. Hot filtration followed by chilling of the clear green toluene filtrate produced a flocculent reddish precipitate, which appeared to darken somewhat on drying in air. The intensification of color, however, appeared not to be due to any change in the nature of the pigment since dilute aqueous and ethereal solutions of the dried material seem identical with solutions of the original pigment in the same solvents.

The substance is soluble in both acids and bases and can be completely removed from ether-hexane solution by shaking either with cold 20 per cent hydrochloric acid or dilute sodium bicarbonate solution. Redissolution in ether from aqueous solutions, without preliminary concentration, is practical only at pH 4.

Characteristic color changes are noted with change in pH. In dilute bases the color is green yellow; acidification causes a deepening of the green color. Evaporation of the alkaline solution results in golden-yellow residues. Evaporation of acidic solutions formed dark red rustlike residues. Dissolved in concentrated hydrochloric or nitric acids, almost colorless solutions are obtained. Evaporation leaves residues similar to those obtained from dilute acids.

A color test which has been found to be very characteristic of the pigment is conducted as follows: One drop of concentrated hydrochloric or nitric acid is placed on a dry film of the substance. As solution takes place, a purple ring is noticed at the periphery of the expanding drop, which itself remains colorless. Evaporation to dryness leaves the red granular substance mentioned above, which dissolves in a few drops of concentrated ammonia water to form a colorless solution.

The pigment can be adsorbed on norit A from ether-hexane solution, and eluted with alcohol, hot water, acetone, and ammonia-alcohol. The water eluate is opaque to ultraviolet light.

The foregoing data, though preliminary in nature, are presented to give some description of the pigment and the conditions for its formation in order to show that it differs in its properties and mode of appearance from the material described by Mayer. By similar comparisons it has been possible to show that the pigment is neither riboflavin (preparation from Merck) nor folic acid (preparation obtained from General Biochemicals, Inc.). Sufficient properties of the pigment described by Spink and Vivino are not available to permit a satisfactory comparison with those reported here.
SUMMARY

Wild type strains of *Neurospora crassa* have been found to produce a water-soluble greenish-yellow pigment in the presence of $10^{-2}$ moles of PABA as growth proceeds and to concentrate an orange pigment in their hyphae. Conditions for the production of this pigment are presented, and a procedure is described for the isolation of the crude material. In addition, a color test is described, and sufficient properties are listed to show that it differs from the pigment found by Mayer (1944).

REFERENCES


