TYROSINASE INHERITANCE IN *STREPTOMYCES SCABIES*

II. INDUCTION OF TYROSINASE DEFICIENCY BY ACRIFLAVINE DYES

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ABSTRACT

GREGORY, KENNETH F. (Ontario Agricultural College, Guelph, Ontario, Canada), AND JAY C. C. HUANG. Tyrosinase inheritance in *Streptomyces scabies*. II. Induction of tyrosinase deficiency by acridine dyes. J. Bacteriol. 87:1287–1294. 1964.—Growth in minimal medium containing 1 µg of acriflavine per ml resulted in a large increase (up to 62%) in the frequency of tyrosinase-deficient (*tye−*) mutants in all of ten strains of *Streptomyces scabies* and eight unidentified streptomycetes studied. This increased frequency did not result from the selection of preformed mutants, since *tye−* clones were usually inhibited by lower concentrations of acriflavine than were tyrosinase-producing (*tye+*) clones, and no significant difference in mycelial yields occurred between the two types growing in a 1 µg/ml concentration of the dye. The mutations induced by X rays and acriflavine were either allelic or closely linked. This *tye−* phenotype was not caused by the production of an enzyme inhibitor, lack of a cofactor, or defect in the conversion of a prototyrosinase to tyrosinase. *Tye−* mutants formed no detectable tyrosinase under a variety of conditions, including the presence of possible inducers. Mutants were able to oxidize glucose and succinate. The *S. scabies* tyrosinase was heat-labile (half-life at 59 °C = 1.6 min) and not particle-bound. We conclude that acriflavine induces the loss of, or alteration in, a structural gene for tyrosinase production present as an extrachromosomal factor.

Acridine dyes are known to alter or eliminate several cytoplasmically inherited and episomal factors in microorganisms, including a respiratory capability factor in yeast (Nagai, Yanagishima, and Nagai, 1961), the F or maleness factor in *Escherichia coli* (Hirot, 1960), and the epism-mediated multiple drug-resistance factors in Enterobacteriaceae (Watanabe and Fukasawa, 1961b). This paper describes experiments in which tyrosinase-producing (*tye+*) strains of *S. scabies* were converted to tyrosinase-deficient (*tye−*) mutants, at high frequencies, by growth in media containing acridine dyes.

MATERIALS AND METHODS

The cultures and media used were described in the preceding paper (Gregory and Huang, 1964). X-ray induced *tye−* mutants were isolated as described by Gregory and Vaisey (1956). Liquid cultures were incubated on a reciprocal shaker (100 strokes per min) at room temperature (22 to 25°C) except where otherwise indicated. All other incubations were at 30°C.

The acridine dyes used were proflavine (3,6-diaminoacridine sulfate), euflavine (3,6-diamino-10-methylacridine chloride), acridine orange (3,6-bis dimethylaminoacridine hydrochloride), and acriflavine (neutral), a mixture of proflavine and euflavine. The dyes were prepared as 0.1% stock solutions, sterilized at 121°C for 20 min, and added to previously sterilized culture media immediately prior to inoculation of the media.

The effect of acridine dyes on the incidence of *tye−* mutants in growing cultures of streptomycetes was determined as follows. Liquid minimal medium (100 ml per 500-ml flask) was prepared with and without acridine dye (usually acriflavine at 1 µg/ml). Each flask was inoculated with about 10⁶ spores of *S. scabies* or other *Streptomyces* spp., and was incubated, with shaking, for 7 days. The mycelial pellets were washed twice by centrifugation and resuspended.
in 50 ml of 0.02% Tween 20. The mycelial suspension was then treated in a cold Waring Blender for 15 sec. The proportion of \( \text{tye}^- \) mycelial fragments was determined by spreading dilutions of the suspension on the surface of minimal medium plus 0.04% L-tyrosine and complete medium plus 10% skim milk plus 0.02% L-tyrosine. After incubation at 30 °C for 4 days, all \( \text{tye}^+ \) colonies were surrounded by a dark brown pigment (melanin), whereas the \( \text{tye}^- \) colonies did not discolor the medium.

Mycelial extracts for tyrosinase measurements were prepared from mycelia harvested and washed on Büchner funnels containing Whatman no. 541 filter paper. Wet mycelium (1 g) was suspended in 20 ml of cold 0.1 M phosphate buffer (pH 6.0), and treated for 8 to 10 min in a Raytheon 10-kc sonic oscillator. Cell debris was removed by centrifugation at 1,800 \( \times \) g for 15 min. The supernatant fluids were kept at 4 °C until assayed.

Tyrosinase activity was routinely assayed spectrophotometrically (Horowitz and Fling, 1953). In most cases, 2 ml of extract, 2 ml of 0.1 M phosphate buffer (pH 6.0), and 1 ml of freshly prepared substrate, 3,4-dihydroxyphenylalanine (DOPA, 4 mg/ml of phosphate buffer), were used. The rate of formation of DOPAchrome at 30 °C was followed at 475 mμ with a Beckman DB spectrophotometer, using a 1-cm light path, and a Photovolt Varicord 43 strip chart recorder. One unit of tyrosinase was defined as the amount causing an initial rate of change in optical density of 0.001 per min under the above conditions.

**RESULTS**

The effect of growth in 1 μg of acriflavine per ml on the incidence of \( \text{tye}^- \) mutants among the fragmented mycelia of ten independently isolated strains of \( S. \) scabies and eight unidentified, \( \text{tye}^+ \) strains of Streptomyces is shown in Table 1. In all strains, acriflavine increased the incidence of \( \text{tye}^- \) colonies. The proportions of \( \text{tye}^- \) clones obtained from treated cultures, when the platings were made on minimal medium (glucose-asparagine-mineral salts), ranged from 2 to 62% (mean, 20.2%); the proportions from nontreated cultures ranged from 0.0 to 7.6% (mean, 1.8%). Both treated and control cultures yielded fewer \( \text{tye}^- \) colonies when the platings were made on complete medium (minimal with casein hydrolysate and yeast extract) plus skim milk. Almost ten times more \( \text{tye}^- \) colonies arose from the fragmented mycelia of control cultures (1.8%) than from their spores (0.2%). Cultures grown in complete medium plus acriflavine did not show an increase in the proportion of \( \text{tye}^- \) mutants compared with control cultures.

Optimal concentrations of acriflavine, ranging from 0.5 to 1.0 μg/ml, were observed in each of three strains studied. Acriflavine orange and the two components of the acriflavine preparation used in these studies, viz., euflavine and proflavine, were equally effective in increasing the incidence of \( \text{tye}^- \) mutants. The optimal concentration with strain A26, in each case, was 1.0 μg/ml. Since none of these three dyes resulted in as high

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**Table 1. Frequency of tyrosinase-deficient mutants in fragmented mycelia of Streptomyces scabies and other tyrosinase-producing streptomycetes grown in minimal medium with and without 1 μg of acriflavine per ml**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimal plating medium</th>
<th>Complete + milk plating medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acriflavine-treated</td>
<td>Not treated</td>
</tr>
<tr>
<td>( S. ) scabies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-2</td>
<td>4</td>
<td>1.8</td>
</tr>
<tr>
<td>PA-3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PA-4</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>PA-5</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>PA-10</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>PA-12</td>
<td>16</td>
<td>0.8</td>
</tr>
<tr>
<td>PA-29</td>
<td>62</td>
<td>6.4</td>
</tr>
<tr>
<td>A-9</td>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td>A-26</td>
<td>37</td>
<td>2.2</td>
</tr>
<tr>
<td>A-30</td>
<td>3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unidentified species</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S-23</td>
<td>8</td>
<td>1.3</td>
<td>10</td>
<td>0.8</td>
</tr>
<tr>
<td>S-27</td>
<td>9</td>
<td>2.2</td>
<td>10</td>
<td>1.1</td>
</tr>
<tr>
<td>S-40</td>
<td>7</td>
<td>1.3</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>S-41</td>
<td>15</td>
<td>0.7</td>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td>S-42</td>
<td>17</td>
<td>7.6</td>
<td>13</td>
<td>3.2</td>
</tr>
<tr>
<td>S-43</td>
<td>10</td>
<td>0.2</td>
<td>4</td>
<td>0.1</td>
</tr>
<tr>
<td>S-44</td>
<td>35</td>
<td>0.0</td>
<td>5</td>
<td>1.6</td>
</tr>
<tr>
<td>S-47</td>
<td>58</td>
<td>2.4</td>
<td>18</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>20.2</td>
<td>1.8</td>
<td><strong>12.3</strong></td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Each value is the mean of two to four experiments performed at different times.
an incidence of *tye*− mutants as did the bicomponent acriflavine preparation, acriflavine was used in the subsequent experiments.

Two mechanisms could account for the increased yield of *tye*− clones resulting from acriflavine treatment. The dye might have selectively inhibited *tye*+ cells and thereby favored the growth of spontaneous *tye*− mutants. Alternatively, the dye might have acted as a general or a specific mutagenic agent. To distinguish between these hypotheses, the acriflavine tolerances of 45 *tye*+ and 25 *tye*− clones, derived from X-irradiated spores of *S. scabies* A26, were determined. These clones had had no previous contact with acriflavine.

Spores from these cultures showed wide variations in their tolerances to acriflavine in minimal agar medium (Fig. 1). At each concentration tested, a higher proportion of *tye*− clones was inhibited than of *tye*+ clones.

The dry weights of mycelia produced by 14 *tye*+ and 14 *tye*− clones, after 7 days of shaking in liquid minimal medium containing 1 µg of acriflavine per ml, expressed as percentages of the yields in the absence of the dye, are shown in Table 2. The effects of the dye ranged from marked inhibition to stimulation with both *tye*+ and *tye*− clones. An analysis of variance revealed no significant difference between the two groups in their growth response to acriflavine.

To investigate the possibility that the dye might favor one particular class of *tye*− mutants, tests for allelism were made between the *tye*− allele in *S. scabies* A26-59 (auxotrophic for ar-ginine and methionine, resistant to 20 µg of streptomycin per ml, and *tye*−) and the *tye*− alleles in each of three *tye*− mutants isolated after acriflavine treatment, and five mutants isolated after X irradiation. Spores from the mixed growths of A26-59 with each of the other *tye*− strains on complete medium were plated on minimal medium containing streptomycin. Yields of prototrophic, streptomycin-resistant recombinants ranged from 70 to 2,800 per ml of spore suspension. From all these plates, only a single *tye*+ colony, probably a contaminant, was found. The various *tye*− mutants were either allelic or very closely linked, since no significant recovery of *tye*+ recombinants occurred among the recombinant progeny.

Since acriflavine did not appear to be selectively favoring the growth of *tye*− mutants, experiments were performed to see whether it was acting as a general mutagen. The incidence of revertants to nutritional independence of phenylalanine and nicotinic acid in a strain of *S. scabies* (A26-40) requiring these growth factors, and of mutation to low-level streptomycin resistance (4.5 µg/ml) in strain A26, was compared for cultures grown in the presence and absence of acriflavine. The numbers of these mutants in

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**TABLE 2. Growth of tyrosinase-producing and tyrosinase-deficient clones of Streptomyces scabies, isolated after X irradiation of strain A26, in presence of acriflavine (1 µg/ml)**

<table>
<thead>
<tr>
<th>Strain (X26)</th>
<th>Tyrosinase-producing</th>
<th>Tyrosinase-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>X26-5</td>
<td>135.8</td>
<td>X26-16</td>
</tr>
<tr>
<td>X26-6</td>
<td>133.3</td>
<td>X26-10</td>
</tr>
<tr>
<td>X26-4</td>
<td>121.6</td>
<td>X26-31</td>
</tr>
<tr>
<td>X26-21</td>
<td>116.2</td>
<td>X26-2</td>
</tr>
<tr>
<td>X26-11</td>
<td>116.2</td>
<td>X26-17</td>
</tr>
<tr>
<td>X26-12</td>
<td>116.0</td>
<td>X26-8</td>
</tr>
<tr>
<td>X26-18</td>
<td>100.3</td>
<td>X26-27</td>
</tr>
<tr>
<td>X26-19</td>
<td>95.5</td>
<td>X26-26</td>
</tr>
<tr>
<td>X26-23</td>
<td>51.5</td>
<td>X26-28</td>
</tr>
<tr>
<td>X26-22</td>
<td>49.5</td>
<td>X26-29</td>
</tr>
<tr>
<td>X26-13</td>
<td>45.2</td>
<td>X26-24</td>
</tr>
<tr>
<td>X26-14</td>
<td>39.4</td>
<td>X26-25</td>
</tr>
<tr>
<td>X26-33</td>
<td>28.6</td>
<td>X26-32</td>
</tr>
<tr>
<td>X26-20</td>
<td>2.6</td>
<td>X26-9</td>
</tr>
</tbody>
</table>

* Growth relative to growth in absence of acriflavine.

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**FIG. 1. Inhibition by acriflavine of 45 tyrosinase-producing and 25 tyrosinase-deficient clones derived from Streptomyces scabies A26 after X irradiation.**
fragmented mycelial preparations could not be
determined directly because of background
growth. Spores harvested from mycelia, which
had been fragmented and cultured on complete
agar medium, did not contain significantly
different proportions of nutritional revertants in
treated and control cultures. The low incidence
of these mutants made precise comparisons
impossible, but threefold or greater differences
would have been readily detected. The frequency
of streptomycin-resistant mutants was lower from
the acriflavine-grown cultures (2.7 × 10⁻⁷)
than from the control cultures (4.8 × 10⁻⁶).

**Nature of the tyrosinase-deficiency mutation.**
Experiments were designed to distinguish among
the following possible sites of the tyrosinase-
deficiency mutation: (i) a structural gene for
tyrosinase synthesis, (ii) a regulatory gene, (iii)
a gene producing a cofactor required for tyro-
sinase activity, (iv) a gene influencing the con-
version of a prototyrosinase to tyrosinase, as in
*Neurospora* (Horowitz and Shen, 1952; Fox and
Burnett, 1959), (v) a gene producing an inhibitor
of tyrosinase, (vi) a factor controlling the activity
of oxidative enzymes in general [like the respira-
tory-deficiency mutation in yeasts (Nagai et al.,
1961)], and (vii) a factor required for the organi-
ization of the enzyme within the cell. Mammalian
tyrosinase is found in the particulate fraction of
cell homogenates (Seiji et al., 1963), and cyto-
plasmically inherited factors often affect the
structural organization of enzymes (Catchexide,

To distinguish among these possibilities,
high yields of tyrosinase were needed. Both in-
cubation temperature and medium composition
markedly affected tyrosinase production. Tyro-
sinase activity could not be detected in either the
growth media or the extracts from sonically
disrupted mycelia of cultures grown at 30 °C, but
was present in cultures grown at room tem-
perature (ca. 23 °C). This difference may have
been due to the lability of the enzyme. The half-
life of crude tyrosinase from *S. scabies* A26 at
59 °C was 1.6 min, and the half-life of the “heat-
labile” type of *Neurospora* tyrosinase at this
temperature was reported to be 3 to 4 min (Hor-
witz and Fling, 1953). Inactivation followed
first-order kinetics.

Media prepared with tap water yielded seven
times as much tyrosinase as did media prepared
with distilled water. The active component of
the tap water appeared to be calcium (Fig. 2). Most
of the tyrosinase was found in the medium rather
than in the mycelium. The amount of tyrosinase
produced (retained plus excreted) and the dry
weight of mycelium formed were inversely related
at various calcium concentrations. Mycelial
growth in the presence of 50 to 250 μg of CaCl₂
per ml was ca. 15% of the growth occurring in
the absence of calcium.

Although tyrosinase is a copper-containing
enzyme (Dressler and Dawson, 1960), the addi-
tion of 1 and 5 μg of Cu++ (as CuSO₄) per ml of
medium had no effect on tyrosinase production.
The higher concentration partially inhibited
growth.

Dialysis of mycelial extracts of strain A26
against 0.1 m phosphate buffer (pH 6.0) was found
to increase the enzyme activity two- to threefold,
suggesting the presence of a dialyzable inhibitor.
To test the possibility that an inhibitor might be
present in high concentrations in tyrosinase-
deficient mutants, equal volumes of mycelial
extracts from A26 and each of two *tye*⁻ mutants
were mixed together, and tyrosinase activities
were measured. Extracts from A26 grown at
room temperature and at 30 °C were similarly
mixed and tested. The results (Table 3) indicated
that a dialyzable inhibitor of tyrosinase was
present in all extracts, both those with and those
without tyrosinase activity. The amount of
inhibitor in the mycelial extracts of deficient
strains was not sufficient to abolish the activity
of tyrosinase in the mixtures and appeared to be
similar to the amount present in extracts from

![Fig. 2. Effect of calcium content of medium on
tyrosinase activity in the culture supernatant (un-
shaded areas) and in the mycelium (shaded areas)
of *Streptomyces scabies* grown in minimal medium
for 3 days.](http://jb.asm.org/)

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TABLE 3. Effect of mixing mycelial extracts from tyrosinase-producing and tyrosinase-deficient strains of Streptomyces scabies

<table>
<thead>
<tr>
<th>Mycelial extract*</th>
<th>Initial activity† (units/ml)</th>
<th>Activity after dialysis† (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A26 alone</td>
<td>39</td>
<td>86</td>
</tr>
<tr>
<td>AF26-2 alone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A26 + AF26-2</td>
<td>13</td>
<td>66</td>
</tr>
<tr>
<td>X26-36 alone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A26 + X26-36</td>
<td>21</td>
<td>78</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A26 grown at room temp</td>
<td>94</td>
<td>140</td>
</tr>
<tr>
<td>A26 grown at 30 C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A26 (room temp) + A26 (30 C)</td>
<td>64</td>
<td>140</td>
</tr>
</tbody>
</table>

* Strain A26 = tye⁺; strain AF26-2 = tye⁻ isolated after growth in acriflavine; strain X26-36 = tye⁻ isolated after X irradiation. Extracts were mixed in equal volumes. Cultures were grown on complete medium plus 50 μg of CaCl₂ per ml.
† Activities expressed as units per ml of the original enzymatically active extract present.
‡ In Experiment 1, all incubations were at room temperature.

A26 grown at 30 C. Most of the inhibitory activity was removed by dialysis at 4 C for 24 hr. Dialysis of extracts from the mutant strains did not reveal tyrosinase activity.

Fox and Burnett (1959) reported that incubation of crude mycelial extracts of N. crassa at temperatures of 5 C or higher increased the enzymatic activity with L-DOPA through the activation of an enzyme precursor, protyrosinase. When mycelial extracts of A26 were stored at 5 C and assayed for tyrosinase activity at 12-hr intervals, the activity remained unchanged for 4 days but decreased slightly by the fifth day. When extracts from tye⁺ and tye⁻ strains were mixed, incubated at room temperature, and sampled at four 30-min intervals, no change in tyrosinase activity occurred.

The possibility that the tyrosinase of S. scabies might be organized in particulate components was explored by the use of differential ultracentrifugation of crude mycelial extracts of strain A26 in a Spinco preparative refrigerated ultracentrifuge. The enzymatic activity of the various fractions, after each was restored to the original volume, is shown in Fig. 3. Most of the enzyme was present in the "soluble" fraction, i.e., the supernatant after centrifugation at 100,000 × g for 60 min. The fact that the total tyrosinase activity recovered was greater than the initial tyrosinase activity can probably be accounted for, at least in part, by the absence of inhibitor from the particulate fractions.

The ability of two tye⁻ mutants and the tye⁺ parent to oxidize various substrates was determined by standard manometric methods (Umbreit, Burris, and Stauffer, 1957). Mycelia from 1-day-old cultures grown in shaken complete medium were washed, fragmented by a motor-driven Teflon pestle glass homogenizer for 30 sec, and shaken in 0.03 M phosphate buffer (pH 5.8) for 3 hr at 30 C, so as to reduce the endogenous respiration. Substrates were then added (12.5 μmoles per flask), and oxygen uptake was read at 10-min intervals for 2 hr. The parent strain oxidized L-tyrosine, L-DOPA, glucose, and sodium succinate. All substrates showed rates of oxygen uptake greater than the endogenous from the first readings but the rates with L-DOPA and sodium succinate increased further after 30 min. The tye⁻ mutants did not oxidize either L-tyrosine or L-DOPA but did oxidize glucose and sodium succinate.

Oxidation of p-cresol and catechol by "cell-free" mycelial extracts of A26 and two tye⁻ mutants and by commercial mushroom tyrosinase (Nutritional Biochemicals Corp., Cleveland, Ohio) was determined by the manometric
method of Mallette and Dawson (1947). Catecholase activity was also tested by the sensitive chronometric method of Miller et al. (1944). Rapid oxygen uptake occurred on p-cresol with mushroom tyrosinase and A26 extracts, but none was detected with the tye- extracts. The mushroom tyrosinase was more active on catechol than on p-cresol, but no trace of catecholase activity was found in any of the S. scabies extracts.

Horowitz and Shen (1952) reported that two types of tye- mutants of N. crassa, ty-1 and ty-2, could be induced to form tyrosinase by cultivation in media containing DL forms of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, whereas a high sulfate concentration repressed tyrosinase synthesis. No difference in tyrosinase production occurred when strain A26 was grown in medium containing 0.0015% MgSO4-7H2O instead of the usual 0.02% concentration. Neither of two tye- strains produced a detectable amount of tyrosinase under these conditions. No significant difference in mycelial yield or tyrosinase activity of A26 resulted from the addition of 0.25 mg/ml concentrations of DL-tryptophan or DL-phenylalanine; DL-tyrosine increased mycelial growth threefold but decreased tyrosinase activity per milligram of mycelium to one-third of the activity in the control culture. No enzyme was detected in the mycelia of the mutant strains grown in these media.

**DISCUSSION**

Acriflavine markedly increased the normally high (average, 1.8%) incidence of tyrosinase-deficient plating units found in the fragmented mycelia of S. scabies cultures. The phenomenon appears to be a general one, since all ten strains of S. scabies and eight strains of other tyrosinase-producing streptomycetes gave this response. Since the presence of acriflavine did not favor the growth of tye- clones over tye+ clones, the increased frequency of tye- colonies cannot be attributed to the selection of preformed mutants. The fact that tye- mutants tended to be inhibited by lower concentrations of acriflavine than did tye+ clones probably accounts for the existence of an optimal concentration of acridine dye for this phenomenon. Concentrations above the optimum would have inhibited the mutants more than the parental type.

It is not possible to state conclusively that acriflavine induced the tyrosinase-deficiency mutation to the exclusion of other possible mutations. It resulted in no detectable increase in the proportion of spores representing back mutations for two nutritional deficiencies or of mutants resistant to streptomycin. Accurate determinations of mutation rates per nuclear division cycle in the streptomycetes, however, are hampered by formidable technical problems.

The mutagenic action of acriflavine appears to be limited to genes which are not linked to a major portion of the genome. Acridine dyes prevented the replication of vegetative bacteriophage but did not affect the chromosomally bound temperate phage (Bertani, 1957). Although acridine orange converted F+ clones of E. coli to stable F- forms, it did not eliminate the F factor attached to the chromosome in Hfr males (Hirota, 1960). Acridine dyes eliminated the episome-mediated multiple drug-resistance factors from species of Enterobacteriaceae (Watanabe and Fukasawa, 1961b). Sulfonamide resistance, however, in a strain of Shigella flexneri which could not transfer this characteristic by conjugation was not eliminated by acridine dyes. The various resistance factors in Enterobacteriaceae represent more than one gene locus, since these factors can be transduced individually (Watanabe and Fukasawa, 1961c) but are transferred together by conjugation (Watanabe and Fukasawa, 1961a). The size of this linkage group may account for the fact that it is eliminated by acridine dyes at a lower frequency (ca. 4%) than most other epimemes. F' strains of E. coli, strains in which a fragment of the genome has become attached to the F factor, usually spontaneously lose the merogenote at frequencies greater than 10^-3, and this frequency is increased by acridine orange (Pittard, Lootit, and Adelberg, 1963). One such F' strain, harboring an F-merogenote bearing several loci governing the syntheses of arginine, methionine, isoleucine, and valine, however, was not affected by acridine orange. The elimination of the tye+ characteristic from a large proportion of the mycelial fragments of acriflavine-grown S. scabies suggests that a genetic factor involved in tyrosinase capability by this species is extrachromosomal and linked to few or no other genes.

The data tend to eliminate possible sites of action other than the structural gene for tyrosinase synthesis. If any cofactor required for tyrosinase activity were lacking in the mutants, then mixtures of mutant and parent extracts...
should have shown increased activity. Similarly, an increase in activity should have followed incubation of such mixtures if a system capable of converting protorosinase to tyrosinase were lacking in the mutants. No indication of a protorosinase was found in either mutants or parent. Although mycelial extracts of the mutant strains carried a dialyzable inhibitor of tyrosinase, its concentration was similar in the parental strain and its removal by dialysis did not reveal tyrosinase activity in the mutants. Since the tyrosinase of *S. scabies* is not particle bound, the tye− phenotype cannot result from a defect in the organization of tyrosinase within mutant cells. The mutation does not affect respiratory enzymes in general, since the oxidation of glucose and sodium succinate was unimpaired. No trace of tyrosinase activity was detected from tye− strains grown in the presence of the aromatic amino acids, which were capable of inducing tyrosinase production by strains of *N. crassa* bearing mutations of two regulatory genes for tyrosinase production. The exclusion of a regulatory gene as the site of action of acriflavine is thus indicated but not proven.

Tyrosinase catalyzes two types of reactions involving molecular oxygen (Bright, Wood, and Ingraham, 1963). One reaction is an oxidation of a monophenol (such as *p*-cresol and L-tyrosine) to an ortho diphenol; the second is an oxidation of an o-phenol (such as catechol and L-DOPA) to an orthoquinone. Since both reactions involve an uptake of oxygen, and tye− mutants showed no O2 uptake on either L-tyrosine or L-DOPA, both functions were missing in these mutants. Unlike the tyrosinases from most sources, the *S. scabies* tyrosinase was inactive on catechol.

The data presented in this paper support the hypothesis (Gregory and Shyu, 1961; Gregory and Huang, 1964) that the structural gene for tyrosinase production by *S. scabies* is carried in a small genetic unit or “plasmid” unlinked to most other genes.

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**LITERATURE CITED**


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