THE EFFECTS OF GENETIC CHANGES ON TYROSINASE ACTIVITY IN GLOMERELLA

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ONE of the most significant and interesting problems in biology concerns the relationship between genes and enzymes. Recently considerable attention has been directed towards the hypothesis of genic action which states that the primary function of a gene lies in the control of enzyme synthesis (Horowitz 1949; Beadle 1945, 1948; Haldane 1942; Wright 1941). In support of this concept several investigators have established correlations between the activity of an enzyme and the genetic constitution of an organism. However, the particular kind of correlation established varies with the different investigations. For example, Mitchell and Lein (1948) have shown that a single gene change completely prevents any measurable activity of the enzyme which catalyzes the coupling of serine and indole to form tryptophane in Neurospora. Similarly, Sawin and Glick (1943) working with atropinesterase from the blood serum of rabbits showed that this enzyme was inherited as an incompletely dominant trait. Homozygous recessive rabbits produced serum with no atropinesterase activity while the serum from the heterozygotes had about half the enzymatic activity of the serum from the dominant homozygotes. Thus, in this case, a single gene seems to exert an effect on the quantity of an enzyme produced.

On the other hand, the work of Winge and Roberts (1948) on maltose fermentation by Saccharomyces cerevisiae indicates a polygenic control of enzyme synthesis. They found that any one of three non-allelic genes enabled S. cerevisiae to ferment maltose, presumably by bringing about the synthesis of maltase. A somewhat different kind of polygenic control of an enzyme, involving the degree of enzyme activity, is shown by the investigations of Russell and Russell (1948) on the intensity of dopa oxidase activity in the hair bulbs of the skin of mice. The dopa oxidase activity was found to be proportional to the intensity of yellow pigment in the hair and to be under the control of several different genes.

These examples of the genetic control of enzyme synthesis all involve measurements of the degree of enzymatic activity. Such differences in enzymatic activity may be attributed either to differences in the amount of the enzyme present or to different efficiencies of the same quantity of enzyme. Different efficiencies may in turn be explained by associated changes of the chemical milieu (pH changes, for example) in which the enzyme is acting or to inherent differences in the enzyme molecule itself. It is this last interpretation which forms the basis of the hypothesis (cf. Beadle 1945) that the gene determines the specificity of the enzyme. Some of the support for this hypothesis stems

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from the demonstrated qualitative relationship between genes and specific protein antigens in the different blood groups. A similar kind of relationship may exist between genes and the protein enzymes. In fact, one investigator (SCHUURMAN 1937) has concluded that a qualitatively different enzyme is produced in the mealworm, *Tenebrio molitor*, as the result of a change in a single gene. Extracts prepared from the genetically different adult mealworms catalyzed the production of differently colored pigments from the same chromogen. However, since neither the chemical composition of the pigments nor the chemical steps in their synthesis are known, the enzymatic differences observed in the mealworm cannot be considered a conclusive verification of the one gene-one specific enzyme hypothesis.

These varied investigations on gene-enzyme relationships, taken together, seem to suggest that genes may control both the specific properties of an enzyme and also the quantity produced. In an effort to gather more information about gene-enzyme relationships, the present investigation was directed towards correlating changes in genetic constitution with the amount of tyrosinase activity in the fungus *Glomerella*.

**MATERIALS AND METHODS**

*Characteristics of Tyrosinase*

Several investigators have shown that the enzyme tyrosinase possesses properties which make it particularly useful for investigations of the genetic control of enzyme synthesis. Perhaps the most distinctive characteristic of tyrosinase is its ability to catalyze two quite different oxidations—that is, 1) the insertion of a hydroxyl group into monohydric phenols ortho to the one already present, and 2) the oxidation of many o-dihydric phenols to their corresponding o-quinones. Although some investigators doubt that tyrosinase is a single enzyme, NELSON and DAWSON (1944) and their collaborators have, over a period of several years, accumulated a large body of convincing evidence to show that tyrosinase is a single protein entity.

The relative rates of the two catalytic activities of tyrosinase extracted from the common mushroom *Psalliota campestris* are variable and depend upon such factors as the physical nature of the tyrosinase complex and the presence of other proteins. For example, the addition of gelatin to purified preparations of the enzyme alters the ratio of activities on the two kinds of substrates. Moreover, different methods of purification apparently change the physical nature of the tyrosinase, because, as NELSON and DAWSON (1944) have found, purified preparations with different ratios of activities also exhibit differences in their kinetic, substrate, inactivation, stability, and electrophoretic characteristics. These differences suggest that the different kinds of tyrosinase differ in the size or surface character of the enzyme molecule. It seems reasonable to suppose that such altered surface properties might also be induced by genetic changes which affect the synthesis of the enzyme complex. In fact, purified tyrosinase from the wild mushroom *Lactarius piperatus* is not identical with samples obtained from Psalliota. DALTON and NELSON (1939) were unable to
alter the ratio of catalytic activities of tyrosinase from Lactarius even by the same methods which were successful in the case of preparations from Psalliota. These results indicate that different kinds of tyrosinase may be produced by organisms of different genetic constitutions.

An additional reason for using tyrosinase in genetic studies stems from the extensive data on the inheritance of melanin pigmentation in mammals and birds. The initial steps in the formation of these pigments are apparently under the control of tyrosinase or dopa oxidase—an enzyme which is similar to, if not identical with, tyrosinase. Moreover, a large number of mutant genes have been found which affect the quantity and quality of the melanin pigments (Wright 1942). These genetically controlled changes in pigmentation can also be correlated in some instances with changes in dopa oxidase activity (Russell and Russell 1948; Ginsburg and Kaplan 1947; Ginsburg 1944). Thus the activity of tyrosinase seems to be particularly susceptible to genetic changes.

Tyrosinase in Glomerella

Several different plants and animals are known to produce tyrosinase (Kastle 1910; Nelson and Dawson 1944). However, few of these organisms have characteristics which make them convenient tools for research in genetics. In an effort to find a suitable fungus for this investigation many black colored fungi were picked up at random and tested for their ability to produce tyrosinase, the assumption being that black pigment might indicate the presence of tyrosinase. Eventually a black-colored fungus, rich in tyrosinase, was found as a contaminant on an agar plate. This fungus was identified as a strain of the ascomycete Glomerella cingulata (Stoneman) Spauld. and v. Schr. by Dr. Diehl of the United States Department of Agriculture. Confirmation of this identification was obtained by crossing the fungus with Edgerton's plus B strain of Glomerella, which was kindly supplied by S. J. P. Chilton of Louisiana State University. Edgerton, Chilton, Lucas, and Wheeler during the last few years (1944, 1945, 1946, 1948, 1949) have published a series of informative papers on the genetics of Glomerella and on methods of handling this fungus in the laboratory. Much of their technique and some of their strains of Glomerella have been used during the present investigation.

Strains of Glomerella

When Edgerton's plus B strain of Glomerella was crossed with the strain first obtained as a contaminant on an agar plate, the progeny segregated into four types. These four kinds of segregants were obtained both by isolating individually the eight ascospores from a single ascus and by isolating colonies at random from plated out ascospores. These four types, when grown on a complete medium,² are distinguishable by the different degrees of black pig-

² NH₄ tartrate 5 grams glucose 20 grams
   NH₄NO₃ 1 gram agar 15 grams
   K₂HPO₄ 1 gram yeast extract 5 grams
   MgSO₄·7H₂O 0.5 gram malt extract 5 grams
   NaCl 0.1 gram H₂O 1000 ml
   CaCl₂ 0.1 gram
mentation of the mycelium. Evidently, two pairs of genes are responsible for these pigment differences. If these pairs of genes are designated $A, A'$ and $B, B'$, then $AB$ is very black, $AB'$ is gray (Edgerton's plus B), $A'B$ is also gray though easily distinguished from $AB'$, and $A'B'$ is much whiter than the others although it produces some black pigment. Gene $A$ or a closely linked gene also controls the formation of perithecia, only conidia being produced by strains having its allele $A'$ (cf. Chilton, Lucas, and Edgerton 1945). This genetic analysis was further confirmed by crossing strain $AB'$ with strains $A'B'$ and $AB$. In crosses of this kind only the parental types were found among the offspring, thus indicating a single gene difference between these types.

Previous investigators (Edgerton 1914) have used the terms plus and minus to designate the mating types of Glomerella. These designations now seem inappropriate, however, in view of the fact that crosses occur not only between plus and minus types but also between two plus types and between two minus types. Such crosses, although very infertile, were clearly demonstrated by using different pigment mutants of the same mating type. The occasional perithecia which formed along the line of contact between colonies of the two different pigment mutants frequently contained ascospores which produced the double mutant or standard (wild) type colonies. Thus crossing between the two strains must have occurred. In general, the only requirement for crossing between related strains of Glomerella is that at least one of the partners to the cross must carry the factor ($A$) for perithecia formation. However, the degree of fertility of the cross depends upon other genetic factors—a subject which will be dealt with more extensively in a later paper.

**Induction of Mutations in Glomerella**

Since tyrosinase catalyzes at least two steps in the formation of the black pigment of Glomerella, it seemed reasonable that mutations affecting tyrosinase would be manifested as alterations in the quantity or quality of the pigment formed. This belief was reinforced by the knowledge that the formation of melanin pigments in other organisms is controlled by a large number of different genes.

Numerous mutant strains of Glomerella were obtained by irradiating the uninucleate conidia of strain $A'B$ with ultraviolet light. The conidia were obtained from cultures grown on 2 percent Difco corn meal agar or upon Westergaard's (1947) synthetic medium, since the complete medium is very unfavorable for spore formation. The irradiated spores were then plated out in serial dilution cultures, and after small colonies formed, mycelial transfers were made to agar slants of complete medium. Within a few days the mycelia become large enough so that changed morphological and pigmentation characters may be easily recognized. With optimal doses of ultraviolet irradiation the number of visible mutants was usually between 15 and 30 percent of all the colonies. Although this percentage is satisfactorily high, a technique was developed for increasing the percentage of mutants. This technique was based on the fact that some irradiated spores germinate later than others, possibly because of greater absorption of the ultraviolet irradiation. Among the colonies
which germinate late, the percentage of mutants is distinctly higher than among those which germinate early. Consequently, the irradiated spores were allowed to develop in a liquid nutrient solution for periods of time ranging up to 48 hours. The mycelia of the germinated spores were then filtered off through cotton and the remaining ungerminated spores plated out. By this treatment the percentage of mutant colonies among those picked was generally raised to more than 50 percent.

Preparation and Measurement of Tyrosinase in Glomerella Mutants

Nutritive and other environmental conditions under which the fungus is grown exert significant effects upon the tyrosinase activity of the mycelium. Therefore when comparisons were to be made between strains, the cultures were always prepared in the same way and grown under standard conditions. The cultures were initiated by small mycelial transfers to the center of an agar slant containing 8 ml of complete medium and then grown for 8 days at 25°C. The mycelium, which forms a dense felt-like mat on the surface of the agar, was easily harvested by peeling it away from the agar substratum and scraping off any adhering pieces of agar with a dull scalpel. Enzyme extractions were made by thoroughly macerating the frozen mycelium in a homogenizer with sand, using 10 ml of phosphate buffer (pH 7.0) for each gram (wet weight) of the mycelium. The enzyme solution was then filtered and diluted, if necessary, to bring the activity within a convenient range for measurement. The temperature of the enzyme solution, during the preparative procedure, was at all times kept several degrees below room temperature. Although this procedure is subject to some uncontrolled variations, the results were essentially the same in repetitions of the experiments. Moreover, the fluctuations in activity which were observed in different enzyme preparations from any one mutant were not of the same order of magnitude as the differences between the mutants reported here.

Tyrosinase activity in the various mutant strains of Glomerella was determined by two methods: by measurements of 1) the rate of pigment (halla-chrome) formation and 2) the rate of oxygen uptake during the enzymatic oxidation of tyrosine and dopa. The colorimetric determination of tyrosinase activity was made in a Klett-Summerson colorimeter using .02 M tyrosine and .02 M dopa as substrates. Air was bubbled continuously through the enzyme-substrate mixtures with high activity except for the few seconds required to make readings. Readings were taken every minute during the first ten minutes of the reaction and every five or ten minutes thereafter until the rate of the reaction leveled off (fig. 1). For preparations with low enzyme activity readings were taken only at five or ten minute intervals and continuous aeration was not provided. Comparisons of enzyme activity (table 1) were made by comparing the averages of the highest rates of activity per minute achieved in triplicate determinations with each preparation.

A less sensitive but more precise determination of tyrosinase activity was obtained by measuring the initial rate of oxygen uptake in a Warburg constant
Figure 1.—Curves showing pigment formation during the oxidation of dopa or tyrosine at room temperature and pH 7.0 as catalyzed by tyrosinase from three strains of Glomerella. Two ml of tyrosinase solution were used for each .2 ml of .02 M substrate. The numerical values for pigment are readings $\times 10^{-3}$ taken with a Klett-Summerson colorimeter equipped with a blue filter. The curves level off because of enzyme inactivation. The enzyme preparations from $A'B-308$ were diluted to one tenth the original strength to bring the activity within the scale of the graph.
volume respirometer. The Warburg flasks, of about 15 ml capacity, contained .2 ml of 10 percent KOH in the center well, .2 ml of .02 M substrate (tyrosine or dopa) in the side arm, and 2 ml of the enzyme solution in the main part of the flask. After temperature equilibration was reached at 30°C, the reaction was initiated by tipping the substrate from the side arm into the flask. Readings of O₂ uptake were taken at 2 minute or 5 minute intervals depending upon the length of time the oxygen uptake curve remained linear. When dopa was used as a substrate for the crude enzyme preparations, the rate of the reaction remained essentially constant for about 10 minutes in most cases (see fig. 2). When tyrosine was the substrate there was a lag period ranging up to 20 minutes with enzyme preparations of low activity. Enzyme preparations with very high activity however exhibited no lag period, probably because of the formation of small quantities of dopa in the crude enzyme preparation, since it is known that small amounts of dopa will eliminate the lag period when added to tyrosine-tyrosinase mixtures. Comparisons of the relative activity

<table>
<thead>
<tr>
<th>GENETIC CONSTITUTION OF STRAINS*</th>
<th>PIGMENT FORMATION PER MINUTE**</th>
<th>RATIO OF ACTIVITIES DOPA/TYROSINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WITH DOPA</td>
<td>WITH TYROSINE</td>
</tr>
<tr>
<td>A'B</td>
<td>720</td>
<td>60</td>
</tr>
<tr>
<td>AB</td>
<td>2,000</td>
<td>90</td>
</tr>
<tr>
<td>A'B'</td>
<td>3,400</td>
<td>175</td>
</tr>
<tr>
<td>A'B</td>
<td>675</td>
<td>25</td>
</tr>
<tr>
<td>AB-203</td>
<td>70</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AB'-203</td>
<td>202</td>
<td>6</td>
</tr>
<tr>
<td>A'B-203</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>A'B'-203</td>
<td>1</td>
<td>0</td>
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<tr>
<td>AB-507</td>
<td>73</td>
<td>2</td>
</tr>
<tr>
<td>AB'-507</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>A'B-507</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>A'B'-507</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>AB-200</td>
<td>32</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AB-206</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>A'B-209</td>
<td>61</td>
<td>5</td>
</tr>
<tr>
<td>A'B-212</td>
<td>24</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A'B-216</td>
<td>29</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A'B-218</td>
<td>85</td>
<td>7</td>
</tr>
<tr>
<td>A'B-228</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>A'B-300</td>
<td>4,400</td>
<td>230</td>
</tr>
<tr>
<td>A'B-305</td>
<td>5,720</td>
<td>270</td>
</tr>
<tr>
<td>A'B-308</td>
<td>12,500</td>
<td>700</td>
</tr>
</tbody>
</table>

* The allelic pairs A, A' and B, B' were present in the original strains. The numbered genes are mutant genes induced by ultraviolet irradiation.

** The numerical values for pigmentation are based on readings made with a Klett-Summerson colorimeter equipped with a blue filter. No ratio of activities is listed for preparations with very low activities since the calculated ratio in such instances is not accurate enough to be significant.
Figure 2.—Curves showing oxygen uptake during the oxidation of .2 ml of .02 M substrate (dopa or tyrosine) at 30°C and pH 7.0 as catalyzed by 2 ml tyrosinase solutions from three strains of Glomerella. The enzyme preparations were made in the same way except that preparations from A'B-308 were diluted to one tenth their original concentration to bring their activity within range of the other preparations.
of the enzyme preparations for the different mutants were made (table 2) by comparing the averages of the highest rates of activity achieved in triplicate determinations with each preparation. The initial rate of the reaction was the highest when dopa was used as the substrate, but with tyrosine as the substrate the highest rate of reaction occurred after the lag period. Highly active enzyme preparations were diluted until the maximum rate of oxygen uptake did not exceed 8μl/minute since at higher rates the oxygen supply became limiting under the conditions of the experiment.

**Table 2**

*Manometric comparison of tyrosinase activity in extracts from mutant strains of Glomerella.*

<table>
<thead>
<tr>
<th>Constitutions</th>
<th>Microliters of Oxygen uptake per 10 minutes*</th>
<th>Ratio of Activities based on A'B as 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Dopa</td>
<td>With Tyrosine</td>
</tr>
<tr>
<td></td>
<td>With Tyrosine</td>
<td>Dopa/Tyrosine</td>
</tr>
<tr>
<td>A'B</td>
<td>74</td>
<td>33</td>
</tr>
<tr>
<td>AB</td>
<td>212</td>
<td>40</td>
</tr>
<tr>
<td>AB'</td>
<td>271</td>
<td>65</td>
</tr>
<tr>
<td>A'B'</td>
<td>77</td>
<td>27</td>
</tr>
<tr>
<td>AB-203</td>
<td>12.0</td>
<td>0</td>
</tr>
<tr>
<td>AB'-203</td>
<td>45.2</td>
<td>6.1</td>
</tr>
<tr>
<td>A'B-203</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>AB'-203</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>AB-507</td>
<td>14.9</td>
<td>3.5</td>
</tr>
<tr>
<td>AB'-507</td>
<td>3.0</td>
<td>.9</td>
</tr>
<tr>
<td>A'B-507</td>
<td>1.9</td>
<td>.6</td>
</tr>
<tr>
<td>A'B'-507</td>
<td>2.2</td>
<td>.7</td>
</tr>
<tr>
<td>A'B-200</td>
<td>11.0</td>
<td>1.4</td>
</tr>
<tr>
<td>A'B-206</td>
<td>4.4</td>
<td>3.0</td>
</tr>
<tr>
<td>A'B-209</td>
<td>21.5</td>
<td>7.9</td>
</tr>
<tr>
<td>A'B-212</td>
<td>6.7</td>
<td>2.3</td>
</tr>
<tr>
<td>A'B-216</td>
<td>4.4</td>
<td>2.0</td>
</tr>
<tr>
<td>A'B-218</td>
<td>23.8</td>
<td>6.5</td>
</tr>
<tr>
<td>A'B-228</td>
<td>12.4</td>
<td>3.5</td>
</tr>
<tr>
<td>A'B-300</td>
<td>688</td>
<td>148</td>
</tr>
<tr>
<td>A'B-305</td>
<td>776</td>
<td>160</td>
</tr>
<tr>
<td>A'B-308</td>
<td>1040</td>
<td>511</td>
</tr>
</tbody>
</table>

* Rates listed are averages of the highest rates achieved in triplicate determinations with each preparation. Rates greater than 80 were obtained by multiplying the measured rate by the dilution factor.

Miller and Dawson (1941) have pointed out the inadequacies of the manometric technique in measuring tyrosinase activity and have developed a more accurate chronometric technique based on the time required for the oxidation of a given amount of ascorbic acid by the ortho-quinone formed during the enzyme reaction. During the present investigation the manometric technique was preferred because of the possibility that the crude enzyme preparations from the different mutants might contain significantly different
amounts of compounds which might be oxidized by the orthoquinone. Such reducing substances would probably not affect the initial oxygen uptake significantly although they could greatly change the results obtained by the chronometric method.

RESULTS

One large class of mutants, obtained by the ultraviolet irradiation of conidia, exhibited different kinds or degrees of melanin pigmentation ranging all the way from black through various shades of gray, brown, red and yellow to white. Only the mutants of the black-gray-white series are dealt with in this paper. In this series of mutants the amount of black pigment in the mycelium was usually a fairly good indicator of the tyrosinase activity found in a water extract of the macerated mycelium. Although the mutants listed in tables 1 and 2 exhibit striking differences in tyrosinase activity, it should be noted that a consideration of all the strains so far tested indicates an almost continuous gradation of activity from mutant $A'B-507$ which has the least activity to mutant $A'B-308$ which has the highest activity. The diminished tyrosinase activity of some of the mutants, particularly as measured by pigment formation, might conceivably be due to the production of inhibitors of tyrosinase rather than to a lowered production of tyrosinase itself. In fact several investigators have shown that pigment formation in crude tyrosinase-substrate mixtures may be influenced by a number of compounds independently of the enzyme concentration. Therefore, enzyme preparations from strains showing conspicuous differences of activity were mixed in an effort to discover any pigment-inhibiting effects in the preparation with low activity. Such mixtures exhibited no more than a dilution effect on the preparation with the high activity, regardless of whether pigment formation or oxygen uptake was used as a measure of enzyme activity. Thus it seems likely that the different amounts of tyrosinase activity in the various mutants represent real differences in enzyme concentration.

From tables 1 and 2 it may be seen that several enzyme preparations with very low activity on dopa have no recorded activity on tyrosine. These data alone might lead to the conclusion that several mutant genes in Glomerella result in the inability of the fungus to synthesize a true tyrosinase. However, colorimetric assays for enzyme activity on tyrosine have given indications of at least very small amounts of tyrosinase activity in each of these mutants.

Particularly noteworthy are strains $A'B''-203$, $A'B-200$, and $AB$, which, in comparison to other strains, exhibit markedly different ratios of activities on the two substrates tyrosine and dopa. Although the presence of many mutant genes can be correlated with great fluctuations in the tyrosinase activity of Glomerella, with few exceptions the ratio of activities remains fairly constant. The exceptions may represent changes in enzyme specificity or may be due to closely associated metabolic products which depress one activity more than the other. On the other hand, this change in the ratio of activities may indicate that tyrosinase is indeed two enzymes. However, the fact that the great majority of the mutants with changed amounts of tyrosinase activity exhibit
about the same ratio of activities as the standard (wild) type strongly supports
the view that tyrosinase is a single protein complex with two different catalytic
activities.

A few of the pigment mutants tested for enzyme activity were also bio-
chemical mutants which in addition exhibited changed morphological charac-
ters. Especially interesting are mutants 203 and 507. Although the standard
type does not require any amino acid or vitamin for growth these two mutants
require tryptophane. They are conspicuously different in tyrosinase activity,
morphology, fertility, and even in their tryptophane requirement. Neither will
grow on anthranilic acid nor on indole—precursors of tryptophane in Neuro-
spora. In fact, they both accumulate indole or some very similar compound as
indicated by the odor of the medium on which they have grown. In addition, a
chemical test for indole (Fearon 1944) gave a strong positive reaction with the
medium from cultures of 507 and a somewhat weaker reaction with the medium
from cultures of 203. Quantitative colorimetric measurements of the indole
accumulated in the medium of cultures of A'B-507 indicated a concentration
of indole of about .01 mg per ml of medium. Medium on which the wild type
has grown gives a negative result with the indole test. The fact that 507 ex-
hibits no growth whatever in the absence of tryptophane, while 203 will grow
to a very slight extent on a minimal medium is perhaps correlated with the
different degrees to which they accumulate indole. Moreover, strain 507 is
completely self-sterile, whereas strain 203 produces a limited number of
conidia. The mutant characteristics of 507 and 203 are transmitted as a unit
and are presumably due to a single gene. Strain 507, in particular, must be due
to a single gene change because it reverts to the standard type at a very high
rate. Thus a single gene change appears to be responsible for changes in tyro-
sinase activity, tryptophane requirement, pigmentation, morphology, and
fertility.

The accumulation of indole by mutants 203 and 507 suggested the possi-
bility that the observed changes in tyrosinase activity might be due to the
abnormal concentration of this intermediate metabolite. Therefore tubes of
complete medium were made up with .05 mg of indole per ml of medium—a
concentration of indole five times as great as that found in the mutant cultures.
When strain A'B was grown on this indole-enriched medium, the mycelium
developed less pigment and contained only about 50 percent of the normal
tyrosinase activity. By comparison, the presence of mutant gene 507 reduces
the tyrosinase activity almost to zero. It seems unlikely, therefore, that the
accumulation of indole by strains 203 or 507 can alone account for their
greatly diminished tyrosinase activity. Whatever effect indole may have it
appears to be exerted on the production of tyrosinase rather than on the
activity of the enzyme itself. This was shown by the addition of indole to a
standard tyrosinase-substrate mixture. The presence of the indole did not
significantly alter the rate of pigment formation as compared to an equivalent
tyrosinase-substrate mixture without indole. This influence of indole on tyro-
sinase production gives some additional support to the view that the various
changes in tyrosinase activity induced by mutation are due to the synthesis of different amounts of tyrosinase by the mutants rather than to the presence of inhibitors of tyrosinase.

Genetics of Glomerella Mutants

The single gene nature of the hereditary changes responsible for the enzyme differences shown in tables 1 and 2 was established by the usual technique of crossing the mutant to the standard (wild) type. In a few instances the eight spores from a single ascus were isolated by free hand. In all cases the spores proved to be either four mutant and four standard, or all eight were alike. In other words, the characteristics of each mutant were always inherited as a group as if they were due to a single gene. Where the eight spores were found to be alike it seemed reasonable to assume that no crossing had occurred, particularly in view of the fact that perithecial strains form ascospores homothallically. Of course, what appear to be unit factors may be closely linked groups of factors. Only data from very large numbers of offspring can render this possibility implausible. Since direct manual isolation of single spores is very time-consuming, a simpler procedure was used in testing large numbers of spores for evidence of crossing over. Perithecia which were formed by crossing between the standard type and the mutant were crushed on a glass slide. The ascospores were then pipetted off and plated out in dilution cultures. After the spores germinated, mycelial transfers were made to agar slants. In this way the genetic constitution of several hundred spores was easily studied. For each mutant strain listed in tables 1 and 2, at least a hundred colonies which had segregated from a cross with the standard type were examined. In the case of some mutants several hundred cultures were examined, but in all instances the mutant character was inherited as if due to a single gene. Some cultures were found to be double mutants by this technique but none of these double mutants is listed in table 1 or 2.

The non-allelic nature of the mutants was shown by forming double mutants or by segregating out the wild type from a cross between two mutants. In some fungi, such as Neurospora, the formation of heterocaryons may also be used as a test for allelism. However, the few attempts which were made to form heterocaryons in Glomerella were unsuccessful as one would expect if the hyphal cells are unicellular (Ullstrup 1938; Wheeler, Olive, Ernest, and Edgerton 1948). All the mutant genes discussed here have not yet been tested for allelism. However, genes A and B have been shown to be non-allelic with each other and with all the other genes in tables 1 and 2. Thus from these data alone at least three loci are involved in the control of tyrosinase activity. Moreover, four additional non-allelic genes (not included in the tables) have been shown to affect both tyrosinase activity and the color of the mycelium. Therefore, at the present time at least six different loci have been shown to affect tyrosinase activity, and it is presumed that most, if not all, of the remaining mutants also represent distinct loci. It seems reasonable to conclude on the basis of these data that tyrosinase activity in Glomerella is affected by many different genes.
which probably act by altering the normal balance of biochemical reactions in such a way as to reduce or increase the synthesis of tyrosinase.

The complexity of the genetic control of tyrosinase synthesis is illustrated by the different effects produced by various combinations of mutant genes. For example genes 203 and 507 have in common the property of preventing the synthesis of tryptophane and also of greatly diminishing the production of tyrosinase. However, these genes may be readily distinguished by the different effects produced in combination with the allelic pairs $A, A'$, and $B, B'$. The strain $AB'-203$ has nearly four times the tyrosinase activity of $AB-203$ (see table 2). By contrast, strain $AB'-507$ has only $1/5$ as much tyrosinase activity as $AB-507$. Thus gene $B$ is more effective than its allele $B'$ in promoting tyrosinase synthesis in the presence of mutant gene 507, whereas the allele $B'$ is more effective than gene $B$ in the presence of gene 203. In combination with $A'B$ or $A'B'$ both 507 and 203 diminish tyrosinase activity almost to the vanishing point. It is interesting to note that neither gene $A$ nor $B$ nor their alleles $A', B'$ exert any effect upon the tryptophane requirement induced by the presence of either gene 203 or 507.

**DISCUSSION**

The data obtained in this investigation indicate that the synthesis of the enzyme tyrosinase is under the control of a large number of different genes. The mechanism of this control, however, still remains obscure. Whatever the mechanism of control may be, it must be able to produce a finely graded series of quantitatively different effects in order to explain the gradations of tyrosinase activity in Glomerella mutants. Quantitative variations in the production of essential growth substances may also be observed among the biochemical mutants of Glomerella and Neurospora, although more attention has been given to those mutants which produce very little, if any, of some essential growth factor. In explanation of the biochemical mutants of Neurospora the hypothesis has been advanced (Beadle 1945) that the gene impresses upon the enzyme molecule that specific configuration which determines its catalytic capacities. This hypothesis explains very well a graded series of quantitative effects produced by a series of allelic genes, since allelic genes could impress configurations of different catalytic efficiencies upon the enzyme molecule. Similarly, different quantities of a single enzyme produced through the cooperative activity of many non-allelic genes can also be attributed to an altered efficiency in carrying on any one of the gene-controlled contributory reactions. Thus the data on tyrosinase activity in Glomerella mutants do not contradict the one gene-one specific enzyme hypothesis. Neither do the data contradict the hypothesis that the specificity of an enzyme is the logical culmination of its characteristic synthesis, in which case there would be no single “tyrosinase” gene acting like a template to impress a specific catalytic configuration upon an already formed protein molecule. Further studies with purified preparations of tyrosinase from the Glomerella mutants exhibiting different ratios of activities may provide critical data for evaluating these two
hypotheses. The admittedly inadequate data from the crude enzyme preparations do suggest the possibility, however, that genetically-induced alterations in the metabolic pattern of the cell may qualitatively change the nature of the enzyme formed. In this connection it is interesting to note the work of Caspaki and Richards (1948) on the proteins of \( a^+a^+ \) and \( aa \) Ephestia moths. The mutant Ephestia, which is unable to transform tryptophane into kynurenone, has a measurably higher tryptophane content than the wild type. The authors conclude from their measurements that part of the increased tryptophane content of \( aa \) Ephestia is stored in the proteins; thus, the normal protein make-up of the cells must be changed.

It is generally assumed that an additional growth factor requirement in a mutant strain of Glomerella or Neurospora is due to the absence (or very low activity) of an enzyme which catalyzes some step in the synthesis of the growth factor. This assumption implies that in the case of the tryptophane-requiring, essentially tyrosinaseless mutants \( A'B-203 \) and \( A'B-507 \) a single gene change has effectively prevented the synthesis or activity of two distinct enzymes (cf. Horowitz 1949, for discussion of number of functions per gene). Furthermore, this double effect does not appear to result from any simple inhibiting action of accumulated precursors of tryptophane on either the synthesis or activity of tyrosinase as was shown by the addition of indole and anthranilic acid to the medium of standard cultures. The absence of inhibiting precursors is further suggested by the fact that genetic changes can significantly increase tyrosinase synthesis without affecting the tryptophane requirement. Of course, such genetic changes might conceivably act by removing some inhibiting precursor of tryptophane synthesis.

It should be noted again that the amount of tyrosinase in the mycelium varies greatly with the age of the mycelium and with the conditions of nutrition, aeration, and temperature under which it is grown. Such great variability in tyrosinase content indicates that the enzyme is of no great importance to the life of the fungus. It is perhaps not surprising, therefore, that genetically-induced changes in the normal biochemical pattern of the cell should also result in great fluctuations in the synthesis of tyrosinase. In other investigations of biochemical genetics moreover, it is also quite possible that the synthesis of other enzymes is similarly dependent upon the metabolic pattern of the cell, and therefore, correlations of genetic constitution with enzyme activity are not conclusive demonstrations of any specific, direct relationship between the gene and the enzyme.

In many ways the polygenic control of tyrosinase activity seems very similar to the action of the numerous suppressor and enhancer genes found in Drosophila (Bridges and Brehme 1944) and in Neurospora (Houalah and Mitchell 1947; Emerson 1948), since enhancer and suppressor genes are likewise non-allelic genes affecting the same character. However, in Drosophila and Neurospora the various suppressor and enhancer genes for any one character have not yet been shown to act through a single enzyme system although such a mechanism of action seems reasonable. In Glomerella, most of the genes
affecting pigmentation do act through the single mechanism of changing the concentration of tyrosinase, although it is highly improbable that the primary action of all of these genes is directly upon the enzyme.

**SUMMARY**

1. The object of this investigation was to study the genetic control of enzyme synthesis. The enzyme tyrosinase, by reference to the work of other investigators, was shown to have characteristics which make it particularly favorable for a study of the relationship between genes and enzymes. Different kinds of tyrosinase are found in different organisms, and the activity of tyrosinase (or dopa oxidase) appears to be modifiable by many different genes.

2. The ascomycete, *Glomerella cingulata*, was found to be a rich source of tyrosinase. Mutant strains of Glomerella were obtained by ultraviolet irradiation of the conidia. Standard genetic procedures were used in demonstrating the non-allelic, single gene nature of the mutant strains used in this investigation. Many of these mutant strains exhibited different degrees of melanin pigmentation and contained different amounts of tyrosinase, thus demonstrating the polygenic control of tyrosinase activity.

3. Two distinct Glomerella mutants with almost no tyrosinase activity were also unable to synthesize tryptophane, presumably because of the absence of an enzyme essential for tryptophane synthesis. In these mutants a single gene change apparently controls the activity of two distinct enzymes.

4. The many genes influencing melanin pigmentation in Glomerella resemble, in their mode of action, the suppressor and enhancer genes described in other organisms. Although the genetically-induced changes in pigmentation in Glomerella are usually correlated with changes in tyrosinase activity, it is highly improbable that the primary effect of these genes is directly upon tyrosinase itself.

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**LITERATURE CITED**


TYROSINASE IN GLOMERELLA


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