IMMUNOGENETIC STUDIES OF TYROSINASE SPECIFICITY

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MEASUREMENTS of tyrosinase activity in mycelial extracts of the fungus Glomerella have shown wide variations in activity which are correlated with genotype, with variations in the environmental conditions under which the organism is grown (MARKERT 1950), or with the age of the mycelium (SUSSMAN and MARKERT 1953). Any one or a combination of two or more of the following hypotheses might be advanced to explain these differences in tyrosinase activity: 1) The various mycelia may synthesize different quantities of tyrosinase. 2) The enzymatic efficiency of the tyrosinase molecule might be altered, particularly by the genetic changes. 3) Enzymatic activity might be altered by changes in associated substances (e.g., inhibitors) regulating the effective activity of the tyrosinase molecule.

Serological examination of protein extracts of Glomerella was undertaken in an effort to test these hypotheses and in particular to examine the possibility that genetically induced changes in tyrosinase activity might have altered the structure of tyrosinase in a serologically detectable fashion. Serological properties of tyrosinase from standard type Glomerella extracts have previously been described (OWEN and MARKERT 1954). In these previous studies Glomerella tyrosinase proved to be an effective antigen in rabbits. Antibodies against tyrosinase rapidly inactivated the enzyme and eventually precipitated the enzyme from solution. The enzymatically active surface of the tyrosinase molecule is strongly implicated in these antibody-tyrosinase reactions, because of the rapid inactivation of the enzyme in the presence of antibody and because of the effects on inactivation produced by varying the relative concentrations of enzyme, substrate, and antibody. The present report gives results of an extension of these serological tests to extracts derived from mycelia of different genotypes grown under various environmental conditions. An examination was also made of the response of tyrosinase from other species (Neurospora, Solanum, Psalliota and Tenebrio) to antisera against Glomerella tyrosinase.

MATERIALS AND METHODS

Glomerella cultures. The standard type Glomerella culture is designated A^1B^1 and differs as indicated by changes at two loci from wild type Glomerella (A^+B^+) (see WHEELER and MCGAHEN 1952 for terminology). Mutations were induced in standard type conidia by ultraviolet irradiation, X-irradiation,

[1] This work was largely performed while the first author was a Merck Fellow of the National Research Council.

or neutron bombardment (MARKERT 1952). Mutants exhibiting changes in melanin formation were then screened for possible variations in the tyrosinase content of their mycelia. Several mutants with tyrosinase activities varying from almost no activity to greatly enhanced activity were then selected for the preparation of extracts to be examined serologically.

Preparation of extracts. The procedure generally used in preparing extracts was as follows: Small mycelial transplants from each Glomerella type were inoculated to the center of about 50 Petri dishes (100 x 15 mm) each containing 30 ml of complete medium (see MARKERT 1950 for media). The cultures were then grown at 25°C for 10 days by which time the mycelia had generally spread over the entire surface of the medium. The harvested mycelia were freed from the agar medium and ground in a phosphate buffer (pH 7.0) in a colloid mill for 20 to 30 min. The resulting brei was centrifuged at 13,000 g for 30 min. and the supernatant then dialyzed against buffer for 24 hrs. All extractive procedures were carried out in the cold. The dialyzed supernatant was frozen and lyophilized, and the resulting dry powder when redissolved was used as the extract for all enzyme and serological tests.

In addition to the surface cultures, submerged aerated liquid cultures of several of the Glomerella types were also prepared. Extracts were made from these liquid cultures according to the procedures employed for the surface cultures.

Neurospora (mutant no. 84605a, cf. HOROWITZ and SHEN 1952) extracts were prepared in the same way except that the mycelia were obtained exclusively from liquid cultures. In these liquid cultures the medium was distributed in 20-ml aliquots to 125-ml flasks and was made up of minimal medium supplemented with 10 γ tyrosine and 2 mg cysteine per 20 ml of medium. Cultures were grown for 10 days at 25°C with no shaking or aeration. The Neurospora mycelia floated on the medium in contrast to the Glomerella which grew submerged in liquid culture.

Extracts of Solanum, Psalliota and Tenebrio were made by grinding frozen tissue with a mortar and pestle, washing with acetone, and then suspending the acetone-dried powders in buffer. The suspensions were then centrifuged at 13,000 g for 30 min. and the remainder of the extractive procedure carried out as outlined for the Glomerella cultures.

Enzyme measurement. Tyrosinase activity was measured in a Klett-Summerson colorimeter equipped with a No. 42 (blue) filter; the rate of pigment (dopachrome) formation proved to be a satisfactory index of the concentration of tyrosinase. The reaction mixture generally contained 0.5 ml of .02 M L dopa, 1 ml of serum, 1 ml of enzyme solution, and 3 ml of buffer (pH 7.0) to make a total volume of 5.5 ml. Readings were taken at 10-second intervals beginning 10 seconds after mixing and the tyrosinase activity was calculated on the basis of the maximum reaction achieved in 40 seconds.

Throughout this report tyrosinase activity measurements are recorded in terms relative to the activity of the standard type extract (S) which is given an index value of 100. In absolute terms, one milligram of extract (S) reacting

in a volume of 5.5 ml containing an excess of dopa produced a change of one unit on the Klett colorimeter scale in 5.7 seconds. Accordingly, one milligram of extract S-3 with an index value of 1000, would produce a change of 10 colorimeter units in 5.7 seconds or of 1 unit in 0.57 seconds.

Preparation of antisera. The extracts, at a concentration of 10 mg of the dried powder per ml of physiological saline or distilled water, were injected intravenously into rabbits, 0.5 ml per injection. After 9 injections (Monday, Wednesday and Friday of three successive weeks) the rabbits were given a 10-day rest, then bled by cardiac puncture. Usually only one bleeding, of 50 ml whole blood, was taken at this time. After a further rest of at least two weeks the rabbits were reimmunized with an intraperitoneal injection of 2 ml of the extract at 10 mg/ml, then two intravenous injections of 0.5 ml on alternate days thereafter. Bleedings on the tenth and eleventh days after the last injection provided the " Number 2," or reimmunization antisera. Later reimmunizations to provide " Number 3 " and " Number 4 " antisera were similarly performed.

RESULTS

The details of preparative procedures are important if quantitative comparisons are to be made of the enzyme activities of different preparations. It has previously been noted that the tyrosinase activity in a Glomerella mycelium is dependent upon such environmental conditions as the nutrient composition of the medium, temperature, and the nature of growth—whether surface or submerged (MARKERT 1950). Temperature and nutrition also affect tyrosinase

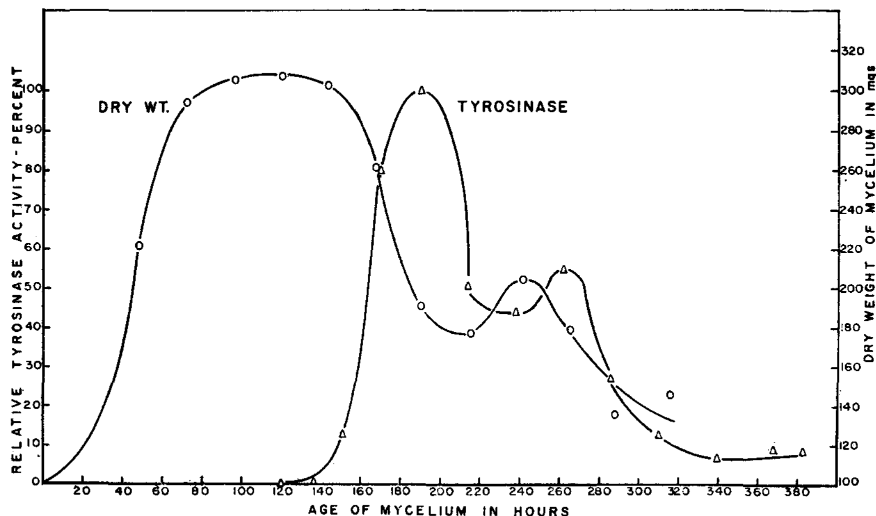

FIGURE 1.—Curves showing the formation of tyrosinase during growth of the mycelium. Growth measured as dry weight of the mycelium on a single Petri dish (15 × 100 mm) containing 30 ml of complete medium. Growth initiated by flooding surface medium with a dense spore suspension. Extracted tyrosinase activity measured colorimetrically. Points on tyrosinase curve are averages of three experiments.

activity in Neurospora (HOROWITZ and SHEN 1952). The age or stage of growth of the mycelium is of crucial importance in Glomerella. SUSSMAN and MARKERT (1953) found that tyrosinase synthesis starts at the time growth ceases, increases rapidly to a maximum and then declines to a much lower, relatively stable, level (fig. 1). The growth conditions for Glomerella cultures used in this investigation differ somewhat from those employed by SUSSMAN and MARKERT; nevertheless the mycelia were generally harvested near the peak of their tyrosinase activity in accord with the cultural conditions under which they were grown. However, growth conditions and the time of harvesting were varied for several standard type cultures with the result that extracts of different enzyme activities were obtained. These cultures of identical genotype (A^1B^1) are designated in table 1 as A^1B^1(S), A^1B^1(S-1), A^1B^1(S-2), A^1B^1(S-3). The A^1B^1(S) culture was grown under the same conditions as the mutant types on solid medium and is therefore used as a standard for comparing tyrosinase activities.

The type of growth, whether surface or submerged, appears to exert the most striking effect on the tyrosinase activity of the mycelium. Submerged cultures fail to develop large amounts of activity, although mutants with very high activity in surface culture (e.g., A^1B^1 318) do develop a small amount of tyrosinase activity in submerged culture (table 1).

Recently HOROWITZ and FLING (1953) in an investigation of Neurospora tyrosinase found that the effects of temperature on tyrosinase activity depended

TABLE 1

Relative tyrosinase activity of Glomerella mutants.

Mutant designation	Growth medium*	Tyrosinase activity**	Antisera	Antibodies induced
$A^1B^1$308	solid	6570	DH	+
$A^1B^1$318	solid	3100	DL	+
$A^1B^1$318	liquid	10	DI	0
$A^1B^1$65	solid	1100	DD	+
A^1B^1(S-3)	solid	1000	....	–
$A^1B^1$537	solid	985	DF	+
A^1B^1(S-2)	solid	970	BT	+
A^1B^1(S-1)	solid	642	CR	+
A^1B^1(S) standard	solid	100	DJ	0
A^1B^1	liquid	< 1	CT	0
$A^1B^1$218	solid	48	DG	0
$A^1B^1$212	solid	8	DK	0
$A^1B^1$216	solid	5	DM	0
$A^1B^1$216	liquid	< 1	CQ	0
$A^1B^1$503	solid	4	DE	0
84605a (Neurospora)	liquid	3	DN	0
$A^1B^1$507	solid	< 1	DA	0
$A^1B^1$507	liquid	< 1	CP	0

*Solid medium produced surface growth and liquid medium submerged growth except for Neurospora which grew partly submerged and partly on the surface.

**Relative activity calculated with the standard type (A^1B^1) as 100.

upon the strain used, some strains producing tyrosinase when grown at 35°C and others not. All the Glomerella mutants so far tested respond alike to temperature, that is, high temperatures drastically inhibit the development of tyrosinase activity under conditions otherwise conducive to maximum activity. Consequently all the cultures used in this investigation were grown at 25°C, a temperature conducive to tyrosinase development.

The effects of nutrition on tryrosinase development are complex and not consistent for all genotypes. That is, a nutritionally minimal medium supports the formation of high tyrosinase activity in mycelia of some genotypes while causing low activity in others. For most genotypes a complete medium was most conducive to the development of the maximum tyrosinase activity and this type of medium was therefore used in the agar Petri-dish cultures. A minimal medium was used for the liquid cultures in which a low tyrosinase activity was desired.

TABLE 2

*Tyrosinase activity of extract S-3 in presence of extracts from mutants with low activity.**

	Extract designation					
	A^1B^1503	A^1B^1218	A^1B^1318**	A^1B^1212	A^1B^1216	A^1B^1**
Δ colorimeter reading at 2 min.	89	138	104	78	81	88
Activity of added extract	5	46	17	5	9	1
Activity due to extract S-3	84	92	87	73	72	87

*Reaction medium composed of 1 ml (at 0.8 mg/ml) of extract S-3 from standard type, 1 ml (at 20 mg/ml) of low activity extract, 1 ml M/50 DL dopa, 2 ml buffer. Pigment formation measured in a Klett colorimeter as discussed in *Methods*.

**Cultures grown in liquid medium.

In comparing the enzymatic activity of the extracts from the various genetic types shown in table 1, the standard type has been assigned arbitrarily an activity of 100. It thus becomes apparent that under identical environmental conditions, single gene changes may reduce tyrosinase activity to essentially zero (mutant A^1B^1 507) or increase the activity over 65 times (mutant A^1B^1 308).

The wide variations in tyrosinase activity in these extracts is apparently due to large molecules since dialysis of the extracts does not change their relative activity. Variations in tyrosinase activity in extracts must be attributed either to variations in the number or kinds of tyrosinase molecules or to the presence of other large molecules which act as inhibitors. The possibility that low levels of tyrosinase activity in some extracts are due to the presence of inhibitors may be tested by mixing an active extract with the relatively inactive extracts. Accordingly, 1 ml of extract S-3 from standard type (at 0.8 mg/ml) was mixed with 1 ml (at 20 mg/ml) of a variety of relatively inactive extracts.

After one hour at 37°C the mixtures were placed overnight at about 4°C, then centrifuged and the supernatants added to 1 ml M/50 DL dopa + 2 ml of buffer. The pigment formation at 2 minutes for each mixture is given in table 2. These readings are corrected by subtraction of the small activity present in the extracts with low activity. Although some variation in effective activity of the S-3 extracts is thus apparent in the presence of a variety of extracts with low activity, this variation shows no correlation with the level of activity found in the low activity extract. It may reasonably be concluded, therefore, that large molecular inhibitors are not a significant factor in reducing the activity formed in these mutant extracts. This conclusion is further supported by serological data presented later in this report.

In a previous paper OWEN and MARKERT (1954) demonstrated that antiserum made against standard type Glomerella extracts will inactivate and precipitate the tyrosinase in such extracts. Accordingly, antisera were produced against extracts from various mutants exhibiting a wide range of tyrosinase activities and also from the standard type grown on surface and in submerged

TABLE 3

Antibody activity of antisera.

Antiserum	Inducing extract	Tyrosinase activity of extract	Antibody activity* of antiserum
CR_4	A^1B^1(S-1)	642	1000
DD_2	$A^1B^1$65	1100	1300
DF_2	$A^1B^1$537	985	2400
DH_2	$A^1B^1$308	6570	6000

*Measured in terms of the number of tyrosinase units inactivated by 1 ml of the antiserum under standard conditions.

cultures. These antisera were then tested for enzyme inactivating and precipitating ability. All of the antisera produced an extensive precipitation when mixed with any of the extracts. However, the presence of specific antibodies against tyrosinase in an antiserum appears to depend upon the level of tyrosinase activity in the inducing extract. It may be seen in table 1 that all extracts with tyrosinase activities of less than 642 failed to induce antibodies to tyrosinase even though some tyrosinase was contained in the injected extract. Furthermore, the antibody activity of an antiserum appears to depend somewhat upon the level of enzymatic activity in the extract injected. Thus the extract with the highest enzymatic activity—A^1B^1 308—induced the formation of an antiserum with the highest titer of antibody against tyrosinase (see the examples listed in table 3).

As previously shown, antisera against standard extracts rapidly inactivate and slowly precipitate tyrosinase. Figure 2 illustrates the inactivating effect of antibody to homologous tyrosinase. Within a few seconds after the addition of extract to a mixture of antiserum and substrate, the enzymatic activity is reduced between 50 and 90%. The extent of reduction depends upon the par-

ticular antiserum and extract used and upon the relative concentrations of enzyme, substrate, and antiserum. Previous incubation of the enzyme with an active antiserum before substrate is added effectively reduces initial tyrosinase activity; in some instances the tyrosinase activity is completely destroyed, although generally a small fraction of the activity persists. Cross-reactions between heterologous antisera and an active extract from standard type (fig. 3) show that the activity of an antiserum is not specific for its homologous extract; it reacts equally well with the tyrosinase in other extracts. Antisera to mutants with no tyrosinase activity (A^1B^1 216 in fig. 3) fail to inactivate tyrosinase; such antisera precipitate materials from the crude enzyme extracts but do not affect the tyrosinase. The failure of such extracts to induce the

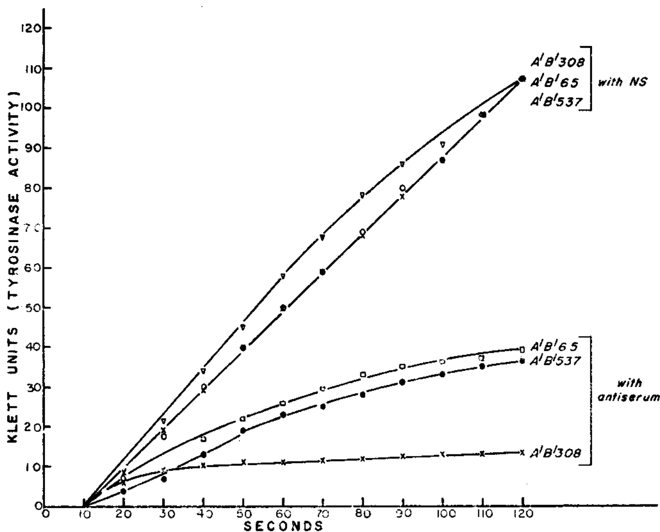

FIGURE 2.—Curve showing the reaction of tyrosinase from three mutants in normal serum and in their antisera. Reaction mixture contained 1 ml serum, 3 ml of buffer, .5 ml dopa, and 1 ml enzyme solution (diluted to make the three mutants approximately equal). Readings were started 10 seconds after mixing.

formation of antibodies against tyrosinase is further evidence that they do not contain tyrosinase which is simply masked by the presence of large molecular inhibitors. Such inhibited tyrosinase molecules if they existed might be expected to function as antigens, at least in the production of precipitating antibodies, even though their enzymatic activity was suppressed. We should note, however, that an enzymatically inactive material related to β-galactosidase in *E. coli* functions as an antigen only in combining with antibodies, not in inducing them (COHN and TORRIANI 1952, 1953).

These quantitative reactions do not critically exclude the possibility that antibodies from different antisera may have some specific differences corresponding to specific differences among the tyrosinase molecules from different mutant extracts. Further tests for specificity were made by means of absorp-

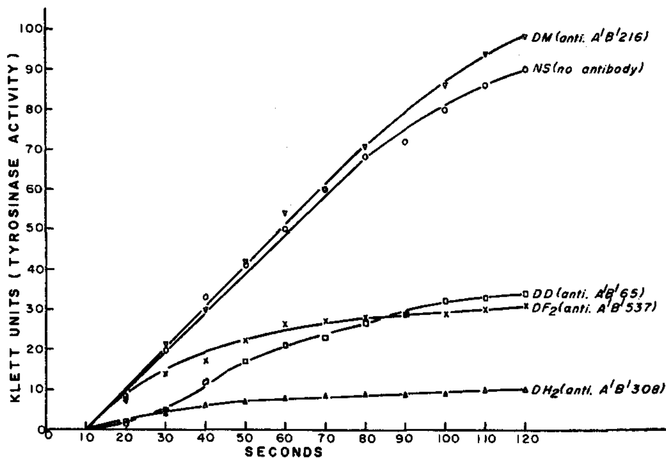

FIGURE 3.—Curves showing the reaction of tyrosinase from standard type in normal serum and in various antisera. Antiserum DM was against a mutant with almost no tyrosinase activity.

tion techniques. Figure 4 shows the results of absorbing active antisera with active extracts and with enzymatically inactive extracts. In table 4 are shown the results of absorbing several different antisera with a variety of enzymatically active extracts. Because the extracts differed in enzymatic activity, the amount of the extract used in the absorption tests was adjusted so that equal total activities for each absorbing extract were employed. It is apparent from table 4 that the heterologous and homologous extracts are equally effective in absorbing antibodies to tyrosinase. It appears, therefore, that the antibodies to tyrosinase in the various antisera do not distinguish among the tyrosinases of different type and mutant cultures. The absorbed antisera are like normal control sera with regard to their effects on tyrosinase.

TABLE 4

Cross-reactions of antisera and tyrosinase from different mutants.

Antiserum	Inducing extract	Tyrosinase activity of injected extract	Absorbing* extract	Remaining antibody activity to tyrosinase
CR_3	S-1	642	S-3	None
			A^1B^165	None
			A^1B^1308	None
			A^1B^1318	None
DD	A^1B^165	1100	S-3	None
			A^1B^165	None
DH	A^1B^1308	6570	A^1B^1308	None
DL	A^1B^1318	3100	S-3	None
			A^1B^1318	None

* At calculated equivalence as discussed in text.

Absorption of active antisera by enzymatically inactive extracts resulted in extensive precipitation but the antibodies against tyrosinase were not precipitated and the supernatant retained its capacity to inactivate tyrosinase. Similarly the antisera made against inactive extracts precipitated many constituents from an enzymatically active extract, but the tyrosinase in the extract was neither inactivated nor precipitated. The enzymatic activity remained in the supernatant and was subject to later inactivation and precipitation on the addition of an antiserum containing antibodies to tyrosinase.

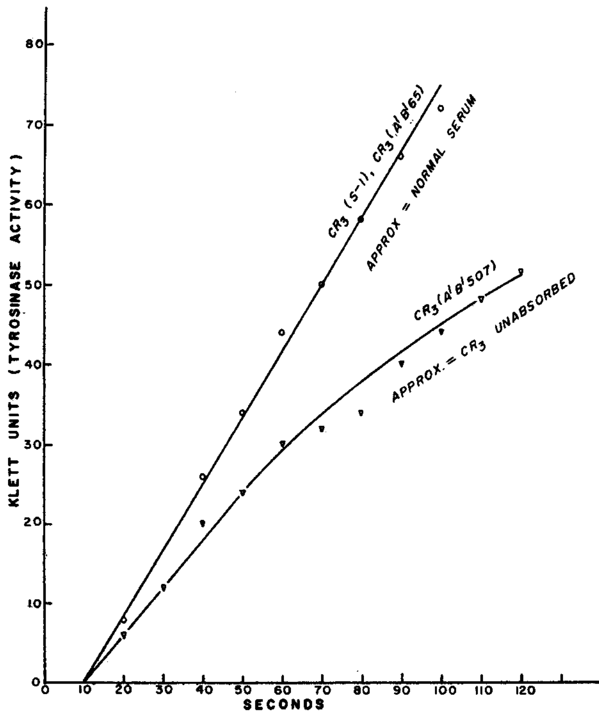

FIGURE 4.—Tyrosinase activity in antiserum CR_3 unabsorbed, and after absorption with extracts from A^1B^1(S-1), $A^1B^1$65, and $A^1B^1$507. Absorption with an active extract renders the antiserum equal to a normal serum, but absorption with an inactive extract does not change the antibody titer to tyrosinase.

The cross-precipitation in absorption experiments demonstrated that the extracts of Glomerella contained antigens common to or similar in all of the extracts. However, the presence of an antigen which will induce antibodies against tyrosinase appears to correspond exactly with the presence of at least a minimum amount of the enzyme activity itself. Similarly, the presence of an antigen which will precipitate the antibodies evoked by the active enzyme depends upon the presence of the enzyme itself. This study therefore provides no evidence of enzymatically inactive materials serologically related to tyrosinase in any of the diverse preparations tested.

This conclusion is further substantiated by the absence of any comple-

mentary inactivation of tyrosinase in mixed antisera. The tyrosinase extracts used in testing for complementary inactivation were from mutants A^1B^1 65, A^1B^1 537 and A^1B^1 308, and from the standard type (extract S-3). Antisera employed were the homologous antisera CR_4 (anti-standard type), DD_2 (anti 65), DF_2 (anti 537), and DH_2 (anti 308). The tyrosinase activities of the extracts and the antibody titers of the antisera were initially quantitatively different. However, by the use of appropriate dilutions it was possible to balance the antibody content of an antiserum against the enzymatic activity of its homologous extract. Then any of the antisera (or their mixtures) at proper dilution could be substituted for one another in inactivating the various enzyme extracts or their mixtures. The different antisera therefore appear to act at the same points in interfering with enzyme activity, and not to summate by acting at independent points. This further implies that there are no serologically recognizable differences among the tyrosinase molecules formed by the various mutant types of Glomerella.

The equivalence points of the various tyrosinase-containing extracts may be determined (OWEN and MARKERT 1954), and a comparison of these equivalence points should serve as a measure of the relative efficiencies of the tyrosinase molecule in the various extracts as an enzyme and as an antigen (table 5). The antigen excess edge of the equivalence zone, at which significant enzymatic activity begins to appear in the supernatants of precipitin tests, was also used to compare the precipitating antibody content of different antisera with reference to several extracts. It was evident that in any particular

TABLE 5

Relative enzymatic and serological activity of tyrosinase in equivalence quantities[1] of extracts as determined for various antisera.

Antiserum designation	Extract designation	Mgs of extract at equivalence	Total tyrosinase activity	Relative tyrosinase activity[2]	Relative serological activity[3]
CR_3	S-3	2.6	2600	1.0	1.0
	$A^1B^1$308	0.4	2600	6.6	1.0
	$A^1B^1$318	0.8	2500	3.1	1.0
DH_2	S-3	6.0	6000	1.0	1.0
	$A^1B^1$308	0.8	5300	6.6	1.1
DL	S-3	1.6	1600	1.0	1.0
	$A^1B^1$318	0.5	1600	3.1	1.0
DD_2	S-3	1.3	1300	1.0	1.0
	$A^1B^1$65	1.3	1400	1.1	0.9
DF_2	S-3	2.4	2400	1.0	1.0
	$A^1B^1$537	2.6	2600	1.0	0.9

[1]The equivalence quantity is the amount of extract required to neutralize the antibodies in 1 ml of antiserum.

[2]Approximate tyrosinase activity per mg of extract compared to extract S-3 which is taken as 1.0.

[3]Antibody-neutralizing activity of the tyrosinase present at equivalence in each extract compared to the antibody-neutralizing activity of the tyrosinase in extract S-3 which is taken as 1.0.

antiserum, the edge of antigen excess appeared at nearly the same number of units of enzymatic activity regardless of which extract was used as a source of the enzyme. The determination of these equivalence points is subject to some error and the differences indicated by the data in table 5 are probably not significant. The absence of striking differences in these equivalence points of different preparations supports the conclusion that the tyrosinase molecules in the different mutant extracts are antigenically identical. The similarity in equivalence points also demonstrates that the activity of tyrosinase molecules is essentially independent of other constitutents of the extract. Variations in enzyme activity in different mutant extracts can therefore be considered an

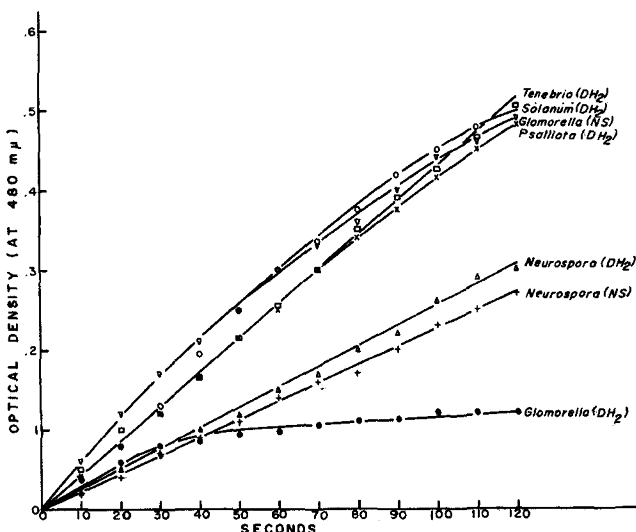

FIGURE 5.—Curves showing the tyrosinase activity of extracts from five species in normal serum and in antiserum to Glomerella tyrosinase. Reactions in normal serum for Psalliota, Solanum and Tenebrio are not plotted since they were almost exactly the same as in antiserum DH_2. Only the Glomerella tyrosinase is drastically inhibited by the antiserum. Readings for the Neurospora tyrosinase were taken at 10-minute intervals and the optical density readings were .1 of the values shown on the graph.

accurate measure of the relative numbers of tyrosinase molecules present in the extracts.

The very rapid inactivation of Glomerella tyrosinase by antibody and the suggested kinetic competition between antibody and substrate dopa (OWEN and MARKERT 1954) strongly imply that the enzymatically active surface is directly involved in the antigen-antibody union. If antigenic and enzymatic surface were identical, then tyrosinase from other species might react with antibodies against Glomerella tyrosinase. Presumably enzyme surfaces are complementary to the substrate surface; since the active enzymatic surface combines with the same substrates in the case of tyrosinases from different species, it might perhaps be expected that these active surfaces would also react with the same antibodies. Accordingly, tyrosinase was extracted from

the fungus Neurospora, the mushroom Psalliota, the potato Solanum, and the mealworm Tenebrio. In addition to the crude extract from Psalliota a commercial, purified preparation with 4,200 catecholase units of tyrosinase activity per ml was used.

The tyrosinase activities of these various preparations were adjusted by dilution with buffer until they were nearly identical in activity. Then a reaction mixture was prepared consisting of 1 ml of 0.02 M DL dopa, 1 ml of dialyzed serum (normal serum, or antisera DH_2 or CR_4) and 1 ml of the enzyme solution. Measurements of dopachrome formation were taken at 10-second intervals in a spectrophotometer (Beckman model B) set at 480 mμ. The Neurospora tyrosinase activity was so low that measurements were taken at 10-minute intervals. The results are illustrated in figure 5. The reactions in normal serum are not graphed for the Psalliota, Solanum or Tenebrio tyrosinases since these reactions were almost identical to the reactions in antiserum. The antisera DH_2 and CR_4 were alike in not affecting tyrosinase activity from non-Glomerella species. The purified commercial Psalliota tyrosinase and the crude extract of Psalliota were equally indifferent to the antibodies against Glomerella tyrosinase, and it is apparent that there is no cross reaction between the antibodies to Glomerella tyrosinase and the tyrosinases from any of the other four species. That antiserum DH_2 is very effective against Glomerella tyrosinase is, however, clearly shown in figure 5.

The availability of antisera containing antibodies that combined with tyrosinase as well as with other antigens found in extracts of Glomerella mycelia prompted an effort to repeat the work of EMERSON (1944) on the induction of mutations by antibodies. Emerson used the fungus Neurospora in his investigations. The conidia of Glomerella, being haploid and uninucleate, provide especially favorable material for measuring the mutagenicity of various agents since the treated spores may be plated out and the mutants evaluated directly without the necessity of first passing through a sexual cross. Accordingly, the conidia of Glomerella were treated with antisera to Glomerella mycelial extracts by the procedures previously reported to induce mutations.

The hypothesis that antibodies may act as mutagens is an attractive one because of the relationship between certain antigens (e.g., blood group antigens) and genes. If a structural relationship exists between the gene and its product—an enzyme or antigen—then the presence of a structurally related antibody at the time of gene replication might conceivably interfere with normal replication in such a way as to produce a gene with an altered structure—that is, a mutation. EMERSON tested this hypothesis by treating *Neurospora* conidia with antisera against culture filtrates, mycelial extracts, or fractions of such extracts. Mutants appeared among the isolates following each type of treatment while no mutants were found among controls. Combining all the data from various types of antiserum treatment EMERSON found 25 mutants out of 695 treated isolates and no mutants out of 276 control isolates.

The present investigations with Glomerella conidia gave quite different

TABLE 6

Treatment of Glomerella conidia with antiserum containing antibodies to tyrosinase.

Treatment	Spore concentration × 10^6/ml	Total isolates	Mutants
67% antiserum CR 24 hrs at 2 °C.	1.2	139	0
67% antiserum CR 24 hrs at 25 °C.	1.2	137	0
67% normal serum 24 hrs at 2 °C.	1.2	94	1
67% normal serum 24 hrs at 25 °C.	1.2	95	0
Untreated controls	1.2	80	0

results. The data are summarized in table 6. It should be noted that neither the normal serum nor the antiserum had any adverse affect on the viability of the conidia. Isolated colonies were evaluated for mutant characteristics by visual inspection only, but this method is sufficient for detecting both morphological and nutritionally-deficient mutants since all nutritional mutants of Glomerella are recognizably different from the standard type even on complete medium. Furthermore, any tyrosinase mutants would have been conspicuous by their altered pigmentation. The results of this experiment, therefore, lend no support to the hypothesis that antibodies are mutagenic. The single mutant found had not been treated with antibodies.

DISCUSSION

These serological studies of tyrosinase are informative with respect to five different but related problems.

(1) Genetic and environmental control of enzyme synthesis.

It has been previously shown (MARKERT 1950) and is again demonstrated here that single gene changes at many different loci alter the tyrosinase activity in extracts from the mycelia of *Glomerella*. At the inception of this investigation three plausible mechanisms were suggested to explain the changed enzymatic activity: (a) gene changes affected the biochemical environment of the tyrosinase molecules in cells and in extracts so that the measurable tyrosinase activity was changed, or (b) gene changes resulted in a qualitative alteration of the enzymatic efficiency of the tyrosinase molecule—that is, different kinds of tyrosinase were produced by different mutants, or (c) gene changes modified the quantity of enzyme synthesized in the Glomerella mycelium. Of these possible explanations, the present study supports the third for the following reasons.

First, the experiments with mixed extracts indicate that there is nothing in the inactive extracts that inhibits active extracts added to them, and nothing in the active extracts that enhances enzymatic activity in inactive extracts

added to them. The differences in tyrosinase activity of these extracts appears to depend on their tyrosinase contents and not on the environments in which tyrosinase functions. Furthermore, the serological studies offer no evidence for inhibitors with antigenic properties comparable to those suggested by DULIÈRE and ADANT (1934) in their study of mealworm tyrosinase.

Second, the tyrosinases of the different extracts appear to be identical as antigens, both in the induction of antibodies and in reactions with antibodies. If the precipitin equivalence tests may be considered to reflect the number of enzyme molecules at equivalence with a given antiserum, then the uniformity of equivalence activity values for various extracts tested against a given antiserum indicates that this "equivalence number of molecules" has virtually identical activity in all of the extracts tested. The differences in activity per mg of extract, therefore, appear to depend on differences in the amount, not in the quality or activity of tyrosinase. The great reduction of tyrosinase activity found in extracts of Glomerella mycelia grown in submerged culture as compared to surface culture is also apparently due to a reduction in the amount of tyrosinase synthesized by the mycelium. The serological characteristics of extracts with very little enzyme activity are the same regardless of whether the reduced enzyme activity is due to genetic or environmental changes. So far, the effects of environment upon the development of enzyme activity have been studied serologically only in the case of submerged and surface cultures. Other environmental effects—temperature, nutrition, etc.—may of course change enzymatic activity by some mechanism other than simple change in the number of enzyme molecules synthesized. It should be noted that HOROWITZ and SHEN (1952) found that Neurospora, when grown on a sulfur-sufficient medium, synthesizes a dialyzable inhibitor of tyrosinase.

(2) Antigenic uniqueness of tyrosinase.

The antigenic properties of tyrosinase appear to be unique. No other antigen present in enzymatically inactive extracts of Glomerella either precipitates antibodies against tyrosinase, or induces antibodies that will inactivate or precipitate tyrosinase. Such inactive extracts do not appear to possess a "precursor" or other protein having a serologically detectable relationship to the finished enzyme molecule. In this regard the Glomerella tyrosinase system appears to be different from the β-galactosidase of *E. coli* studied by COHN and TORRIANI (1952, 1953). These investigators found that an enzymatically inactive protein related, possibly as a precursor, to β-galactosidase would precipitate a large part of the antibody to the active enzyme, although the inactive protein was not itself an effective antigen.

(3) Correpondence of antigenic and enzymatic surface of tyrosinase molecule.

The preceding conclusion may have to be modified if the site of the antigenic configuration of tyrosinase is, in fact, also the site of the enzymatic surface, for then the enzymatic and antigenic properties of the tyrosinase molecule would be inseparable. Three kinds of data suggest the positional

equivalence, at least in part, of these two surfaces. First, the extremely rapid inactivation of tyrosinase by antibody suggests the direct involvement of the enzymatically active surface of the molecule in the antigenic-antibody combination. Secondly, the close correspondence in absorption tests between combining capacity and enzyme activity suggests that these two properties of the extracts simply measure the amount of tyrosinase in the extracts. And thirdly (cf. OWEN and MARKERT 1954), the kinetic competition between substrate and antibody in reacting with the tyrosinase molecule strongly suggests an association between the substrate and the antibody combining sites.

(4) Species specificity of tyrosinase.

Since tyrosinases derived from different species catalyze the oxidation of the same substrates, it seems reasonable to suppose that their enzymatic surfaces are very similar if not identical. However, their antigenic properties are distinctly different—that is, Glomerella tyrosinase is antigenically distinct from Neurospora, Solanum, Psalliota and Tenebrio tyrosinase. Whether the tyrosinases from these last four species are individually distinct has not yet been tested but the presumption is that they too will be found to be distinguishable from one another. This conclusion is supported by the early work of GESSARD (1902a, 1902b) who demonstrated the species specificity of tyrosinase by showing that both cephalopod (cuttlefish) and mushroom tyrosinases were inhibited by homologous antisera, but not by the heterologous antisera. ADAMS (1942) similarly showed that rabbit antiserum against *Psalliota campestris* tyrosinase failed to precipitate the tyrosinase prepared from the related mushroom *Lactarius piperatus*. This species specificity of tyrosinase seems to preclude a simple identity between the antigenic and enzymatic surfaces of tyrosinase.

Studies of the serology of enzymes (cf. SEVAG 1951, for general discussion) have demonstrated that certain enzymes exhibit species specificity in serological tests whereas others show cross-reactions. For example, MILLER *et al.* (1949) demonstrated that antiserum to yeast hexokinase inhibited yeast hexokinase but not rat brain hexokinase. Similarly, KREBS and NAJJAR (1948) produced inhibitory antisera to yeast D-glyceraldehyde 3-phosphate dehydrogenase by injection of the purified yeast enzyme into rabbits and chickens, but such antisera did not cross-react with the equivalent enzyme from rabbit skeletal muscle.

On the other hand HOUSEWRIGHT and HENRY (1947) showed that anti-penicillinase produced by injection into rabbits of penicillinase from *B. cereus* inactivated penicillinase from both *B. cereus* and an additional organism, *S. aureus*. HOWE and TREFFERS (1953) found cross-reactions between certain related enzymes (lysine decarboxylase reacted with antisera to arginine decarboxylase) but were able to distinguish serologically between the lysine decarboxylases from three distinct coliform organisms.

In terms of current conceptions of the bases of complementary specificity (cf. PAULING in LANDSTEINER 1945) firm combinations, very slightly reversible if at all, between antibody and enzyme, suggest surface-specific forces

distributed over a relatively large area. In contrast, substrate-enzyme combinations represent a very small area, freely reversible and subject to competitive inhibition by small molecules of appropriate structure. Perhaps the most reasonable explanation of the failure of cross-inactivation of tyrosinase is one that pictures the enzymatic surface as being a part of a larger antigenic surface. The enzymatic surfaces of various tyrosinases could then be identical while species distinctions would be imposed by variations in the remainder of the antigenic surface of the tyrosinase molecule.

(5) Non-mutagenicity of antibodies to tyrosinase.

Several investigators have used antisera in attempts to demonstrate mutagenicity of antibodies, with conflicting results. EMERSON (1944) and ANDERSSON-KOTTÖ (1951) reported positive results using antiserum-treated Neurospora conidia. Unfortunately, in EMERSON's investigation the effects of serum (as contrasted with the antibodies in serum) were not adequately controlled since the control isolates were not treated with normal serum (except for 12 isolates—too few to be significant). In ANDERSSON-KOTTÖ's work the frequency of mutation observed was too small to be more than suggestive. Only 3 mutants were found out of 169 isolates treated at 23°C in 100% antiserum. Treatments at other temperatures and with other spore types yielded negative results. On the other hand FOX and ZIEBUR (1952) reported negative results in their attempt to induce mutation at the inositolless locus in Neurospora by means of antibodies. Their results are of doubtful significance, however, in view of the fact that their serological tests failed to demonstrate antibodies specific for the inositolless locus. In any event neither antiserum nor normal serum were shown to have any mutagenic action at this locus.

In the tests with Glomerella conidia, both normal and antiserum effects were tested, with negative results. The single mutant found after normal serum treatment was consistent with expectations based on the spontaneous mutation rate in Glomerella. The number of spores examined in the Glomerella investigation was fewer than in EMERSON's study, but the frequency of mutation found by EMERSON after antiserum treatment (3.6%) would, if the same frequency held in Glomerella, have yielded about 10 mutants, whereas none was found. This negative result has added significance in view of the ease with which a wider variety of mutants are recognizable in Glomerella as compared to Neurospora. Furthermore, the antiserum was proved to contain antibodies to tyrosinase, and changes affecting this enzyme would have been especially noticeable. Similar negative results were obtained by RYAN *et al.* (1953) who found that antibodies against the enzyme β-galactosidase in *E. coli* were non-mutagenic. Normal γ-globulin was also shown to be non-mutagenic.

SUMMARY

1. The tyrosinase activity extractable from a Glomerella mycelium is dependent upon the genotype of the fungus and upon the environmental conditions under which the mycelium is grown.

2. Crude extracts of Glomerella mycelium are effective antigens when in-

jected into rabbits. When the injected extracts contain an amount of tyrosinase in excess of a rather high threshold, the antisera obtained rapidly inactivate and slowly precipitate tyrosinase from all Glomerella extracts with tyrosinase activity. Extracts with little or no tyrosinase activity (from mutants or submerged cultures) fail to induce antibodies against tyrosinase.

3. Genetic or environmentally induced changes in the tyrosinase activity of a mycelium are related to changes in the number of tyrosinase molecules synthesized by the mycelium rather than to changes in the nature of the tyrosinase molecule. This point is supported by several lines of evidence including the fact that the amount of tyrosinase activity found in an extract closely parallels the ability of the extract to combine with antibodies against tyrosinase.

4. The enzymatically active surface of the Glomerella tyrosinase molecule appears to be a part of the antigenically active surface.

5. The antigenic surfaces of tyrosinase molecules are species specific since no cross reactions were found between antibodies to Glomerella tyrosinase and tyrosinase from Psalliota, Solanum, Neurospora, and Tenebrio.

6. Antisera containing antibodies to Glomerella tyrosinase are not mutagenic when applied to Glomerella conidia.

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