EFFECT OF ENVIRONMENTAL CONDITIONS UPON TYROSINASE ACTIVITY IN GLOMERELLA CINGULATA[1]

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PREVIOUS EXPERIMENTS with *Glomerella cingulata* have established that tyrosinase activity first appears in plate culture about 120 hr. after inoculation and that its formation is probably adaptive in nature (Sussman and Markert, 1953). On the other hand, cytochrome oxidase activity is present at all times in the organism and in roughly equivalent quantities. In an earlier investigation (Markert, 1950) it was noted that the amount of tyrosinase in the mycelium of *Glomerella* varies with the conditions of nutrition, aeration, and temperature under which the mycelium is grown. More recently, other workers have called attention to the importance of temperature (Horowitz and Shen, 1952) in the formation of tyrosinase in *Neurospora,* and in the formation of other fungous enzymes (Fries, 1953). The following experiments were designed, therefore, to explore the effects of these factors and others upon tyrosinase formation in *Glomerella.*

METHODS.—The organism used in these experiments was the standard type (A^1B^1) of *Glomerella cingulata* which is a conidiating double mutant from the wild type. Surface cultures were grown from mass conidial inoculum, usually on 30 ml. of agar medium in Petri dishes, and incubated at 20°C. until harvested. Where divergence from these techniques occurred, mention will be made before the results are presented. The "complete" *Neurospora* medium (Ryan, 1950) was used, except where otherwise noted.

Mycelia were harvested and handled as described by Sussman and Markert (1953). Enzyme extracts were made by grinding the weighed mycelial mats in 1/15 M phosphate buffer at pH 7.2 with a glass homogenizer. The "brei" was centrifuged at 100 × g for 15 min. after which the supernatant fluid was collected and used.

Tyrosinase activity was determined colorimetrically by the method of Markert (1950) as modified (Sussman and Markert, 1953) to employ a Beckman spectrophotometer. In this investigation the model DU was used in place of the model B. The reaction mixture consisted of 1 ml. of 0.02 M DL-dihydroxyphenylalanine (dopa), 0.1 ml. of the enzyme preparation, and 1.5 ml of 1/15 M phosphate buffer at pH 6.5. In certain instances, less enzyme was used because of the preparation's high activity; when this was done, enough buffer was added to bring the total volume to 3.0 ml. The substrate was added at zero time and readings were made at 480 mμ at 10-sec. intervals. The change in optical density during this time was used in computing tyrosinase activity. Previously (Sussman and Markert, 1953), check runs with this material using manometric techniques had established that no reducing substances were interfering with the determinations by preventing color formation. Also it was assumed that no endogenous inhibitor of the enzyme, such as is present in *Neurospora,* can be found in *Glomerella* since the several inactive preparations checked by the methods of Horowitz and Shen (1953) showed no such substance.

The enzyme extracts were standardized on the basis of their total nitrogen content. These determinations were made by means of a direct nesslerization after sulfuric acid and peroxide digestion, according to a modification of the method of Koch and McMeekin (1924). The average weight of two mycelial mats dried at 105°C. overnight was used as a measure of the growth of the organism.

In later experiments, the extracts were standardized on the basis of the mg. protein in the extract. The method used was that of Kalckar (1947) wherein the difference between the extinction of the solution at 280 and 260 mμ was computed after the use of constants to correct for the specific absorption of nucleic acids and proteins at these wavelengths. In no case did the nucleic acid content of the enzyme preparations, computed from the ratio of the extinctions at the same wavelengths, exceed 20 per cent.

RESULTS.—*Effect of amount of medium upon tyrosinase activity.*—Occasional inconsistencies in the time of inception of tyrosinase activity had been noted previously (Sussman and Markert, 1953). In checking back over the techniques used, a likely possibility was that the amount of medium used influenced enzyme formation. In order to investigate this possibility, plates containing 10, 20, 30, and 60 ml. of complete medium were prepared, inoculated, and incubated as usual. Samples were harvested periodically for enzyme analysis and for dry weight determinations and the results plotted in fig. 1. In order to check whether lack of tyrosinase activity was due to the actual absence of the enzyme or to the presence of an inhibitor in the extracts, dialysis experiments were performed, but no increase in activity resulted. Moreover, the addition of inactive extracts to an active one did not result in any decrease in the expected activity. It is immediately obvious that the amount of medium has effect upon the time of inception and a lesser effect upon the titer of tyrosinase activity. The fact that the maximum titer shows considerable variation is not surprising since it has been shown that

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the peak of tyrosinase activity is rather sharp so that it is easy to miss the peak, unless samples are taken at very short time intervals (Sussman and Markert, 1953). In 10 ml. of medium *Glomerella* forms tyrosinase shortly after 100 hr. but when 60 ml. of medium are used, the enzyme is formed only after 220 hr. Other experiments were performed which showed that the maximum titer reached in 10, 20, 30, and 60 ml. of medium was 1300, 1430, 1200, and 1400 respectively calculated as (opt. dens./min./100 N) $\times 10^3$. It seems clear therefore that approximately the same amount of activity is obtained in all cases but that the inception of activity is delayed in the culture grown in larger amounts of media. These data also establish that the cessation of growth coincides with the time of induction of tyrosinase activity, as was previously shown (Sussman and Markert, 1953).

The previous experiments suggested two possibilities: first, that the increased amount of medium permitted more growth and thereby delayed the inception of tyrosinase activity; and second, that the mere increase in the depth of the medium was somehow responsible for the observed differences.

These possibilities were investigated by the use of doubled and trebled amounts of all the components of the complete medium, except for the agar and water. Thirty ml. of medium were used and the plates were inoculated, incubated, and harvested as before. Although the peak of growth was reached at about 142 hr. in the doubled medium, and at about 172 hr. in the trebled one, no tyrosinase activity occurred in either case even after as long as 600 hr. of incubation.

That no inhibitor of tyrosinase were present as in *Neurospora* was shown by experiments wherein enzyme preparations from these samples were added to preparations of known activity. Since there was no effect upon the rate of oxidation it was concluded that there was no endogenous inhibitor present in *Glomerella* extracts.

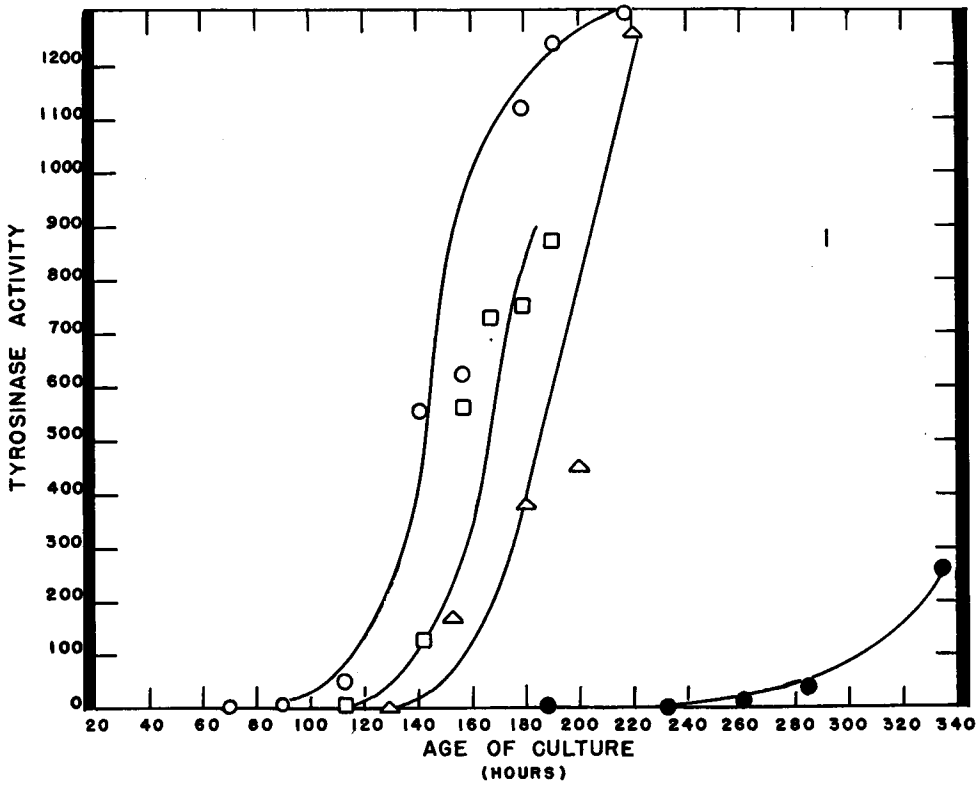
TABLE 1. *Tyrosinase activity in cultures started at 30°C. and moved to 20°C. at various times after the start of incubation*

Age at harvest (hours)	Tyrosinase activity (△ opt. dens./min./mg. protein) $\times 10^3$			
	Cultures grown at 30°C. and moved to 20°C. after:			Control (grown continuously at 20°C.)
	44 hrs.	84 hrs.	130 hrs.	
95	0	1810	.....	0
108	0	1530	.....	0
121	0	1720	.....	.....
133	10	1410	.....	.....
145	850	1300	690	1000
156	750	1040	760	.....
169	.....	870	810	990
180	.....	700	720	.....
192	1210	170	930	280

Effect of temperature upon tyrosinase activity.—Horowitz and Shen's (1953) results showing that no tyrosinase is produced when *Neurospora* is grown at 35°C. was in accord with qualitative observations of the growth of *Glomerella* (Markert, 1950). Experiments designed to explore this effect disclosed that temperature did indeed have a marked effect upon both the time of inception of enzyme activity and upon the amount. These results are presented in fig. 2. Experiments were also carried out at 33°C. and 36°C., but since results were identical with those at 30°C., only the latter are presented. The maximum enzyme titer decreases progressively with increased temperature. At 30°C. and above, no tyrosinase is produced at any time during the growth of the organism. This is so despite the fact that there is no inhibition of growth until temperatures above 34°C. are used. Indeed, the only effect is upon the rate of growth since the maximum is reached at 60 hr. instead of at 110 hr. as in cultures grown at 20°C. The decrease in maximum titer is accompanied by earlier appearance of enzyme. For example, measurable activity is obtainable at 109 hr. after inoculation in the case of cultures grown at 22–26°C., but none is obtained until 120 hr. at 20°C.

In order to determine whether the loss of tyrosinase activity at high temperatures was reversible, cultures were grown on complete medium in Petri plates at 30° and 36°. At various times after the start of incubation, plates were removed and transferred to 20°C. Plates were harvested after varying times of incubation and tyrosinase activity measured. The results are given in tables 1 and 2 and show that activity was found in all cultures started at 30°C. and moved to 20°C. This is so even for cultures kept at 30°C. until after the logarithmic phase of growth had ceased (about 130 hr.). That no decrease in activity, as compared to controls at 20°C., was occasioned by incubation at 30°C. is shown for the 44-hr. and the 84-hr. samples. In fact, there appears even to be some increase in such activity, as well as a speeding of the time of inception of this activity. For example, there is high activity in cultures which were harvested at 95 hr., after 84 hr. of prior incubation at 30°C. In contrast, the controls showed no significant activity until after 133 hr. It should be pointed out that, in this case, activity appeared in inactive cultures after 11 hr. at 20°C.

The situation is somewhat different for those cultures grown at 36°C. and transferred to 20°C. If incubation at 36° is continued beyond 48 hr., no tyrosinase is produced after transfer to 20°. However, if cultures are transferred before this time a somewhat reduced amount of activity is obtained. Mycelial transplants were also taken from cultures that had been grown at 36°C. for over 200 hr. and


TYROSINASE ACTIVITY
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AGE OF CULTURE
(HOURS)
1

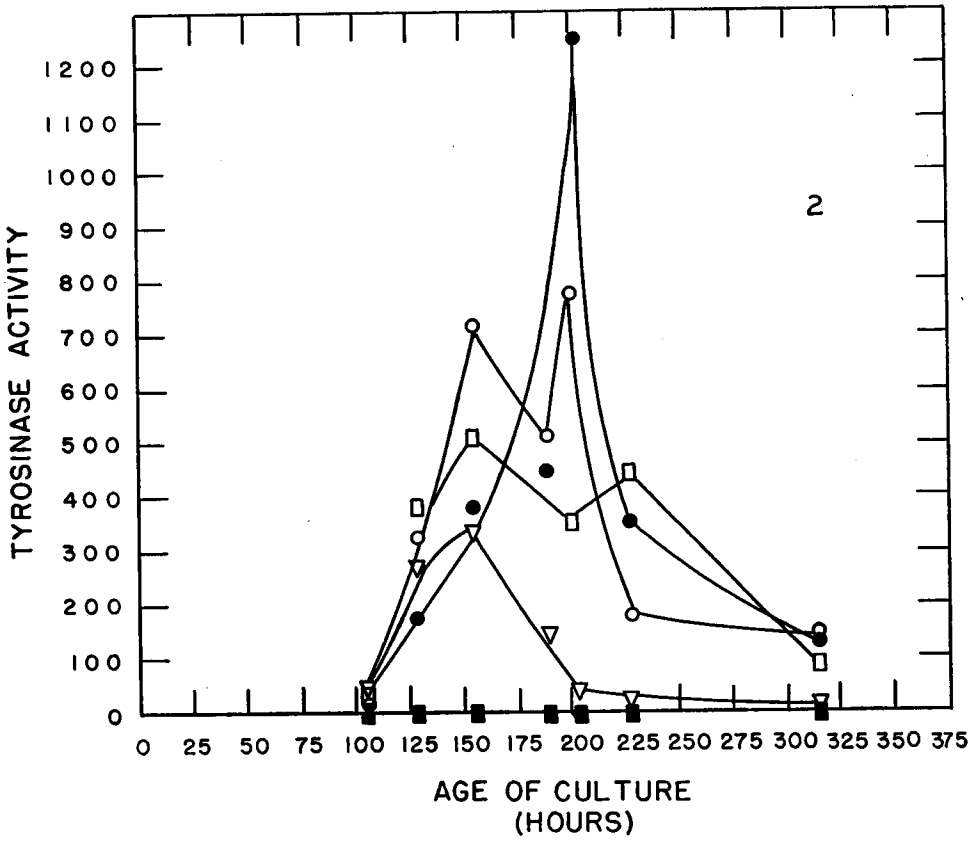
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TABLE 2. *Tyrosinase activity in cultures started at 36°C. and moved to 20°C. at various times after the start of incubation. All cultures were harvested after a total of 212 hr. of incubation*

Hours grown at 36°C.	Tyrosinase activity (△ opt. dens./min./mg. protein) × 10^3
10	690
22	530
36	630
48	10
72	0

inoculations were made on complete medium. In all instances where the transplants were incubated at 20°C., tyrosinase activity was obtained. Up to now, therefore, tyrosinase activity has always reappeared in the first generation after heat treatment.

Sussman and Markert (1953) had shown that the inception of tyrosinase activity in the standard type and certain mutants of *Glomerella* seemed to coincide with the beginning of autolysis. It was suggested, therefore, that high temperatures of incubation might be interfering with the autolytic process and thereby preventing the synthesis of the enzyme. To test this hypothesis, the growth of the standard type on 30 ml. of "complete" medium was measured at 20°, 30°, and 36°C. and the results given in table 3. Although the rate of growth at 30°C. is faster than that at the other temperatures, no marked difference in the total amount of growth or autolysis was observable.

The question then arose as to whether the failure of the enzyme to appear at 30°C. was due to the lability of the enzyme itself or to the lability of the agent(s) responsible for its formation. This problem was first approached by exposing duplicate samples of *Glomerella* tyrosinase, extracted as described previously, to temperatures of 20 and 35°C. The activity of these preparations was measured at several times after the beginning of incubation but no significant difference between the two samples was apparent as long as 2 hr. after incubation was begun. Further experiments to decide this point were carried out by growing the organism at 20°C. for periods of time long enough to permit the development of tyrosinase activity. After these times, the cultures were transferred to 30°C. and their tyrosinase activity measured after such incubation. The results of these experiments are given in fig. 3. What is most evident is that exposure to 30° causes an immediate decrease in enzyme activity. This is clear in all cases except in those samples transferred to 30°C. after 120 hr. although even here the increase is so small, when compared to the ultimate titer reached at 20°, as to be negligible. Moreover, the fact that no precipitous loss in activity resulted when cultures were transferred after 120 hr. suggests that the enzyme, once formed, is relatively thermostable. For example, almost half of the organism's tyrosinase activity after 220 hr. is retained during 20 hr. of incubation at 30° and considerable activity still remains after 60 hr. of incubation. However, those cultures transferred to 30° after 120 hr. do show a net loss in tyrosinase activity and it is likely that this decrease is associated with a system, normally present in the organism, that destroys the enzyme (see Discussion).

Effect of carbon source upon tyrosinase activity.—The differences in tyrosinase activity obtained by varying physical factors suggested that chemical factors would also influence this activity. For this reason, a start was made toward delineating some of the factors concerned by varying the carbon source. This was accomplished by using different sugars in a concentration of 0.1 M in 25 ml. of complete medium. The cultures were grown at 20°C. and harvested at various times and their tyrosinase activity and growth determined with the results shown in tables 4 and 5. The dependence of tyrosinase activity upon the type of carbon source is clear from these data since the maximum titers range from 8500 for cultures grown in mannitol to 1 and 2 in those grown in maltose and trehalose. Moreover, activity was manifested in mannitol at a much earlier time than in any of the other sugars. Al-

TABLE 3. *Growth of Glomerella cingulata at various temperatures on "complete" medium. The dry weights of duplicate samples were averaged to provide a measure of growth*

Hours of growth	Amount of growth (dry wt. in mg.) at: 20°C.	30°C.	36°C.
54	248	307	222
66	275	383	260
78	290	358	245
89	300	348	331
102	312	320	288
114	319	322	-----
126	308	315	236
150	288	269	205
179	265	237	202
198	240	238	195
246	200	203	194
294	195	231	-----

Fig. 1–2.—Fig. 1. Effect of different amounts of medium upon tyrosinase formation in *Glomerella*. Tyrosinase activity is expressed as (△ optical density/min./100 mg. N_2) × 10^3. Legend: open circles, 10 ml. of medium; squares, 20 ml., triangles, 30 ml.; closed circles, 60 ml.—Fig. 2. Effect of temperature upon tyrosinase formation in *Glomerella*. Tyrosinase activity is expressed as (△ optical density/min./100 mg. N_2) × 10^3. Each plate contained 25 ml. of "complete" medium. Legend: closed circles, cultures grown at 20°; open circles, 22°; open squares, 24°; triangles, 26°; closed squares, 30°.

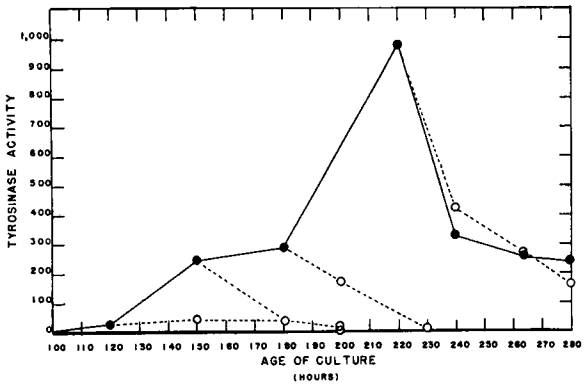

Fig. 3. Effect of incubation at 30° upon tyrosinase activity in *Glomerella*. Solid lines connect points indicating the activity of cultures maintained at 20° throughout, broken lines connect points indicating the activity of cultures moved to 30° after growth at 20°. Tyrosinase activity is expressed as (Δ optical density/min./mg. protein) × 10^3.

though there are differences in the amount of growth on these sugars there is no obvious correlation with the amount of tyrosinase activity.

DISCUSSION.—The experiments described in this paper emphasize that considerable possibility for phenotypic variation in tyrosinase activity occurs in *Glomerella*. Such possibilities are amply demonstrated by the data dealing with the amount of medium upon which the organism is grown. These data suggest that the concentration of nutrients is the important factor rather than the mere increase in the depth of the medium because adding double and triple the amount of medium precludes the formation of the enzyme. Therefore, it is possible that a metabolite (or metabolites) interferes with the formation of the enzyme. That the metabolite concerned is not a sulfur-containing compound of the type studied by Horowitz and Shen (1952) is suggested by the fact that no inhibitor such as was reported by these authors has ever been found in *Glomerella*.

TABLE 4. *Tyrosinase activity in G. cingulata after growth on different carbon sources. Mycelium was grown on 25 ml. of medium for the times listed below*

Carbon source	Tyrosinase activity (Δ opt. dens./min./mg. protein × 10^3) after: 120 hr.	180 hr.	260 hr.
glucose	31	1215	1250
fructose	50	788	1205
mannitol	334	8500[a]	4300
ribose	42	0	437
xylose	16	438	359
sucrose	3	39	182
maltose	2	2	1
trehalose	0	1.2	2

[a] The mycelium of cultures grown in mannitol was purple black in color at this time. Mycelium grown in the other sugars was light grey or cream in color.

TABLE 5. *Growth of G. cingulata on different carbon sources. Mycelium was grown on Petri dishes containing 25 ml. of medium and harvests were made at intervals. Figures are average of two samples*

Carbon source	Dry weight (mg.) after: 120 hr.	180 hr.	260 hr.
glucose	284	222	177
fructose	294	236	197
mannitol	183	215	248
ribose	167	133	168
xylose	346	218	189
sucrose	314	236	223
maltose	218	150	171
trehalose	235	268	336

Further possibilities for phenotypic variation in the tyrosinase content of *Glomerella* are introduced by the effect of temperature upon enzyme formation. Although the enzyme is formed in large amounts in cultures maintained at 20°, none is formed at 30°. That this change is not inherited was demonstrated by transplantation experiments which showed the return of tyrosinase activity at 20° in the first generation after colorless forms were induced at 30°.

An attempt was made to determine whether the enzyme itself or the enzyme-forming system is affected by the change in temperature. However, the interpretation of these data is complicated by the fact that there is an active system that is responsible for the destruction of the enzyme. In support of this assumption are previous data showing that tyrosinase activity declines rapidly after reaching its peak (Sussman and Markert, 1953). Furthermore, it is clear from the results in this paper that the processes responsible for the decrease in enzyme titer (deadaptation?) probably go on concurrently with enzyme synthesis since there is a decrease of activity at 30° even before the peak is reached at 200 hr. (see fig. 3). That this is probably occurring at 20° as well is argued for by the almost parallel decrease in the activity of the samples maintained at 20° and 30° after 220 hr. of growth. A possible interpretation of these data is that tyrosinase synthesis and degradation go on simultaneously after 120 hr. of growth at 20°. Until 220 hr., the rate of synthesis greatly exceeds that of degradation so that a net increase in activity results. However, after this time, the rate of degradation exceeds that of synthesis and a sharp decrease in titer results.

Two important differences between the tyrosinase of *Glomerella* and that of the related ascomycete, *Neurospora*, are brought into focus by these results. First, no dialyzable inhibitor such as was found by Horowitz and Shen (1952) in *Neurospora* has been found to occur in *Glomerella*. This difference

is expressed most obviously in the color of the mycelium, for *Neurospora* remains uncolored while *Glomerella* blackens only after the onset of tyrosinase activity. The other difference concerns the question of whether the failure of these organisms to produce tyrosinase above 30° is due to the thermolability of the enzyme itself or to that of the enzyme-forming system. Horowitz and Fling (1953) concluded from their study of a T^s (thermolabile) strain of *N. crassa* that the tyrosinase of that organism is sensitive to elevated temperatures. However, the evidence obtained from the present experiments tends to rule out such a possibility in *Glomerella*. Thus, no significant difference in activity was found in enzyme extracts kept at 20 and 35° after 2 hr. of incubation. Furthermore, when cultures which had developed tyrosinase activity at 20° were moved to 30°, the rate at which preformed enzyme activity decreased was equivalent to that demonstrated by cultures maintained at 20°. It is therefore concluded that high temperatures prevent tyrosinase synthesis in *Glomerella* by interfering with the enzyme-forming system.

Since the kinetics of tyrosinase formation in *Glomerella* resemble those obtained during the induced biosynthesis of other enzymes the suggestion is that tyrosinase is adaptively formed. It is of interest to note, therefore, that high temperatures could be acting to inhibit the induced formation of tyrosinase in the manner proposed by Knox (1953) who also lists several other cases in which high temperatures act as an inhibitor. Furthermore, since the temperatures used in the present experiments inhibited the formation of tyrosinase without affecting growth there seems to be a measure of specificity involved in the effect.

That the chemical environment of the organism is important in tyrosinase formation is demonstrated by the effect of sugars. Virtually no enzyme was produced in media containing trehalose or maltose while all the other sugars tested permitted the development of tyrosinase. In this connection, it is worth noting that Boyd and Lichstein (1955) found that carbohydrates depressed tryptophanase activity in bacteria while Epps and Gale (1942) found the same to be true for bacterial deaminase and other systems. As in the present work, no correlation could be found with the amount of growth in these various media. Therefore, although the presence of carbohydrates may have a marked influence upon enzyme activity, the reasons for this are still obscure.

SUMMARY

Tyrosinase production in *Glomerella* is shown to be considerably affected by environmental factors like temperature, amount of medium, and carbon source. In the case of the first of these variables, considerable enzyme is produce at 20°C. while at 30°C. it is absent. The results suggest that the higher temperature probably affects the enzyme-forming system rather than the enzyme itself. The concentration of metabolites also affects tyrosinase production since increasing amounts of the "complete" medium, or doubled and trebled amounts of the ingredients reduce or entirely preclude tyrosinase activity in agar cultures. In addition, qualitative changes in the carbon source were shown to influence enzyme production.

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