2. The 2,4-dinitrophenylhydrazone of this aldehyde was prepared.

3. Attempts were made to obtain coronene by heating 2,7-polythionaphthalenedialdehyde with copper powder.

KNOXVILLE, TENNESSEE RECEIVED JULY 17, 1942

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Factors Influencing the Cresolase Activity of Tyrosinase. The Effect of Gelatin and *p*-Cresol Concentration

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Previous workers[2,3,4] have demonstrated that tyrosinase preparations from the cultivated mushroom *Psalliota campestris* show a marked variation in ratio of catecholase to cresolase activity,[5] depending on the procedure used to isolate the enzyme. Since during the process of purification, unless special precautions are taken, the major portion of the cresolase activity is apt to be lost, the ratio of the catecholase to the cresolase activity in the purified preparation is often high (greater than 2). In these so-called "high catecholase" preparations, the catecholase activity is proportional to the copper in the preparation whereas the cresolase activity is not.[3,7] Until recently it was this type of preparation that was used for several investigations in this Laboratory concerning the nature, properties and mode of action of the enzyme tyrosinase.[2,7,8] In 1940 Parkinson and Nelson[4] reported the development of tyrosinase preparations in which the ratio of catecholase to cresolase activity was low (2 or less), and in which both enzymic activities were proportional to the copper content of the preparation. Such preparations have been called "high cresolase" preparations.

The development of different type tyrosinase preparations, having different ratios of catecholase to cresolase activities, has resulted in conjecture as to whether or not the enzyme is in reality one copper protein possessing two types of enzymic action, or is a mixture of two copper proteins each with its own activity. Before any considerable progress can be made toward a solution of this interesting and fundamental problem, it is necessary to have reliable means of characterizing the different type preparations on the basis of their catecholase and cresolase activities. Because of the marked inactivation of the enzyme that is observed particularly during the oxidation of catechol, it has been the practice to measure the enzyme activities in the presence of a "protecting" agent, gelatin being commonly used for this purpose.[2,9] Thus previous studies designed to compare the two types of tyrosinase preparations on the basis of their catecholase and cresolase activities have been made with gelatin present in the reaction medium.[4,10]

Recently, however, it has been found that the presence of gelatin in the reaction medium during the enzymatic oxidation of catechol (catecholase activity) tends to obscure fundamental differences between the different type preparations,[11] and it has been found that gelatin need not be present in the reaction medium in order to obtain a reliable measurement of catecholase activity.[12] These observations made it seem advisable to reinvestigate, from the same point of view, the effect of gelatin on the other characteristic activity of the different type tyrosinase preparations, *i. e.*, the cresolase activity.

The results of such a study, described in detail below, reveal that the increase in rate of oxidation of *p*-cresol (increase in cresolase activity), that is observed when tyrosinase is used in the

(1) Present address: Stamford Research Laboratories, American Cyanamid Company, Stamford, Conn.

(2) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2474 (1938).

(3) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **B125**, 187 (1938).

(4) G. G. Parkinson and J. M. Nelson, THIS JOURNAL, **62**, 1693 (1940).

(5) The ability of the enzyme preparation to catalyze the aerobic oxidation of the dihydric phenol catechol and the monohydric phenol *p*-cresol are referred to as catecholase and cresolase activities, respectively. One catecholase unit and one cresolase unit have been defined as the amount of enzyme required to cause the uptake of 10 cu. mm. of oxygen per minute when acting on 4 mg. of catechol and 4 mg. of *p*-cresol, respectively. For further details see Gregg and Nelson.[6]

(6) D. C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2500 (1940).

(7) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

(8) C. A. Bordner and J. M. Nelson, *ibid.*, **61**, 1507 (1939).

(9) M. H. Adams and J. M. Nelson, *ibid.*, **60**, 2472 (1938).

(10) D. C. Gregg and J. M. Nelson, *ibid.*, **62**, 2506 (1940).

(11) W. H. Miller and C. R. Dawson, *ibid.*, **63**, 3368 (1941).

(12) W. H. Miller and C. R. Dawson, *ibid.*, **63**, 3375 (1941).

presence of gelatin, is dependent on the type of tyrosinase preparation employed and also on certain environmental factors such as the substrate concentration. The variable effect of gelatin in these *p*-cresol–enzyme systems has been found to be complex in nature, and may be such as to obscure or unduly accentuate certain fundamental characteristics of the particular enzyme preparation.

The Enzymatic Oxidation of *p*-Cresol

When a typical high catecholase preparation is used, with and without gelatin in the reaction medium, the enzymatic oxidation of *p*-cresol, as followed by manometric measurements of oxygen consumption, proceeds as shown by the curves of Fig. 1. In these experiments a constant amount of the enzyme was employed and the substrate (*p*-cresol) concentration was varied tenfold, that is from 2.5 to 25 mg. in an 8.0-cc. reaction volume. In contrast to the enzymatic oxidation of catechol which starts at a maximum rate,[11] it can be seen from Fig. 1 that the enzymatic oxidation of *p*-cresol is characterized by an initial lag or induction period in the rate of oxygen absorption. The length of this induction period can be varied in a number of ways and this phase of the reaction has been studied[6,8] in attempts to distinguish more clearly the differences between cresolase and catecholase activity. From Fig. 1 it is apparent that a tenfold variation in *p*-cresol concentration has no pronounced effect on the length of the induction period, either in the presence or absence of gelatin. Only in the case where gelatin is present in the reaction medium (Curves I-G, II-G, etc.) might it be inferred that the induction period is significantly less with the lower substrate concentrations.

With the high catecholase preparation, the rate of oxygen absorption after the initial lag period in the *p*-cresol reaction reaches a maximum value, usually in twenty to thirty minutes. This rate then continues at nearly a constant value whether gelatin is used or not. Thus, in the case where 2.5 mg. of *p*-cresol was used (Curves I and I-G of Fig. 1) it can be seen that the maximum value was maintained until about 75% of the *p*-cresol was completely oxidized.[13]

When a typical high cresolase preparation is used, the general course of the reaction is some-

(13) The complete enzymic oxidation of *p*-cresol results in the absorption of three atoms of oxygen per mole of *p*-cresol,[8] which for 2.5 mg. of *p*-cresol corresponds to an oxygen uptake of 785 cu. mm.

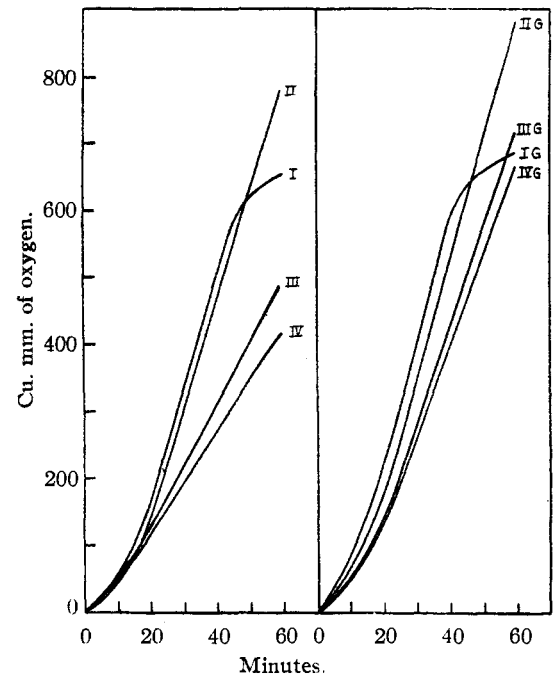

Fig. 1.—Showing the effect of gelatin and substrate concentration on the enzymatic oxidation of *p*-cresol by a high catecholase tyrosinase preparation. Oxygen absorption determined in Warburg Respirometer using flasks of 50-cc. capacity; 120 oscillations per minute; temperature 25°; *p*H 7.1. Total reaction volume was 8.0 cc., consisting of 1.0 cc. 0.2 *M* citrate–0.4 *M* phosphate buffer, 1.0 cc. (5 mg.) of gelatin solution where indicated, 1.0 cc. of *p*-cresol solution of indicated concentration (see data below), 1.2 cc. of a diluted (1:100) high catecholase tyrosinase preparation C144 added from the flask side arm to initiate the reaction, and water to bring to volume. Preparation C144 from the common mushroom, *Psalliota campestris*, had a catecholase to cresolase activity ratio of 13.8 and dry wt. (undiluted) of 2.6 mg. per cc.; contained 0.105% copper and 693 catecholase units per γ copper. Catecholase and cresolase activity were determined using methods previously described.[6,12] Each of the above curves is an average curve obtained from 3 to 5 experiments with readings taken at five-minute intervals. Such experiments are generally more reproducible when gelatin is a component of the system, *i. e.*, the points making up the average curve show less deviation. To compensate for this a greater number of experiments was employed in the systems containing no gelatin. The maximum rate values indicated below are obtained from the average curves over the time range where the oxygen uptake per five-minute interval was maximum and constant to within ±3 cu. mm.

No gelatin			Gelatin	
p-Cresol, mg.		Max. rate, cu. mm./ 5 min.		Max. rate, cu. mm./ 5 min.
2.5	I	88	I-G	93
5.0	II	81	II-G	90
15.0	III	46	III-G	74
25.0	IV	39	IV-G	69

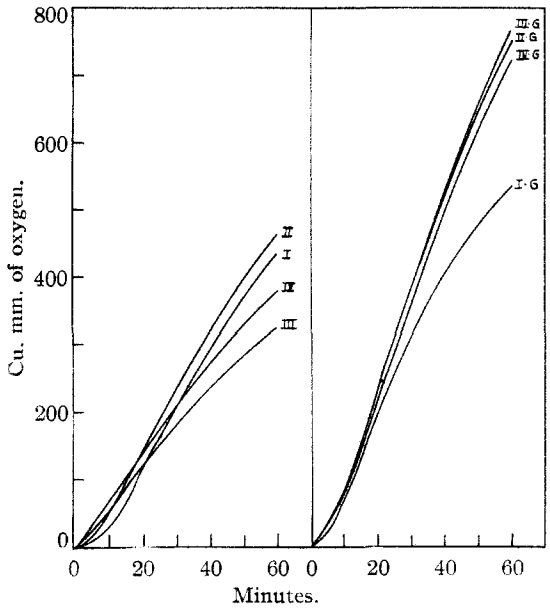

Fig. 2.—Showing the effect of gelatin and substrate concentration on the enzymatic oxidation of *p*-cresol by a high cresolase tyrosinase preparation. Reaction mixture and procedure the same as indicated in the legend of Fig. 1 except that 0.9 cc. of the diluted (1:500) high cresolase preparation C143F2 was used. This preparation has been previously described.[12] See legend of Fig. 1 for method of obtaining maximum rate values shown below.

	No gelatin		Gelatin	
p-Cresol, mg.		Max. rate, cu. mm./5 min.		Max. rate, cu. mm./5 min.
2.5	I	47	I-G	65
5.0	II	48	II-G	79
15.0	III	34	III-G	73
25.0	IV	40	IV-G	69

what different. This is apparent from Fig. 2 where the data plotted have been obtained from experiments exactly analogous to those described in Fig. 1, except that a constant amount of a high cresolase preparation was used. It is to be noted that in a reaction medium containing no gelatin (Curves I, II, etc., of Fig. 2), the length of the induction period is changed rather appreciably by change in substrate concentration. A longer induction period results from the use of lower *p*-cresol concentrations. Gelatin in the reaction medium practically eliminates this effect of substrate concentration on the length of the induction period (Curves I-G, II-G, etc., of Fig. 2). These results are in contrast to the effects of gelatin and substrate concentration on the induction period of a high catecholase preparation (Fig. 1).

With a high cresolase preparation the rate of oxygen absorption, after the initial lag period, approaches a maximum more rapidly, and maintains this rate constant for a much shorter period of time than is the case when a high catecholase preparation is employed (compare Figs. 1 and 2). This is true whether gelatin is present in the reaction medium or not, although gelatin does tend to increase somewhat the time a nearly constant rate of oxygen uptake is maintained with the high cresolase preparation.

For both types of preparations, the most pronounced effect of a tenfold variation in original substrate concentration is, on the maximum rate of oxygen absorption, obtained after the initial lag period (see maximum rate data Figs. 1 and 2). Thus, considering the data obtained using the high catecholase preparation (Fig. 1), it can be seen that when there is no gelatin in the reaction medium (Curves I, II, etc.), a marked lowering of the maximum rate of oxidation results when the original amount of *p*-cresol is increased from 2.5 to 25.0 mg. However, when gelatin is a component of the system (Curves I-G, II-G, etc.), the effect of increasing the original substrate concentration is much less pronounced. Gelatin appears to prevent, to an appreciable extent, inhibition of enzyme action caused by excessive amounts of *p*-cresol. The data obtained with the high cresolase preparation (Fig. 2) show qualitatively the same thing. It should be noted, however, that the inhibitory effect of excessive amounts of *p*-cresol in the absence of gelatin (Curves I, II, etc.) is considerably less pronounced for this type of preparation.

The manner in which the rate of enzymatic oxidation of *p*-cresol varies with the substrate concentration for both types of tyrosinase preparations, and the influence of gelatin on this variation, is shown more strikingly in Fig. 3. The data in Fig. 3 are those of Figs. 1 and 2 (see legend Fig. 3) plotted to show the rates as percentages of the optimum rate obtained in each series of experiments. It is apparent that in the absence of gelatin the rate of oxidation of *p*-cresol with a high catecholase preparation (Curve I) is considerably more sensitive to change in original substrate concentration than is the rate obtained with a high cresolase preparation (Curve II). Of particular interest is the quite different picture obtained when gelatin is present in the reaction medium. Although gelatin does not shift the position of the optimum substrate concentration for either type preparation, its effect on the substrate-rate curve is not the same for both type preparations.

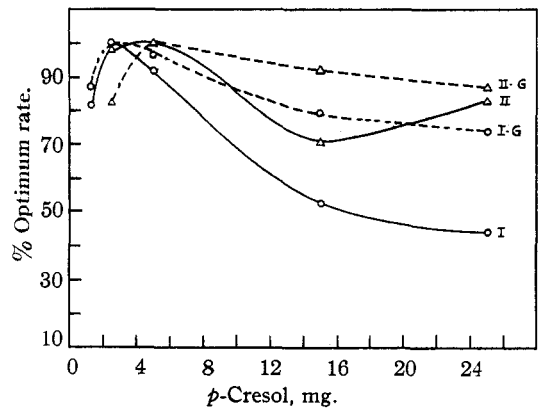

Fig. 3.—Showing the relationship between substrate concentration and cresolase activity when measured in the presence and absence of gelatin. The percentages of the optimum rates obtained with and without gelatin for each type preparation are calculated from the maximum rate data given in legends of Figs. 1 and 2 with one exception. Data obtained as indicated in the legend of Fig. 1 for 1.25 mg. of *p*-cresol have been added to show the optimum *p*-cresol concentration for the high catecholase preparation. Experimental points on the curves are: O, high catecholase; Δ, high cresolase; — — —, gelatin; ———, no gelatin.

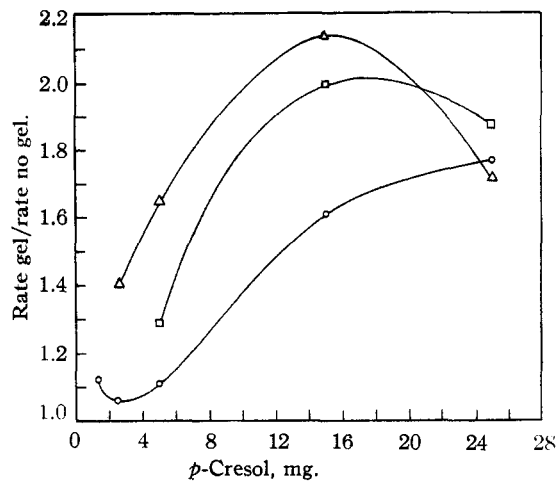

Fig. 4.—Showing that the effect of gelatin on cresolase activity varies markedly with the type of tyrosinase preparation and with the *p*-cresol concentration. For the high catecholase and the high cresolase preparations the ratios (rate gelatin/rate no gelatin) are calculated from the maximum rate data given in legends of Figs. 1 and 2 except for 1.25 mg. of *p*-cresol (see legend Fig. 3). The data shown for the intermediate preparation[14] were obtained as indicated in the legend of Fig. 1 using 0.8 cc. of the diluted (1:100) enzyme preparation C144PbAl. This preparation had a catecholase to cresolase activity ratio of 4.1 and a dry weight (undiluted) of 1.2 mg. per cc. contained 0.106% copper and had 690 catecholase units per γ of copper. Experimental points are: O, high catecholase; Δ, high cresolase; □, intermediate.

This difference in effect of gelatin is particularly noticeable on the low concentration side of the optimum.

Inspection of the rate data in Figs. 1 and 2 shows that when gelatin is present in the reaction medium, the maximum rate of the enzymatic oxidation of *p*-cresol is in all cases greater than that obtained when gelatin is not present. Gelatin causes an apparent increase in cresolase activity. The important point to note in this connection is the fact that the boosting effect of gelatin is not constant, but is variable, depending not only on the type of tyrosinase preparation, but also on the substrate concentration. This statement is emphasized by the data as shown in Fig. 4.

Discussion

Just how gelatin causes the effects described above is not clearly understood at the present time. The mechanism whereby one protein influences the state and action of another presents an interesting and fundamental problem, but it is a problem that cannot be solved until more data on the phenomena are available and more is known about protein structure. A study of the effect of inert protein material on the catalytic action of protein enzymes offers one of the most direct attacks on the problem, for the effects produced by the inert protein can be easily measured. It has been the purpose of this study to emphasize the fact that the effect of gelatin on tyrosinase cresolase activity and characterizing features of that activity is variable and markedly dependent on the type of tyrosinase preparation, *i. e.*, on the state of the tyrosinase protein material.

It has been suggested that the effect of gelatin results from the fact that it tends to prevent inactivation of the enzyme during the course of the oxidation,[9] primarily by preventing the enzyme from entering the air–liquid interface where surface denaturation of the protein–enzyme may occur.[15] Although it is probable that gelatin does "protect" the enzyme to some extent in this manner, certain of the data presented here, and elsewhere,[11] indicate that the function of gelatin is considerably more complex, and involves factors in addition to those which merely prevent inactivation of the enzyme. For the "protection" view of gelatin action to be completely logical and

(14) Tyrosinase preparations having a ratio of catecholase to cresolase activity in the range of 3.5–4.0 frequently are found to have properties intermediate to those characterizing the high catecholase and high cresolase preparations. Such enzyme preparations have been termed "intermediate" preparations.[11, 12]

(15) L. Tenenbaum, Dissertation, Columbia University, 1940.

satisfactory in explaining the very significant increase in cresolase activity that is observed when gelatin is employed in the measurements, one would expect to find considerable evidence of inactivation of the enzyme when gelatin is not employed. Furthermore, one would expect to find correspondingly less evidence of inactivation of the enzyme when gelatin is a component of the system. Yet when the cresolase activity of a high catecholase preparation is measured with no gelatin in the system (Curves I, II, etc., Fig. 1), constant rates of oxidation are observed for periods of over a half hour with the surface of the system changing at least 120 times per minute, *i. e.*, there is no evidence of serious inactivation of the enzyme in the absence of gelatin. With the other type of tyrosinase preparation (high cresolase preparation) there is evidence (short period of constant rate) indicating possible inactivation of the enzyme, when the activity measurements are made in the absence of gelatin (Curves I, II, etc., Fig. 2). However, the change toward a linear oxidation curve, effected by making gelatin a component of the system (compare Curves II and II-G, Fig. 2) appears to be hardly enough to explain a 65% increase in cresolase activity (compare maximum rate data for Curves II and II-G, Fig. 2).

Certain of the data obtained in this study suggest that gelatin increases the enzyme activity by perhaps modifying the enzyme molecule, or more likely by modifying the enzyme substrate relationships during the course of the reaction. Thus the influence of gelatin on the initial phase of the reaction, the induction period, was found to vary with the type of enzyme preparation and the substrate concentration. These same two factors were found also to influence the boosting effect of gelatin on the rate of oxidation obtained after the induction period (see Fig. 4).

It is possible that much may be learned in the future about the enzyme tyrosinase by studying its action in the presence of added protein material, such as gelatin. However, it would appear that when studies are made for the purpose of comparing the various type tyrosinase preparations, in reference to either their cresolase or catecholase activity, the use of gelatin is not to be recommended, at least not until more is known about its complex role in the system. With gelatin present, certain fundamental differences between the various type preparations are likely to be masked or possibly accentuated.

Summary

1. The effect of gelatin and *p*-cresol concentration on the cresolase activity of different type tyrosinase preparations from *Psalliota campestris* has been studied.

2. Over a tenfold range of substrate concentration, gelatin causes an increase in the rate of *p*-cresol oxidation as catalyzed by the enzyme tyrosinase.

3. The increase in rate of oxidation caused by gelatin varies markedly with the type of enzyme preparation and the substrate concentration.

4. The different type tyrosinase preparations show characteristic relationships of substrate concentration to cresolase activity and these relationships are affected differently by gelatin.

5. It appears that the role of gelatin in these systems is considerably more complex than just preventing inactivation of the enzyme during the course of the oxidation.

NEW YORK, N. Y. RECEIVED MAY 8, 1942