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Factors Influencing the Catecholase Activity and Inactivation of Tyrosinase. The Effect of Gelatin and of Catechol Concentration

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The common mushroom *Psalliota campestris* is a convenient source of the enzyme tyrosinase which has aroused considerable interest because of its ability to catalyze two types of aerobic oxidation. This enzyme has the ability not only to catalyze the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones, but also to catalyze the oxidation of certain monohydric phenols to the corresponding *o*-quinone oxidation stage. The enzyme apparently first causes the introduction of a hydroxyl group ortho to the one already present in the monohydric phenol.[1,2] Since catechol has been used widely as a substrate for studying the first type of activity, and *p*-cresol as a substrate for studying the latter type of activity, the two enzymic activities have been referred to as catecholase and cresolase activities.[3,4,5]

The marked inactivation of the enzyme during the enzymatic oxidation of catechol has been recognized for some time.[6,1] Several investigators have attempted to explain this inactivation as due to the influence of reaction products[6,1,7] but later work indicates that the inactivation is not due to any reaction products known to be formed.[8] Adams and Nelson[9] observed that the addition of foreign protein to the enzyme–substrate system decreased the extent of inactivation, and they suggested the use of gelatin for protecting the enzyme during substrate oxidation studies. They also found that when hydroquinone was added to the system the enzyme appeared to suffer less inactivation. Using systems composed of catechol, hydroquinone, gelatin, enzyme and buffer, in which the inactivation of the enzyme was much less pronounced, it became possible to make rate measurements with a fair degree of precision, using the Warburg form of respirometer.[10]

Ludwig and Nelson[9] were the first to demonstrate a stoichiometric relationship between the amount of enzyme used and the oxygen absorbed during the complete inactivation of that enzyme when the enzyme was catalyzing the oxidation of catechol in the presence of gelatin. In other words, the extent of oxidation of the catechol as measured by oxygen absorption, was found to be proportional to the amount of enzyme used. As a measure of the quantity of enzyme, they used the unit proposed by Adams and Nelson[9] and correlated it with the copper content of the enzyme. This unit involves the use of a mixture of catechol and hydroquinone as a substrate for the enzyme. Ludwig and Nelson used only purified high catecholase preparations in their studies.[11] With the development of high cresolase preparations by Parkinson and Nelson,[3] it became apparent that the effect of hydroquinone on the catecholase activity of the high cresolase preparations was different from its effect on the catecholase activity of the high catecholase preparations. These investigators showed that the catechol–hydroquinone method for measurement of catecholase activity could not be applied to the high cresolase preparations. They showed that for a given amount of enzyme copper ($\tau/350$) these high cresolase preparations were more resistant to inactivation than high catecholase preparations during the oxidation of catechol. Furthermore, Gregg and Nelson[12] showed that for the same quantity of enzyme copper the high catecholase preparations had about twice the activity of the high cresolase preparations toward catechol when the comparison was made on the basis of initial rates of oxygen absorption. The minimum

(1) D. Richter, *Biochem. J.*, **28**, 901 (1934).

(2) C. A. Bordner and J. M. Nelson, THIS JOURNAL, **61**, 1507 (1939).

(3) G. G. Parkinson and J. M. Nelson, *ibid.*, **62**, 1693 (1940).

(4) D. C. Gregg and J. M. Nelson, *ibid.*, **62**, 2500 (1940).

(5) 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.[4]

(6) M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **111**, no. 3, 757 (1935).

(7) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937).

(8) B. J. Ludwig and J. M. Nelson, THIS JOURNAL, **61**, 2601 (1939).

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

(10) M. Dixon, "Manometric Methods," University Press, Cambridge, 1934.

(11) Enzyme preparations in which the catecholase but not cresolase activity is proportional to copper content and the ratio of catecholase activity to cresolase activity is high (more than 2 catecholase units to 1 cresolase unit) have been designated as "high catecholase" preparations by workers in this Laboratory. Preparations in which both activities are proportional to copper content and in which the ratio of catecholase to cresolase activity is low (2 or less) have been called "high cresolase" preparations.

(12) D. C. Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2506 (1940).

amount of catechol required to give the maximum measurable rate of oxygen absorption was found for a high cresolase preparation to be about five times that required by a high catecholase preparation.

From the above discussion it is apparent that high catecholase and high cresolase preparations differ in several fundamental respects. It should be pointed out, however, that all of these comparison experiments were conducted using gelatin as a protective agent. The use of a protective agent in experiments designed to compare enzyme preparations having different inactivation characteristics is questionable, unless it can be demonstrated that the protecting agent has the same relative effect in all cases. If this is not the case certain differences between the preparations may become masked or possibly accentuated in part by the protecting agent. The experiments reported in this communication indicate that the relative protecting effect of gelatin varies both with the environment and with the type of enzyme preparation under study.

During a manometric determination of enzyme activity, inactivation of the enzyme may occur through denaturation of the protein by mechanical coagulation at the air–liquid interfaces set up while shaking the manometers.[13,14] It has been suggested that the protective action of gelatin results from the fact that it prevents the enzyme from entering these interfaces.[15] That the role of gelatin is probably more complex than this is evident from an inspection of the data in Table I. These data were obtained by measuring in the presence and absence of gelatin the activity of a given amount of enzyme after it had experienced the indicated twenty minute pretreatment. This pretreatment consisted of allowing the enzyme to stand or to be shaken slowly in water or buffer solutions of varying concentrations. From the experiments in which the measurement of enzyme activity was made without gelatin, it is apparent that slow shaking has little or no effect on the activity of the enzyme during the twenty minute period in water solution. However, while merely standing in buffer solutions the enzyme experiences considerable inactivation during this period. This buffer effect is accentuated by shaking as can be seen in columns 4 and 5. This is particularly demonstrated by the experiment using 3 cc. of buffer where the inactivation was so nearly complete that no measurable rate could be observed.

The experiments in Table I in which gelatin was used during the activity measurements give a different picture of what happened during the pretreatment. Inactivation during this period is indicated in only one case, namely, the one in which the enzyme was shaken in the more concentrated buffer solution. It should be noted that the effect was not so drastic as that indicated by the corresponding experiment measured with no gelatin. Since the experiments with and without gelatin were conducted similarly up to the twenty minute point it is a reasonable assumption that inactivation occurred to the same extent in both cases; and it should be pointed out that the differences in rate measurements obtained with and without gelatin cannot be considered as due to a different extent of inactivation during the actual rate measurement (note discussion of Table II). Therefore, the effect of buffer concentration and shaking on the inactivation of the enzyme is genuine and demonstrated only by those experiments measured in the absence of gelatin. This is further indicated by the fact that in those experiments the inactivation effects show an increase with increasing severity of environmental conditions, whereas in the experiments measured with gelatin there is no such trend of inactivation. Thus it appears that gelatin does not function solely as an inert protector to the active enzyme in the solution, but it may reverse in part at least the inactivation caused by buffer and shaking. If the enzyme has experienced these effects severely enough, it appears that the behavior of the gelatin in this respect, while still definitely apparent, is nevertheless not complete, as indicated by the shaking experiment using 3 cc. of buffer. In view of this effect with a high catecholase preparation in which the gelatin is shown definitely to be more than an inert protective agent, experiments were designed to observe the behavior of all available kinds of tyrosinase preparations in the absence and presence of gelatin. To prevent partial inactivation of the enzyme before zero time due to buffer and shaking effects, the enzyme instead of the substrate was placed in the side arm of the reaction flask and added at zero time.

From Table II, it is seen that when this proce-

(13) Abderhalden and Guggenheim, *Z. physiol. Chem.*, **54**, 331 (1908).

(14) Shaklee and Meltzer, *Am. J. Physiol.*, **25**, 81 (1909).

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

TABLE I[a]

SHOWING THE BEHAVIOR OF A HIGH CATECHOLASE PREPARATION AS MEASURED IN THE PRESENCE AND ABSENCE OF GELATIN AFTER DIFFERENT PRETREATMENT

	Measured with no gelatin				Measured with gelatin			
	Standing		Shaking		Standing		Shaking	
	Rate Cu. mm. O_2 / min.	Total Cu. mm. O_2	Rate Cu. mm. O_2 / min.	Total Cu. mm. O_2	Rate Cu. mm. O_2 / min.	Total Cu. mm. O_2	Rate Cu. mm. O_2 / min.	Total Cu. mm. O_2
Enzyme alone	24	70	22	70	[b]	[b]	25	85
Enzyme and 1 cc. buffer	16	40	10	25	24	70	27	80
Enzyme and 3 cc. buffer	12	25	0	7	24	80	7	15

[a] The period of pretreatment of the enzyme was twenty minutes. The rate of shaking during pretreatment was 60 oscillations per minute. At the start of the measurement the shaking rate was increased to 150–165 oscillations per minute and maintained at that rate until the enzyme was inactivated. Originally the same amount of enzyme was placed in each reaction flask. The buffer used was 0.2 *M* citrate-0.4 *M* phosphate mixture adjusted to *p*H 7.0. The gelatin or gelatin-buffer mixtures were added twenty minutes after the start of the indicated experiment. Catechol was then introduced into the side arm and added to the reaction mixture at zero time, usually ten minutes later. In the case of the mixtures containing no gelatin at any time, the catechol or buffer–catechol mixture was added at twenty minutes (zero reaction time). The concentration of the reactants was varied such that the enzyme during pretreatment was in a 6.0 cc. volume for those experiments using gelatin and a 6.8 cc. volume for experiments without gelatin. The final reaction volume was 8 cc. The amount of gelatin used was 5 mg. The amount of catechol used was 4 mg. The temperature was 25°. Each value given in either of the two top lines is the average result of at least six experiments. Each value shown in the bottom line of data is an average of 3–4 experiments. In computing these averages, the data for all rate measurements have been found to have a deviation on the average of about ±13%. The average total oxygen uptakes are all ±5 cu. mm. The rates are indicative of the amount of active enzyme in the flask at the time of measurement.

[b] Due to circumstances beyond control, these data were not obtainable with the same enzyme preparation (C144F1B). This omission does not affect in any way the general conclusions drawn from the table.

dure was followed, the same maximum rate using 4 mg. of catechol was observed within experimental limits of error whether gelatin was present or not. The gelatin experiments shown in Table II using 4 mg. of catechol were also conducted with the procedure previously used in this laboratory, *e. g.*, the enzyme was placed with the gelatin in the well of the flask and the substrate was added from the side arm at zero time. Since the maximum measurable rate in all cases agreed with those indicated in Table II, the experimental data have not been included. In most of these experi-

TABLE II[a]

SHOWING THE EFFECT OF GELATIN AND EXCESS SUBSTRATE AT *p*H 7.1 ON THE INITIAL RATES OF OXYGEN UPTAKE FOR THREE DIFFERENT TYPES OF TYROSINASE PREPARATIONS

Substrate concentration	Time, min.	No Gelatin			Gelatin			Ratio Gelatin / No gelatin		
		High catecholase	Intermediate	High cresolase	High catecholase	Intermediate	High cresolase	High catecholase	Intermediate	High cresolase
4 mg.	1	24	23	23	23	22	22	1.0	1.0	1.0
	2	17	18	20	20	22	25	1.2	1.2	1.3
	3	7	10	16	13	15	20	1.9	1.5	1.3
25 mg.	1	13	15	21	12	15	18	0.9	1.0	0.9
	2	13	15	15	15	20	26	1.2	1.3	1.7
	3	8	10	13	9	16	21	1.1	1.6	1.6
Ratio 25 mg. / 4 mg.	1	0.5	0.7	0.9	0.5	0.7	0.8			
	2	0.8	0.8	0.8	0.8	0.9	1.0			
	3	1.1	1.0	0.8	0.7	1.1	1.1			

[a] Barcroft differential manometers were used; temperature 25°; buffer used was 1 cc. 0.2 *M* citrate-0.4 *M* phosphate mixture *p*H 7.0; catechol solutions were 4 and 25 mg./cc., respectively (used 1 cc.); 1 cc. of gelatin solution (5 mg.) was used in indicated experiments; total reaction volume made 8 cc. by addition of water. The enzyme solution was placed in the side arm and added to the reaction mixture at zero time. Most of the values shown in the table are an average of about six determinations. Some are the average of five determinations and some are an average of as many as ten experiments. These rate measurements have been found to have a deviation on the average of about ±12%. These rates are given as the number of cu. mm. of oxygen absorbed during one minute. To change to units as defined, divide by ten. The high catecholase preparation was preparation number C144F1B; intermediate preparation C144PbAl; and the high cresolase preparation C143F2.

ments the maximum rate was obtained during the second minute rather than the first. This is quite frequently the case using gelatin, but with measurements in the absence of gelatin the maximum rate is generally obtained the first minute. These experiments then indicate that the new procedure of adding the enzyme from the side arm makes no noticeable difference in the maximum measurable rate. They further show that the presence of gelatin is in reality not necessary using this procedure since the same maximum enzyme activity was obtained both in the absence and presence of gelatin.

In Table II are shown the effects of gelatin and excess substrate on the apparent enzyme activities for three types of tyrosinase preparations. In addition to the high catecholase and high cresolase types of enzyme preparations which have been described before as having properties which are distinctly different, the authors have noticed that frequently enzyme preparations are obtained which possess properties in part like each of the other two types. Unlike the high catecholase and high cresolase preparations, these intermediate preparations occurring at the border line between the two main types do not show consistently the same behavior and so differ slightly but noticeably among themselves. For this reason exact duplication of data with different intermediate preparations is fortuitous rather than the expected. The ratio of catecholase to cresolase activity has been observed as in the vicinity of 3.5–4.0 with these preparations.

In the last three column section of Table II showing data obtained using 4 mg. of substrate it is seen that during the first minute of enzyme action there is no significant difference in the rate whether measured with or without gelatin. During the second minute, gelatin protects all preparations to about the same extent. By the end of the third minute, the protective effect of the gelatin on the activity of the high catecholase preparation has become more pronounced than on the activity of the other two. However, with 25 mg. of catechol, a large excess of substrate, the situation appears quite different. Here the high catecholase preparation is seen to be protected to the least extent during the third minute by the gelatin. With the possible exception of the high cresolase preparation in the absence of gelatin, it is to be noted that the enzyme is restricted in action during the first minute with this excess substrate, the high catecholase preparation being most seriously affected. In the presence of gelatin the high cresolase preparation and to a certain extent the intermediate preparation regain their optimum rate (that obtained with 4 mg. of catechol) during the second minute. The protective effect of gelatin on the rate is shown thus to vary not only with the type of tyrosinase preparation under study but also to exhibit no consistency during a change in environmental conditions of only one factor, *i. e.*, substrate concentration.

These same studies have been carried out at *p*H 5.5 and are summarized in Table III. It is important to note that in the absence of gelatin the results obtained at *p*H 5.5 closely parallel those obtained at *p*H 7.1 (Table II). However,

TABLE III[a]

SHOWING THE EFFECT OF GELATIN AND EXCESS SUBSTRATE AT *p*H 5.5 ON THE INITIAL RATES OF OXYGEN UPTAKE FOR THREE DIFFERENT TYPES OF TYROSINASE

Substrate concentration	Time, min.	No Gelatin: High catecholase	No Gelatin: Intermediate	No Gelatin: High cresolase	Gelatin: High catecholase	Gelatin: Intermediate	Gelatin: High cresolase	Ratio Gelatin/No Gelatin: High catecholase	Ratio Gelatin/No Gelatin: Intermediate	Ratio Gelatin/No Gelatin: High cresolase
4 mg.	1	21	23	26	19	23	21	0.9	1.0	0.8
	2	16	15	20	23	21	24	1.4	1.6	1.2
	3	5	10	16	15	15	20	3.0	1.5	1.3
25 mg.	1	14	17	26	12	16	18	0.9	0.9	0.7
	2	10	12	16	16	18	25	1.6	1.5	1.6
	3	7	10	13	12	16	21	1.7	1.6	1.6
Ratio 25 mg./4 mg.	1	0.7	0.7	1.0	0.6	0.7	0.9			
	2	0.6	0.8	0.8	0.7	0.9	1.0			
	3	1.4	1.0	0.8	0.8	1.1	1.1			

[a] Reaction mixture and procedure the same as indicated in the legend of Table II except that the citrate-phosphate buffer mixture used was adjusted to *p*H 5.1 (final *p*H of reaction mixture 5.4–5.5). Each value shown in the table represents an average of 6 to 8 determinations. Those experiments conducted in the absence of gelatin have a deviation on the average of about ±14%; those using gelatin have a deviation on the average of about ±10%.

TABLE IV

SHOWING THE EFFECT OF GELATIN AND EXCESS SUBSTRATE AT *p*H 5.5 AND 7.1 ON THE OXYGEN ABSORBED DURING THE INACTIVATION OF THREE DIFFERENT TYPES OF TYROSINASE PREPARATIONS

Quantities of enzyme, experimental conditions, and composition of reaction mixtures are as indicated in the legend of Tables II and III. The rate of shaking was maintained at 150–165 oscillations per minute throughout the inactivation study. Those experiments carried out in the absence of gelatin gave results with a deviation on the average of ±12%. Those carried out in the presence of gelatin gave results with a deviation on the average of ±8%.

	Substrate concentration	No gelatin			Gelatin			Ratio $\frac{\text{Gelatin}}{\text{No gelatin}}$		
		High catecholase	Intermediate	High cresolase	High catecholase	Intermediate	High cresolase	High catecholase	Intermediate	High cresolase
*p*H 7.1	4 mg.	60	90	175	80	160	390	1.3	1.8	2.2
	25 mg.	50	75	120	60	170	405	1.2	2.3	3.4
	Ratio 25/4	0.8	0.8	0.7	0.8	1.1	1.0			
*p*H 5.5	4 mg.	50	75	215	80	175	380	1.6	2.3	1.7
	25 mg.	40	60	150	75	150	380	1.9	2.5	2.5
	Ratio 25/4	0.8	0.8	0.7	0.9	0.9	1.0			
Ratio $\frac{p\text{H } 7.1}{p\text{H } 5.5}$	4 mg.	1.2	1.2	0.8	1.0	0.9	1.0			
	25 mg.	1.3	1.3	0.8	0.8	1.1	1.1			

with experiments conducted in the presence of gelatin, the effect of this agent is found to be more pronounced and more variable at the lower *p*H.

It is to be noted from an inspection of both tables that in the presence of gelatin there is a marked tendency for the maximum rate to appear during the second minute. This tendency toward an induction period in the presence of gelatin appears to be accentuated by increased substrate concentration and is more pronounced at the lower *p*H. The contrast between results obtained in the presence and absence of gelatin in this respect is especially apparent when one correlates the results of 325 experiments carried out using three types of tyrosinase preparations under varied conditions. Thus only 26 out of 155 experiments in the absence of gelatin gave a maximum rate the second minute. In the presence of gelatin, however, 91 out of 139 experiments gave the maximum rate the second minute. It is interesting to note that a constant rate was maintained for two minutes in only 31 experiments, 18 of which were carried out in the presence of gelatin.

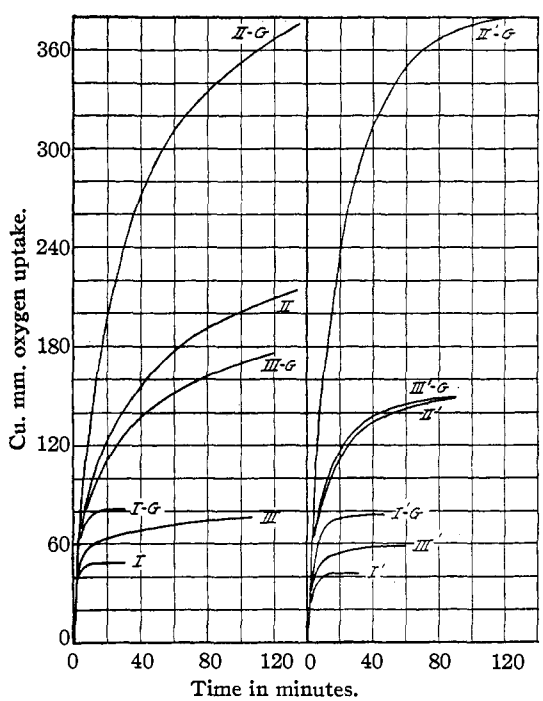

Fig. 1.—Showing the effect of gelatin and catechol concentration on the inactivation of catecholase during the enzymatic oxidation of catechol at *p*H 5.5: Curve I, high catecholase preparation; Curve II, high cresolase preparation; Curve III, intermediate preparation. The G series of curves indicate experiments in which 5 mg. of gelatin was present in the enzyme reaction system. The family of curves shown in the section of the figure to the right (I', II', etc.) represent experiments identical in all respects except that 25 mg. of catechol was used instead of 4 mg. Other experimental details same as for Table IV.

The oxygen absorbed during the complete inactivation of a given amount of enzyme (the inactivation total) during the oxidation of catechol has been used as a means of distinguishing a high cresolase from a high catecholase preparation (3). It seemed of interest to determine the effect of gelatin, *p*H, and excess substrate on this inactivation total. The results of such studies made using three different kinds of enzyme preparations are shown in Table IV and in part by Fig. 1. From the last three column sections of the table, it is seen that the extent of the protective influence of gelatin during the complete inactivation of the enzyme varies with the type of enzyme, the substrate concentration and the *p*H. In contrast to rate measurements at *p*H 7.1 where the high cate-

cholase preparations appeared best protected by the gelatin, the inactivation totals indicate that the high cresolase preparation is offered the greatest protection before complete inactivation. As with the rate measurements, the protective action of gelatin appears more uniform at *p*H 5.1 especially at the higher substrate concentration. From column 7 of this table it appears that gelatin exerts complete protection to the high cresolase preparation against the restrictive action of excess substrate. This is noteworthy since it is seen that in the absence of gelatin, this restrictive action is most apparent with this type of preparation. From a consideration of the lower two lines of data in the table, it is seen that in the absence of gelatin, no striking differences in inactivation totals result from a change in *p*H.

Figure 1 serves not only to illustrate part of the data and conclusions from Table II but more important it indicates the progress of the inactivation of the different enzyme preparations. Thus, during the first two or three minutes the curves appear nearly identical. By seven minutes the curves have taken on definite individual characteristics. It is interesting to note that whereas a high catecholase preparation is almost completely inactivated in ten to fifteen minutes, the other two preparations at this time have caused the absorption of only a third to a half of the oxygen used during their inactivation which requires a period of about seventy-five to one hundred and thirty-five minutes. The position of the curves for the intermediate preparation is seen to be in between those of the two main types. While the inactivation value is closer to that of the high catecholase preparation, the time taken for inactivation as well as the protective action of gelatin appear more to resemble those for the high cresolase preparations. These differences in time required for inactivation, so well illustrated by the curves, offer an explanation to the difference in the protective action of gelatin indicated by rate measurements and inactivation totals. This is discussed below.

Discussion

Many unusual points have been noted concerning the influence of gelatin on the enzyme. One of the more unusual and striking properties it has is its ability to apparently reverse in part at least enzymatic inactivation caused by shaking and buffer salts. These studies were carried out on a type of preparation (high catecholase) which during the enzymatic reaction, at least, is the most easily inactivated of the three types of preparations. The manner in which the experiments were carried out should be noted. The enzyme and gelatin were in contact for ten minutes after the pretreatment period and before the measurement of activity. Therefore whether or not the reversing ability of gelatin is able to exert itself instantaneously has not been determined. However, for the present considerations this point is of little significance. The fact that gelatin can reverse at least certain types of enzyme inactivation raises a question as to its use in the measurement of enzyme activity. If the gelatin were consistent in its behavior toward all kinds of tyrosinase preparations its reversal effect might not be objectionable. However in all the other experiments reported, no such consistency was observed.

The change in procedure of adding the enzyme rather than the substrate from the side arm of the reaction flask at zero time eliminates the possibility of enzyme inactivation due to buffer and shaking during the warm up period in the thermostat. In this way, it is possible to obtain the same maximum rate in the presence and absence of gelatin. These experiments then demonstrate that the use of gelatin as a protective agent is unnecessary to obtain maximum rate. Under such circumstances gelatin does not cause an increase in enzyme activity as measured during the first minute and in many cases gelatin does not allow the maximum rate to be obtained the first minute. What gelatin does do apparently is to exert unequal protectiving effects on the different type preparations as the reaction progresses. This may give a false impression of the differences of the properties possessed by the various enzyme preparations. In addition, gelatin also gives a varied protection against the restrictive action of excess substrate. Thus at *p*H 7.1, with 4 mg. of catechol the protective action of gelatin on the rate for the first three minutes appeared greatest for the high catecholase preparation, and with 25 mg. of catechol the protective effect of gelatin was greatest for the high cresolase preparation. Thus, as shown by rate measurement studies, gelatin masks the effects of environmental changes which may serve in part to differentiate one type enzyme from another.

It has been pointed out that the results of total

inactivation studies with and without gelatin are in part in conflict with those obtained from rate measurements made at the beginning of the enzymic action. That rate measurements indicate gelatin protects a high catecholase preparation the most, and that inactivation measurements indicate gelatin protects a high cresolase preparation the most, is not too surprising when one considers the relative lengths of time necessary to cause inactivation of the two preparations. Whereas a high catecholase preparation inactivates in ten to fifteen minutes, a high cresolase preparation requires about 75 to 135 minutes for inactivation. This means that with these two preparations, the gelatin is acting as a protecting agent for widely discrepant periods, or better and conversely, that in the absence of a protecting agent environmental factors such as buffer and shaking can exert a much greater effect on the inactivation value for the high cresolase preparation. This can become serious enough to offset any differences between the preparations which may have been indicated early in the reaction when the comparison was made over the same time range. However, the use of a protecting agent is undesirable unless it is complete and impartial in its action. From the inconsistency of gelatin behavior, it is more than likely that inactivation totals determined using it do not characterize the highly purified enzyme preparations as such but rather indicate the behavior of the preparations in an environment which does not affect them each to the same extent. However, inactivation totals obtained in the absence of gelatin due to the unequal time for environmental factors to operate may come no closer to characterizing the enzyme on the basis of its inactivation due only to the oxidation of catechol. The inactivation value thus obtained represents rather an indication of the cumulative inactivating effects of buffer, shaking, and the oxidation of catechol operating over a period determined by the resistance of the enzyme preparations to these effects. The most desirable method for the observation of inactivation totals due only to catechol oxidation would be one in which the effect of secondary factors could be reduced to a minimum. This could probably best be done by comparing all enzyme preparations over the same time interval at the start of the reaction and from a reliable determination of their behavior during this period extrapolating to the point of complete inactivation. This method of determining inactivation totals is not practical by the manometric method since rate measurements are not sufficiently reliable and cannot be obtained in sufficient number during the early part of the reaction to warrant such an extrapolation. In consideration of the above discussion of manometric inactivation totals, it would appear that rate measurements give a better indication of the action of gelatin in the enzyme systems.

It is a pleasure to acknowledge the help which we have received from Mr. Stanley Lewis, who made the enzyme preparations.

Summary

1. The protective effect of gelatin against the inactivation of catecholase (tyrosinase) during the oxidation of catechol has been found to be variable, being dependent on the type of enzyme and environmental conditions. For this reason the elimination of gelatin from experiments designed to compare various type tyrosinase preparations is recommended.

2. It has been shown that gelatin can reverse in part inactivation of the enzyme caused by buffer salts and solution agitation.

3. It has been demonstrated that the same activity of the enzyme based on rate measurements can be measured with or without gelatin provided that in the latter case a procedure is used such that the enzyme is not inactivated prior to the start of the measurement.

4. Catecholase activity appears the same at *p*H 5.5 and 7.1. Because the possibility of complicating reactions is greater at *p*H 7.1, the lower *p*H is to be preferred.

5. An intermediate preparation has been compared with the two main types.

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