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The Use of Added Protein in the Determination of the Activity of Tyrosinase

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Tyrosinase is generally defined as the enzyme which catalyzes the aerobic oxidation of certain monohydric phenols such as tyrosine and *p*-cresol, and ortho-dihydric phenols such as catechol and its derivatives. In attempting to isolate the enzyme in a highly purified form, or to determine its relative amounts in tissues, a satisfactory method for its quantitative estimation becomes necessary.

Two principal methods have been used in the past to determine its activity. One involves the determination by chemical means of either the rate of disappearance of the substrate, or the rate of formation of some of the reaction products. The other depends on manometric measurements of the rate of oxygen uptake by the enzyme–substrate system. The latter is the simpler and is sufficiently accurate if conditions are such that the rate of oxygen absorption remains constant for about half an hour. Due to its simplicity the manometric appears to be the favored method. As substrate catechol has been used by Richter,[1] Kubowitz,[2] and Keilin and Mann.[3] Graubard and Nelson[4] preferred to use *p*-cresol in place of catechol, because the rate of oxidation is slower and tends to remain constant over a longer period of time. They used the Warburg form of respirometer[5] with flat-bottomed 50-cc. reaction flasks, the apparatus being run at about 90 complete oscillations per minute; temperature 25°. The reaction mixtures consisted of 4 cc. of 0.1% *p*-cresol, a desired volume of the enzyme solution, 2 cc. of phosphate (0.2 *M*)–citrate (0.1 *M*) buffer, and sufficient water to make the final volume in the reaction flask 8 cc.; *p*H of the reaction mixture was 6.2. The unit of enzyme activity was defined as the amount of enzyme needed to induce the above system to absorb oxygen at the rate of 10 cu. mm. per minute. Their best preparations of tyrosinase had a dry weight of about 0.1 mg. per unit of activity. With preparations of this degree of purity, they found that the rate of oxygen uptake was directly proportional to the amount of enzyme used, provided the latter was in the range of 0.5 to 2 units.

Since then methods of purifying tyrosinase preparations have been improved greatly so that preparations are now available having a dry weight of 0.0015 mg. per unit of activity. In the case of these highly purified preparations of tyrosinase the Graubard and Nelson method was found to be no longer satisfactory, due to the occurrence of considerable inactivation of the enzyme.

In working with highly active preparations of tyrosinase it also was observed that inactivation often occurred when the solutions were diluted. Saul and Nelson[6] observed a similar loss in activity when highly purified preparations of yeast invertase, having a *p*H value on the acid side of the optimum *p*H 4.5, were diluted. When, however, a little protein was added to the enzyme solution, either before or after dilution, this abnormal decrease in activity almost vanished. With this influence of added protein in mind, gelatin was added to dilute solutions of highly purified tyrosinase preparations, and large increases, often several hundred per cent., in activity were noted. When sufficient *p*-cresol was used in the reaction mixture the new value for the activity remained constant throughout a wide range of dilutions.

It will be noticed by means of curves IV and V in Fig. 2 that an increase in added gelatin from 1 to 5 mg. furnishes no additional protection to the enzyme against inactivation. Since in both instances only half (600 cu. mm.) of the theoretical total oxygen uptake (3 atoms, 1244 cu. mm.) occurred it is evident that it is not possible to overcome the inactivation entirely by adding gelatin, and if the oxidation of the *p*-cresol is to be carried to completion, more enzyme would have to be used. The protein contained in the enzyme preparation seems also to exert to some extent an influence on the activity similar to that exerted by gelatin. Thus the data for curve VI in Fig. 2 were obtained by using 2.5 times as much tyrosinase preparation (no gelatin added) as in the experiment represented by Curve I. Yet the ac-

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

(2) Kubowitz, *Biochem. Z.*, **292**, 221 (1937).

(3) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **125B**, 187 (1938).

(4) M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **112**, 135 (1935).

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

(6) Saul and Nelson, *J. Biol. Chem.*, **111**, 95 (1935).

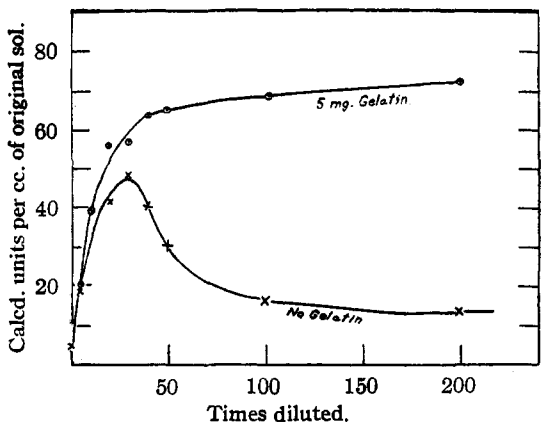

Fig. 1.—Showing change in activity of a tyrosinase preparation (*Psalliota campestris*) on dilution, and the influence on the activity when 5 mg. of gelatin was added to the reaction mixtures (modified Graubard and Nelson method). The increase in activity up to a dilution of 30 times the original volume was probably due to lack of sufficient oxygen. The activity was determined by taking manometric readings of the oxygen uptake at five-minute intervals. Usually a lag occurred in the rate of oxygen uptake at the beginning of the reaction. This was especially true in the case of more highly purified preparations. For this reason the rate of oxygen uptake was allowed to increase gradually until a maximum value was reached. The maximum rate remained practically constant for thirty to sixty minutes and was used in the calculation of the activity in terms of units, as defined by Graubard and Nelson. Temperature 25°. *p*H of reaction mixture 7.0. 1 unit of preparation used had a dry weight of 0.005 mg.

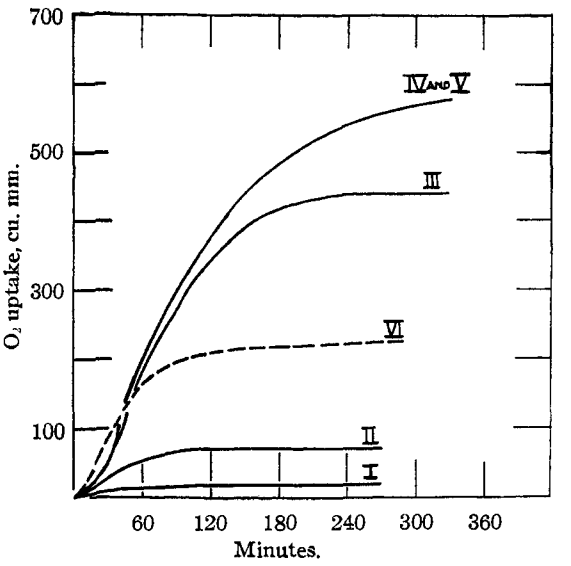

Fig. 2.—Showing the effect of different amounts of gelatin on the activity of tyrosinase. Mg. of gelatin present in the reaction mixtures contained in Warburg flasks: I, 0.0; II, 0.01; III, 0.1; IV, 1.0; V, 5.0. All reaction flasks contained same amount of enzyme, 4 mg. of *p*-cresol, *p*H 7.0 and amounts of gelatin indicated. Total volume 8 cc. Temperature 25°. Activity determined as described in legend of Fig. 1. Theoretical oxygen uptake for 4 mg. of *p*-cresol (3 atoms of oxygen) = 1244 cu. mm. Curve VI differs from Curve I in that twice as much enzyme preparation was used, but no gelatin just as in the case of Curve I.

tivity calculated on the basis of Curve VI was 0.3 unit compared to roughly 0.05 unit when calculated by means of Curve I. In other words, by using 2.5 times as much enzyme the activity was increased about 6 times. Likewise, Curve VI shows a total oxygen uptake (220 cu. mm.) which is about 10 times the total oxygen uptake in the case of Curve I (18 cu. mm.), thereby indicating less inactivation when more enzyme was used. The addition of 0.1 mg. of gelatin (Curve III) brought the initial rate of oxygen uptake nearly up to the maximum value, obtained when 1 mg. of gelatin was used (Curve IV). This explains why Graubard and Nelson were able to get activities roughly proportional to the concentration of enzyme used, since their most active preparation had about 0.1 mg. of protein per unit.

Just why gelatin exerts a protective action on the activity we are unable to state. Almost any protein, in the native or denatured condition, seems to have much the same effect as shown in Table I.

A comparison of four different brands of gelatin gave similar results. Ivory soap had very little effect on the enzyme's activity, whereas agar-agar did cause a noticeable increase of about 0.12 unit. It was not possible to use a greater amount of agar due to its tendency to form a gel. Gum arabic in amounts of 0.1 mg., which is comparable to the amount of protein necessary for maximum protection, exerted practically no influence on the enzyme's activity. Only by using large amounts, 50 mg., was it possible to obtain an effect comparable to that of 0.1 mg. of protein.

TABLE I

Protein	Activity units per cc.
No protein	0.18
Gelatin 5 mg.	.70
Egg albumin 5 mg. (before dialysis)	.40
Egg albumin 5 mg. (after dialysis)	.70
Serum albumin 5 mg.	.69
Hemoglobin 5 mg.	.74
Casein (saturated soln.)	.73
Oxglobin 5 mg. (before dialysis)	.05
Oxglobin 5 mg. (after dialysis)	.69
Catalase (concd. preparation)	.67
Ivory soap 5 mg.	.23
Gum arabic 50 mg.	.67
Agar-agar 1 mg.	.30

TABLE II

SHOWING THE INFLUENCE OF *p*H ON THE ACTIVITY OF TYROSINASE (*Psalliota campestris*) IN THE PRESENCE OF 5 MG. OF GELATIN, TEMPERATURE 25°

*p*H of reaction mixture	4.0	4.5	5.0	5.5	6.0
Activity, units per cc.	0.0	0.20	0.55	0.69	0.76
*p*H of reaction mixture	6.5	7.0	7.5	8.0	
Activity, units per cc.	0.81	0.88	0.90	0.88	

From the data in Table II it will be observed that the change in rate of oxygen uptake is quite marked in the more acid range of *p*H but approaches a maximum around *p*H 7. Beyond *p*H 7 there is danger of autoxidation and for this reason *p*H 7 is the better hydrogen ion concentration for determining activity of tyrosinase toward *p*-cresol, rather than *p*H 6.2 originally used by Graubard and Nelson. Furthermore, the above data indicate no optimum *p*H value for the enzymatic oxidation of *p*-cresol, which is not in line with results reported by Graubard and Nelson. Due to an oversight the last-named investigators failed to mention the use of borate buffer in the more alkaline range. Since then this buffer has been shown to exert a retarding influence on the rate of oxygen uptake, and thus explains the apparent optimum *p*H. Narayanamurti and Ramaswami Ayyar[7] also reported an optimum *p*H value of 6.5 in the oxidation of tyrosine by tyrosinase (from the bean *Dolichos lab lab*). They also used borate buffer for the more alkaline *p*H range.

The data in Table III show that there is a gradual increase in oxygen uptake with increase in concentration of *p*-cresol used. Changing the rate of oscillation of the Warburg apparatus to more than 90 complete oscillations did not influence the rate of oxygen uptake.

(7) D. Narayanamurti and Ramaswami Ayyar, *J. Indian Inst. Science*, **12**, 109 (1929).

TABLE III

SHOWING THE INFLUENCE OF CONCENTRATION OF *p*-CRESOL ON THE RATE OF OXYGEN UPTAKE

Temperature 25°. *p*H of reaction mixture 7.0. Same amount of enzyme used in all experiments. 5 mg. gelatin. Reaction volume 8 cc.

p-Cresol, mg.	0.0	1.0	2.0	3.0	4.0	5.0
Activity, units per cc.	.0	0.36	0.58	0.72	0.83	0.94

In the light of the results obtained in this study it was decided to modify the method of Graubard and Nelson as follows. Reaction mixture: 1 cc. of an aqueous solution containing 4 mg. of *p*-cresol, added to the reaction mixture from the side-arm of the reaction flask at zero time; 2 cc. of (0.1 *M*) citrate–(0.2 *M*) phosphate buffer solution (*p*H of reaction mixture, 7.0); 1 cc. of an aqueous solution containing 5 mg. of gelatin; 1 cc. of enzyme solution and sufficient volume of water to make total reaction volume 8 cc.

Summary

1. The importance of protein concentration in the determination of the activity of highly purified preparations of tyrosinase has been pointed out.

2. The method of Graubard and Nelson for determining the activity of tyrosinase toward *p*-cresol has been modified to conform to the influence of protein.

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On the Nature of the Enzyme Tyrosinase

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In the literature on oxidases from the time of Bourquelot to the present, tyrosinase has been generally considered capable of performing two quite distinct oxidative functions: one, the introduction of a hydroxyl group ortho to the already existing hydroxyl in certain monohydric phenols; and two, the oxidation of certain ortho-dihydric phenols to the corresponding ortho-quinones. Several theories have been proposed to account for these two types of activity. Onslow[1] favored the view that tyrosinase is really a catechol oxidase, and that the oxidation of the monohydric phenols is due to a secondary reaction in which *o*-quinones serve as the oxidizing agent. Richter[2] states "The oxidation of *p*-cresol therefore appears to be essentially a secondary oxidation in which the formation of an *o*-quinone is involved, rather than a direct oxidation of the cresol." Keilin and Mann[3] also believe that the monophenolase activity is due to the catechol oxidase (polyphenolase) plus some "factor" probably of the *o*-quinone type as suggested by Onslow.

Against Onslow's theory may be cited the

(1) M. W. Onslow, "The Principles of Plant Biochemistry," University Press, Cambridge, 1931, p. 138.

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

(3) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **125B**, 187 (1938).