ON TYROSINASE OF *DOLICHOS LABLAB*

I. Methods of Estimation and the Oxidation of Different Substrates

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The presence of an active tyrosinase in the aqueous extract of *Dolichos lablab*, was observed by Narayana in the course of his work on the proteins of Indian pulses. The enzyme was subsequently investigated by Narayananmurti and Iyer.1

Preparation of the Enzyme.—The enzyme is prepared by an aqueous or saline extraction of the dried and powdered seeds of *Dolichos lablab*, the extract after filtration being dialysed to remove globulins. The clear, filtered, light brown solution was fractionally precipitated with ice-cold alcohol. The precipitate between alcohol concentrations 20–60%, was recovered by centrifugation and dissolved in water. The solution after further dialysis, has been employed in the following studies.

In attempting to prepare the enzyme in a state of high purity, the adoption of a reliable method for the quantitative estimation of its activity, is essential. Chemical methods now in vogue, take advantage of the disappearance of tyrosine during the oxidation or of the formation of quinone as the reaction product. The manometric method of determining the O₂ uptake has also been employed. O₂ uptake accompanying the oxidation of a suitable substrate affords a convenient means of measuring the activity of the enzyme preparations.

Rate of Disappearance of Tyrosine during Oxidation by the Enzyme.—This method was developed by Raper,2 and improved considerably by Haehn and Stern.3 It was employed by Narayananmurti and Iyer1 in their studies of the kinetics of the tyrosine-tyrosinase reaction. The method consists in the estimation of the tyrosine, left after removing the enzyme and proteins and the melanin-like oxidation products by suitable treatments from an aliquot of the reaction mixture, by bromination with bromate-bromide mixture. We find that the curves representing the rate of disappearance of
tyrosine for various concentrations of the enzyme (Fig. 1) hold no measurable relation, sufficiently accurate to serve for an evaluation of the activity of the enzyme.

The Manometric Method.—The manometric method of following the absorption of oxygen during the enzymic oxidation of a suitable substrate has been employed with advantage by several workers. The general technique is fully described by Dixon. Richter first attempted an estimation of the enzyme from the rate of O₂ uptake with catechol as substrate, employing a preparation of the enzyme from potato. Graubard and Nelson worked out in detail conditions for an accurate estimation of the activity, and found p-cresol a better substrate on account of the lower and steadier O₂ uptake and observed that at low concentrations of enzyme, the slopes of the O₂ uptake–time curves were directly proportional to the enzyme concentration.

We have investigated the O₂ uptake during the oxidation of several substrates by the enzyme extract from Dolichos lablab, and have standardised conditions for obtaining an accurate measure of the enzyme. Tyrosine appealed as the direct substrate, but results with it were as discouraging as the bromination method. p-Cresol here, too, proved an excellent substrate with a rate of O₂ uptake, over a fairly wide range of enzyme concentrations, proportional to the enzyme.

We have employed the Warburg constant volume manometer with reaction vessels of approximately 15 c.c. total capacity, carrying one side bulb,
The total volume of the reaction mixture was always 2 c.c. and the temperature of the thermostat maintained at 30° C. Brodie's solution was used as the manometric liquid. Thus the readings of the manometer (difference in levels in mm.) are directly proportional to the $O_2$ uptake in cmm. and hence the direct readings in mm. have been taken in all cases for comparison—the absolute volume of $O_2$ utilised being calculated only in cases where that was specifically necessary or desirable. (The constants for the vessels varied from 1.210 to 1.212.) The manometer and attached vessels were shaken at 70-80 complete oscillations per minute, but, between 60-100, there was no appreciable difference in the rate of $O_2$ uptake with the shaking. With $p$-cresol as substrate, conditions of pH, substrate concentration, etc., were standardised for an accurate measure of the enzyme. The substrate concentration (between 0.25 mgm. and 5 mgms.) had practically no influence on the $O_2$ uptake rate. The pH employed was 6.2 (0.2 m. $Na_2HPO_4$—0.1 m. citric acid buffer). The conditions are:

$$\begin{align*}
\text{p-Cresol} & = 1 \text{ mgm.} \\
\text{pH} & = 6.2 \\
\text{Enzyme + Water} & \text{ Total volume 2 c.c.}
\end{align*}$$

A series of $O_2$ uptake curves for different enzyme concentrations are represented in Fig. 2.

There is a short lag period in the initial stages, the duration of which varies with the concentration of the enzyme from 5–20 minutes and
On Tyrosinase of Dolichos lablab—I

...afterwards the oxidation proceeds with practically constant rate of O$_2$ uptake till nearly the end, about 3 atoms of O$_2$ being absorbed per molecule of $p$-cresol oxidised. This steady rate could be noted from the slope of the curve or more directly, from the successive manometric readings themselves. The corrections due to changes in the temperature of the thermostat, or in the atmospheric pressure are, in general, negligible.

The limits of accuracy of the method were determined with one series, the enzyme amounts and corresponding O$_2$ uptake rates being given below:

- 0.7 c.c. - 1.3 mm./min.
- 0.8 c.c. - 1.55 mm./min.
- 0.9 c.c. - 1.85 mm./min.
- 1.0 c.c. - 1.95 mm./min.

Thus results with ± 5% accuracy could be obtained easily. An arbitrary unit of the enzyme could be defined as that amount which promotes O$_2$ uptake (by $p$-cresol under standard conditions) of 10 mm./min. The number of units in relation to the total solids content of a certain preparation of the enzyme is a measure of its purity.

In a study of the nature of the enzyme tyrosinase, in view of recent work on this group of enzymes, it is of great importance to examine the substrate specificity of the enzyme. The existence of a separate enzyme specifically attacking mono-hydroxy phenols has been questioned by many, who hold the oxidation of these to be merely a secondary phenomenon, the enzyme responsible being a di- or poly-hydroxy phenol oxidase. This view is disputed by others. Hence the importance of studying the behaviour of the enzyme towards suitable mono- and di-hydroxy phenolic substrates, at different stages of purification, cannot be overemphasised.

The Oxidation of Catechol.—The dihydroxy phenol, catechol, has been used widely as an experimental substrate in the study of phenolase activity (Richter, Kubowitz, Keilin and Mann). The oxidation of catechol by tyrosinase which is generally rapid, is accompanied by an inactivation of the enzyme. This is brought about by the O-quinone which is formed during the reaction and efforts were directed to eliminate it from the sphere of the reaction. Richter succeeded in securing a fair degree of accuracy in measuring the activity of the enzyme by carrying out the reaction in the presence of aniline, which combines with O-quinone. Kubowitz, on the other hand, introduced a reducing system—thus making the function of catechol a "carrier" of oxygen—using hexose monophosphate with its corresponding apo-dehydrogenase and coenzyme. Other more easily accessible substances could be used as the substrates for "carrier" oxidation, e.g., ascorbic acid and hydroquinone, both employed by Graubard and potassium ferricyanide.
Of the substances so far examined, catechol is most easily and rapidly oxidised by the *Dolichos lablab* enzyme preparation. Next in order comes the other catechol derivative, dihydroxy phenyl alanine, commonly known as "dopa". The course of oxidation follows lines closely similar to that of the enzyme studied by Nelson and Adams. The initial O₂ uptake is very high but tends to decline rapidly unless the proportion of the enzyme to the substrate is very high. There is no conceivable proportionality to the concentration of the enzyme—even during the first minute or two—except as an approximation. With lower concentrations of enzyme, the decline in rate of O₂ uptake is rapid and the O₂ uptake becomes negligible in the course of 10 to 15 minutes. This is presumably due to inactivation of the enzyme. The total O₂ uptake does not prove to be any complete, but corresponds to 2 atoms of O₂ per molecule catechol, when the enzyme concentration is very high. With the larger concentrations of the enzyme, the uptake of the first atom of oxygen is very rapid while the rate is lower during the uptake of the second atom. Neither is the total O₂ uptake during any stage of the oxidation proportional to the concentration of the enzyme. Thus the direct oxidation of catechol cannot, in any way, be employed as a method of measuring the enzyme. Fig. 3 gives the curves for the O₂ uptake by catechol with varying concentrations of the enzyme.
It is thus clear that we are obliged to adopt the method of "carrier" oxidation with catechol as carrier, for measuring the catechol oxidase activity of the enzyme preparation. Both hydroquinone and ascorbic acid are not directly oxidised by the enzyme, but the addition of a trace of catechol to the reaction mixture brings about this oxidation. Alternate oxidation of catechol by the enzyme and reduction of the quinone formed by these substances, is the mechanism that results in the $O_2$ uptake. With either ascorbic acid or hydroquinone as the (secondary) substrate, $O_2$ uptake is proportional to (1) a varying concentration of catechol for a given concentration of the enzyme, within the narrow limits of 0·005 to 0·025 mgm. in 2 c.c., (2) varying concentration of the enzyme for a given quantity of catechol, over a fairly wide range of enzyme concentrations. Curves representing the above results are given in Figs. 4–5. Most suitable conditions for a measurement of the enzyme activity are:

- Hydroquinone or ascorbic acid 2 mgm.
- Catechol 0·02 mgm.
- Buffer pH 6·2
- Water + enzyme

Total volume 2 c.c.

An enzyme unit for the determination of catechol-oxidase activity similar to that previously described for $p$-cresol-oxidase, can be fixed, so
that a comparison of the activities towards the two substrates at different stages of purification, could be made.

The total \( \text{O}_2 \) uptake when ascorbic acid or hydroquinone is oxidised, corresponds to 1 atom oxygen per molecule. The oxidation of ascorbic acid proceeds at very nearly twice the rate of that of hydroquinone, under identical conditions of catechol and enzyme concentration. Extreme care needs to be taken when ascorbic acid is employed as substrate, against traces of extraneous copper in the reaction mixture. Potassium ferrocyanide is also oxidised by the use of catechol as "carrier".

*Products of Oxidation of Catechol: Iodimetric Method of Estimation.*— The enzymic oxidation of catechol was noted to involve the uptake of 2 atoms of oxygen. \( O \)-quinone was suggested to be the product of oxidation and several facts were cited in the evidence for this view, till a conclusive confirmation came from Raper's\(^{10}\) isolation of \( O \)-quinone derivatives from the reaction mixture. Since the formation of \( O \)-quinone requires only one atom of \( \text{O}_2 \), the fate of the second atom taken up remained obscure. After a detailed study of the course of oxidation, Adams and Nelson\(^{9}\) now suggest a scheme of oxidation which should result in the formation of a hydroxy quinone. They have developed a method for following the course of oxidation of catechol, estimating the quinone formed at various stages, by its ability to liberate iodine from acidified potassium iodide. We have also studied this method with a few alterations in the procedure so as to suit a direct comparison of the results with the \( \text{O}_2 \) uptake.

In a 100 c.c. conical flask, are placed 12.5 c.c. of a solution of catechol containing 1 mgm. per c.c. and 3.0 c.c. buffer (0.4 M \( \text{Na}_2\text{HPO}_4 \)-0.2 M citric acid, pH 6.2) and enzyme extract and water, to bring the final volume of the reaction mixture to 25 ml. The flask is stoppered and shaken in a thermostat, maintained at 30° C. Soon after the addition of the enzyme, 2 c.c. of the reaction mixture is removed from the flask and treated with 25 c.c. dilute 2N \( \text{H}_2\text{SO}_4 \). Subsequently 2 c.c. aliquots of the reaction mixture were removed at fixed intervals, treated with dilute \( \text{H}_2\text{SO}_4 \), and the quinone formed estimated iodimetrically. 10 c.c. of 10% potassium iodide was added to the aliquots, the mixture kept in the dark for 15 minutes, and the liberated iodine titrated against \( \text{N}/100 \) thiosulphate.

The course of formation of quinone bodies from catechol follows lines closely similar to that of the enzyme studied by Adams and Nelson. The conversion of catechol into the quinone is quick and quantitative only in presence of large concentrations of the enzyme. The complete conversion corresponds to an \( \text{O}_2 \) uptake equivalent to 1 atom \( \text{O}_2 \) per molecule catechol. But
On Tyrosinase of Dolichos lablab—1

The subsequent reaction which involves a further O₂ uptake observed in the manometer and which proceeds at a lower rate, yields products which do not give corresponding increases in the liberation of iodine. On the other hand, there is a steady decline in the value of iodine titre, which may be attributed to the instability of O-quinone under the experimental conditions. The iodine titre completely vanishes at the end of 3-4 hours. With lower enzyme concentrations, the oxidation to quinone is never complete but the iodine titre, after reaching a maximum value far lower than what should correspond to the complete formation of quinone, falls off. The experimental conditions were chosen so similar, as to have the iodine titre values and manometric readings directly correspond to each other.

\[
\begin{align*}
(1) & \rightarrow (2) \\
\text{and (2) or (3) + 2H I} & \rightarrow I₂ + (3) \\
I₂ &= 2Na₂S₂O₃.
\end{align*}
\]

\[i.e., \text{1 mM. catechol completely oxidised to quinone} \Rightarrow 2 \text{c.c. N. Na₂S₂O₃}
\]

\[i.e., \text{1 mgm. catechol} \Rightarrow 1.82 \text{c.c. N/100 thiosulphate.}
\]

We have moreover applied the same method for following the course of oxidation of hydroquinone through catechol as "carrier", as also of p-cresol, "dopa", tyrosine and phenol. The method is applicable here also, and can be adopted as an alternative to every case where a measure of the enzyme is obtained by the O₂ uptake and quinone is a product of the oxidation. With phenol and p-cresol the initial induction period is indicated by the absence of any iodine liberation during that period.

**Oxidation of other Substrates.**—Phenol is easily oxidised by the enzyme, and the rate of O₂ uptake, after an induction period, remains steady and proportional to the concentration of the enzyme. The rate of the O₂ uptake is higher than with p-cresol, for the same concentration of enzyme. Tyrosine also exhibits an induction period, but the rate of O₂ uptake afterwards is neither steady for a long period nor proportional to enzyme concentration. "Dopa" is oxidised and the course of oxidation is very similar to that of catechol. It can also, similarly, serve as a "carrier" in the oxidation of ascorbic acid or hydroquinone. Among other substrates, the enzyme
oxidises m-cresol and pyrogallol, but o-cresol, vanillin, p-phenylene diamine and resorcinol are not oxidised.

Summary

As a preliminary to a purification and study of the nature of the enzyme, methods for a quantitative estimation of tyrosinase have been standardised. The O\textsubscript{2}-uptake with mono- and di-hydroxy phenols has been studied in this connection. While the direct oxidation of phenol and p-cresol serves admirably as a measure of the enzyme, the oxidation of catechol fails to fulfil the conditions. To secure a steady rate of O\textsubscript{2}-uptake proportional to enzyme concentration, the direct oxidation of catechol cannot be adopted but the oxidation of either ascorbic acid or hydroquinone (or ferricyanide) through the agency of catechol as "carrier", fulfils the requirements. The formation of quinone bodies, the products of oxidation of various substrates by the enzyme, could be followed also iodimetrically.

The general substrate specificity of the enzyme suggests that it is not a "laccase" since the enzyme preparations have been found to be inert towards p-dihydroxy compounds. The fact that the enzyme preparations oxidise several mono- and di-hydric phenols, necessitates a deeper study of the influence of further purifications on substrate specificity.

REFERENCES