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ASSAY OF CATECHOL OXIDASE—A CRITICAL COMPARISON OF METHODS*

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Abstract—Manometric, polarographic, chronometric and spectrophotometric methods for the determination of catechol oxidase activity were critically compared. Where possible, product formation, disappearance of substrate and ratios between oxygen uptake and substrate disappearance were determined. Instability of products and secondary reactions interfered in some of these determinations. Initial rates measured polarographically were eight to twelve times higher than those obtained by the spectrophotometric and chronometric methods and up to 30 times greater than those obtained manometrically. The polarographic method is recommended as the most convenient and accurate method for determining catechol oxidase activity.

CATECHOL oxidase [*o*-diphenol: oxygen oxidoreductase (1.10.3.1)] reacts with two substrates, oxygen and a suitable *o*-diphenol, when its so-called "catecholase" activity is considered. The "Commission on Enzymes" of the I.U.B. recommends that the unit of activity be expressed as the amount of enzyme which catalyses the transformation of 1 μmole of substrate per min under defined conditions. For catechol oxidase, therefore, the transformation of either oxygen or phenol should be measured. The numerous methods employed[1–3] for the determination of catechol oxidase activity, measure either oxygen consumption,[4–7] product formation[8, 9] or the coupled-oxidation of an added reducing agent.[10–14] Many variations of these methods exist, depending on the phenolic substrate and the source of the enzyme investigated. But, despite the variety of methods, in none of them is the transformation of the *o*-diphenol measured directly.

The variety of methods described in the literature[4–14] makes it practically impossible to compare data referring to the reaction rates of different catechol oxidases. In addition, the

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products of catechol oxidase activity are extremely reactive compounds reacting not only with each other but with unchanged substrate and with oxygen.[2, 15, 16] Thus it is essentially impossible to follow the recommendation of the commission on enzymes and measure transformation of substrate.

In the following, four different methods (manometric, polarographic, spectrophotometric and chronometric) for assaying catecholase activity are compared, using a partially purified catechol oxidase prepared from apples.[17]

RESULTS

The principles underlying the four methods compared here are different. The manometric[7] and polarographic methods[7] both measure oxygen uptake but under different conditions, especially as far as rate of oxygen diffusion to the enzyme is concerned. In the chronometric method,[10, 14] where ascorbic acid is present in the reaction mixture, no quinone is allowed to form, and in the spectrophotometric method the reaction is measured only to the stage of 4-methyl-orthoquinone; in the former case therefore no secondary reactions can occur, and in the latter only the very initial rate is measured, so that the secondary reactions have occurred only to a limited extent. In contrast, in the Warburg technique secondary reactions do occur and initial rates cannot be measured with any accuracy.

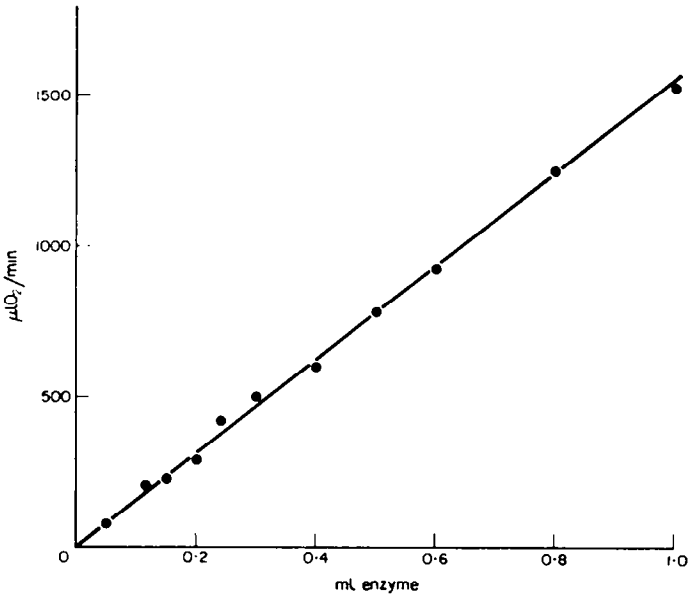

FIG. 1. DEPENDENCE OF RATE OF OXIDATION OF 4-METHYLCATECHOL BY CATECHOL OXIDASE, ON ENZYME CONCENTRATION, USING O_2 ELECTRODE.

Substrate concentration: 5×10^{-3} M.
Phosphate–citrate buffer pH 5·1.

Specific activity of the enzyme was 1300 $\mu l O_2$/mg/protein/min as determined by the Warburg technique at 26°.

The enzyme preparation used contained 50 μg protein/ml.

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Comparison of results obtained using either an O_2 electrode (polarographic) or Warburg technique showed qualitatively good agreement,[7] but considerably higher absolute reaction rates were obtained using the former method.

The rates of reaction as measured by the polarographic oxygen electrode, was independent of the volume of the gas phase above the reaction mixture. This proved that the reaction mixture was fully equilibrated with oxygen. The rate of reaction was strictly linear with increasing enzyme concentration (Fig. 1). One striking difference between the oxygen electrode method and the Warburg one is the rate of stirring, which is much more vigorous

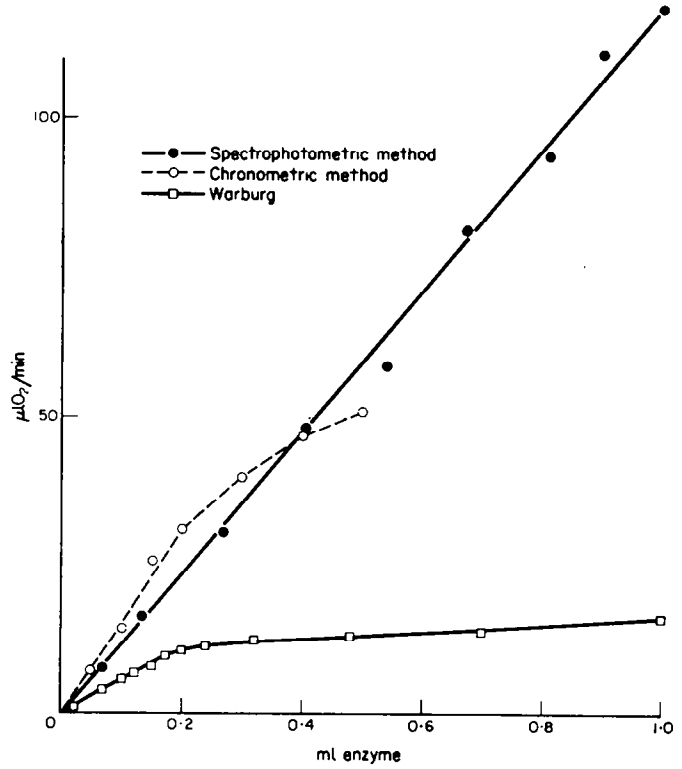

FIG. 2. DEPENDENCE OF RATE OF OXIDATION OF 4-METHYLCATECHOL BY CATECHOL OXIDASE, ON ENZYME CONCENTRATION, USING DIFFERENT METHODS OF DETERMINATION.

Substrate concentration: 5×10^{-3} M.
Phosphate–citrate buffer pH 5·1.
Enzyme preparation and specific activity as in Fig. 1.

in the former. The effect of shaking rate on the reaction rate, using the Warburg technique, was studied and it was found even at the maximal shaking rate (150/min) linearity with enzyme concentration could not be achieved (Fig. 2). In the Warburg method it is almost impossible to determine accurately an initial rate, and undoubtedly equilibration between the liquid and gas phase is poor. In the chronometric method the rate of stirring also proved to be important and the results given were obtained using vigorous mechanical stirring, using a magnetic stirrer. Hand shaking gave similar results as rapid stirring. When the rate of stirring was reduced, poorer results were obtained. In the spectrophotometric method, the rate of stirring and the resulting aeration also considerably affects the reaction rate.[8]

Using the same enzyme preparation the four methods were compared (Figs. 1 and 2).

The rates of reaction using the spectrophotometric and the chronometric methods were similar, but the former method showed better linearity than the latter. The Warburg method showed poor linearity and gave the lowest rate of oxygen uptake, the rate being 2·7 times higher in the chronometric method with small amounts of enzyme. Smith and Kruger[3] reported a ratio of 2·4. The polarographic method gave values eight to twelve times higher than the chronometric method.

Since the methods are based on three different principles it is not clear whether the ratio moles O_2 taken up to moles *o*-diphenol oxidised is the same in all of them. A fall off in the reaction rate before the reaction has gone to completion, may be caused by various factors and different ones may operate in the various methods. Simultaneous measurements of oxygen uptake, or a parameter indicating appearance of product and the disappearance of substrate were therefore made. Disappearance of substrate was determined after stopping

TABLE 1. OXYGEN CONSUMPTION AND 4-METHYLCATECHOL DISAPPEARANCE DURING MEASUREMENT OF CATECHOL OXIDASE ACTIVITY USING THE WARBURG TECHNIQUE OR OXYGEN ELECTRODE

	Warburg				Oxygen electrodes				
Substrate concentration mM	Time (min)	μmoles O_2 consumed (1)	μmoles substrate disappeared (2)	Ratio (1)/(2)	Time (min)	μmoles O_2 consumed (3)	μmoles substrate disappeared (4)	Ratio (3)/(4)	Ratio according to O_2 only*
1·25	45	0·94	0·56	1·68	8	4·42	1·36	3·25	1·77
	60	1·33	0·67	1·99					
2·5	35	2·50	1·70	1·42	5	9·50	3·70	2·56	1·90
	45	2·60	1·76	1·48					
	12	2·24	2·24	1·00	0·5	2·70	2·63	1·02	
5·0	18	3·57	2·69	1·29	0·9	4·67	4·00	1·18	1·20
	75	5·40	3·60	1·50	1·8	7·85	4·57	1·72	
	100	5·85	3·75	1·56	2·8	9·82	4·83	2·03	

0·1 ml enzyme in total reaction mixture of 2·0 ml, 0·05 M phosphate-citrate buffer pH 5·1. Substrate: 4-methylcatechol.

* Ratio μmoles O_2 consumed to μmoles substrate oxidized—assuming all substrate oxidized when O_2 consumption has ceased.

the reaction with boiling 80% ethanol. Since in the chronometric method no *o*-diphenol is oxidized, the O_2/diphenol oxidized ratio can only be calculated, and for this purpose it is usually assumed to be 0·5 mole of oxygen per mole of phenolic substrate. The results for the Warburg and oxygen electrode techniques are shown in Table 1, and the comparative data for the spectrophotometric technique are shown in Table 2.

From Table 1 it can be seen that in both the Warburg and polarographic methods molar ratios near one are only obtained during the very initial stages and at the higher substrate concentrations. Furthermore, in the case of the oxygen electrode, calculations based on the assumption that all the substrate had disappeared (column 6) gave lower ratios than the ones determined experimentally (column 5). In addition it must be remembered that from the findings of Forsyth *et al.*[18,19] it appears that dihydric or tetrahydric phenols are among the

[18] W. G. C. FORSYTH and V. C. QUESNEL, *Biochim. Biophys. Acta* **25**, 155 (1957).
[19] W. G. C. FORSYTH, V. C. QUESNEL and J. B. ROBERTS, *Biochim. Biophys. Acta* **37**, 322 (1960).

first reaction products. These will react with the molybdate reagent, used to estimate the amount of substrate in the reaction mixture. Thus an error is introduced which lowers the amount of substrate which is apparently oxidized. There is no simple way to avoid this difficulty.

Lastly it should be noted, that when the oxygen electrode is used, an additional source of possible error occurs. When the reaction has apparently reached completion, addition of further enzyme caused an additional O_2 uptake, suggesting incomplete oxidation of substrate.

From the result in Table 2 it can be seen that only during the first 30 sec of the reaction is the ratio of product formed to substrate oxidized near the expected value of 1. As the reaction proceeds this ratio drops, indicating secondary reactions of the product.

TABLE 2. 4-METHYLORTHOQUINONE FORMATION AND 4-METHYLCATECHOL DISAPPEARANCE DURING SPECTROPHOTOMETRICAL MEASUREMENT OF CATECHOL OXIDASE ACTIVITY

Time (min)	μmoles quinone formed (1)	μmoles substrate disappeared (2)	Ratio (1)/(2)
0·30	0·256	0·261	0·98
0·90	0·512	0·653	0·78
1·56	0·768	1·133	0·68
3·30	1·024	1·568	0·66
8·00	1·408	2·464	0·57

4-Methylcatechol concentration 5 mM; 0·1 ml enzyme; total volume of reaction mixture 3·2 ml, 0·05 M phosphate-citrate buffer pH 5·1.

DISCUSSION

A comparison of the methods shows that the manometric method gave the lowest absolute rates of O_2 uptake and of rate of transformation of *o*-diphenol. The chronometric and spectrophotometric methods gave rates eight to twelve times higher than the manometric one, while the polarographic method gave rates again higher by a factor of 2–2·5.

Various artefacts have been eliminated as being the cause of the high rates of this latter method. Although the electrodes themselves have a definite O_2 uptake, this is of the order of 4 μl/min which is negligible when rates of hundreds of μl/min are being measured. The possibility that electrical artefacts are involved e.g. current flow due to the presence of the quinone were also shown to be incorrect by direct measurement.

The ratio of oxygen uptake to phenolic substrate oxidized, as recorded in the literature are extremely variable. Forsyth and Quesnel[18] and Dawson and Tarpley[16] record values of 1·0–1·25 g mol. O_2/g mol. catechol oxidized. Wright and Mason[15] and Mason and Wright[20] report ratios of 1·0–1·67 for catechol and 1·7–2·3 for 3,4-dihydroxyphenylalanine (dopa), depending on pH, buffer and substrate concentration. In none of these cases was the disappearance of substrate measured. Instead the reaction was allowed to go to completion, i.e. till no further O_2 uptake was noted and it was assumed that all the substrate had disappeared. It is not clear whether this is in fact the case in the different methods used. Our results also show that this ratio differs in the different methods, depends on substrate concentration and the period during which the measurements are made.

In two methods, the polarographic and the manometric, we found, as also reported in the

[20] H. S. MASON and C. I. WRIGHT. *J. Biol. Chem.* **180**, 235 (1949).

literature, that the ratio oxygen uptake/substrate transformed changed during the reaction, being lower during the initial stages of the reaction and also changes with the substrate concentration. Moreover, ratios calculated from oxygen uptake do not agree with those determined directly, by measurement of substrate disappearance. As mentioned there is no simple way of accurately determining the ratios, but ratios as high as 2 moles O_2/mole substrate oxidized were measured. It is impossible to account for these ratios on the basis of the mechanism of oxidation usually proposed for catechol oxidase. Even if secondary oxidations occur, as they undoubtedly do, those previously described do not account for the high ratios observed. Data in the literature indicate that ratios are higher when dopa is oxidized, than in the case of catechol.[15, 20] In the experiments described here 4-methylcatechol was the substrate; there is nothing to suggest that this compound should differ greatly from catechol, and 4-substituted orthoquinones are more stable in water than orthoquinone itself.[16]

The high molar ratios of O_2 consumed/substrate oxidized, as obtained in the oxygen electrode method are not sufficiently great to account for the much higher absolute rates of oxygen uptake obtained using this method. When determining rates using this method, only the initial rates were considered, when the ratios did not deviate greatly from one, during the first 30 sec of the determination.

Certain drawbacks are inherent in each of the methods described. One of these is probably due to the low affinity of the enzyme for oxygen, which makes high rates of stirring, to attain good equilibration, essential.[21] Part of the difference between Warburg and polarographic techniques is no doubt due to this. In addition, the initial rates are measured easily in the latter and cannot be measured in the Warburg.

The fact that in the chronometric method a coupled oxidation is measured without product formation sharply differentiates this method from the spectrophotometric one. In the latter, product formation is measured directly, but due to the instability of the product this can be done only for a short initial rate lasting less than 30 sec.

It is clear that due to the nature of the reaction the amount of substrate transformed, whether oxygen or *o*-diphenol, cannot be determined since secondary reactions invariably occur. The recommendation of the commission on enzymes cannot be conveniently followed. The most convenient and probably most accurate method of determination is apparently the polarographic method. It permits the determination of initial rates, accurately, and with large numbers of replicates which can be handled rapidly.

EXPERIMENTAL

A partially purified catechol oxidase from apple chloroplasts, prepared according to Harel *et al.*[20] was used.

Warburg and Oxygen Electrode Techniques

Both methods were used as already described,[7] except that the total volume of the reaction mixture was 2·0 ml consisting of 0·5 ml 2×10^{-2} M 4-methylcatechol and 1·5 ml enzyme in phosphate–citrate buffer 0·05 M,pH 5·1.

Chronometric Method[10, 14]

To a 25 ml Erlenmeyer flask were added 9 ml phosphate-citrate 0·1 M pH 5·1 containing $5{\cdot}5 \times 10^{-5}$ M EDTA, 1·0 ml sodium ascorbate (0·5 mg/ml) and 0·1 ml *p*–phenylenediamine

[21] W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, *Manometric Techniques* Chap. I. Burgess, Minneapolis (1957).

(1 mg/ml). After equilibrating for 5 min at 25°, 0·4 ml enzyme solution was added. At zero time, 0·5 ml 4-methylcatechol were added to give a final concentration of 5×10^{-3} M. The reaction mixture was either shaken by hand or stirred with a magnetic stirrer. *p*-Phenylenediamine was added in order to sharpen the end point, as the appearance of the bright yellow colour of the quinone is difficult to detect. The end point as determined in presence of *p*-phenylenediamine was always observed 1–3 sec earlier than in its absence.

The Spectrophotometric Method

To 2·4 ml enzyme in phosphate-citrate buffer 0·05 M pH 5·1, 0·8 ml 4-methylcatechol was added at zero time. The increase in absorptivity at 395 mμ (the λ_{max} of the corresponding quinone) was followed using a Bausch Lomb spectronic 505 recording spectrophotometer equipped with time rate accessory and a V.O.M. 5 recorder. In ether the λ_{max} occurs at 375 mμ, but in water the peak shifts to 395 mμ, and rapidly disappears. For this reason only the initial rates, for 30–60 sec were measured. The enzymic reaction mixture had an identical absorption spectrum with that of synthetic quinone, between 200 and 500 mμ.

4-*Methylbenzo*-2,3-*quinone*

The quinone was prepared[22] by shaking 2·5 g 4-methylcatechol with 10·5 g freshly precipitated silver oxide in 110 ml ether and 8 g anhydrous sodium sulphate for 30 min. The silver oxide was removed by filtration.

The relation between 4-methylbenzo-2,3-quinone concentration and absorptivity at 395 mμ in H_2O was determined using a Beckman D.U. spectrophotometer. The molar extinction coefficient of the quinone both in ether and in water is 1350.

Determination of 4-*Methylcatechol*

4-Methylcatechol was determined using the sodium molybdate method.[23] The enzymic reaction was stopped by addition of boiling 8 ml 80% ethanol per 2 ml reaction mixture. To 5 ml of this mixture 6 ml water and 1 ml 5% sodium molybdate in 50% ethanol were added. The mixture was made up to volume and the absorptivity read at 350 mμ after standing for 15 min at room temperature using a suitable control.

Standards were run along with each experiment.

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[23] L. W. MAPSON, T. SWAIN and A. W. TOMALIN, *J. Sci. Food Agric.* **14**, 673 (1963).