# A nonenzymic oxygen uptake and its implications in the assay of 4-hydroxyphenylpyruvate dioxygenase by an oxygen electrode

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*Summary.* The oxygen electrode provides a rapid, convenient assay for 4-hydroxyphenylpyruvate dioxygenase. However, due to the occurrence of a nonenzymic oxygen consumption when reducing agents were mixed with *a,a'*-dipyridyl, its use is restricted to species which do not require reducing agents or necessitates the addition of catalase to the reaction mixture.

Hepatic tyrosine aminotransferase (EC 2.6.1.5) is the primary factor which normally determines blood tyrosine levels[2]. However, the 2nd enzyme in the catabolic pathway of tyrosine, 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27), may become the controlling factor in certain conditions due to its unusual kinetic properties[3]. Although it has been widely accepted that ascorbic acid and $Fe^{2+}$ are involved in the conversion of 4-hydroxyphenylpyruvate to homogentisate their roles are unclear. Certain purification procedures[4] cause reversible inactivation of 4-hydroxyphenylpyruvate dioxygenase (analogous to reaction inactivation[5]). Thus the apparent requirement of ascorbic acid may merely reflect a need for enzyme reactivation. Metal assays do not support the presence of iron in the enzyme[6].

With the exception of cumbersome Warburg manometry 4-hydroxyphenylpyruvate dioxygenase activity has almost exclusively been studied by the enol-borate method. However, Goodwin[7] suggests that less than 50% of the apparent enol-borate activity in extracts from fresh liver is due to the 4-hydroxyphenylpyruvate dioxygenase. In the present investigation a Clark-type oxygen electrode has been used to develop a rapid continuous assay for 4-hydroxyphenylpyruvate dioxygenase. This method brought to light a hitherto undocumented nonenzymic interaction between components of the assay mixture, and led to certain conclusions on the phenomenon of reaction inactivation.

*Materials and methods.* Pig livers were obtained from freshly killed animals (abattoir) and immediately chilled before returning to the laboratory. All the chemicals used, except standard laboratory reagents, were obtained from Sigma (London) Chemical Company.

*Enzyme preparation.* All operations were carried out at 4 °C. the liver was homogenized in a Waring Blender for 2 min with 3 vol. of ice cold 0.15 M KCl in 0.002 M NaOH. The resulting homogenate was passed through 1 layer of cheesecloth to remove gross particles and then centrifuged at 20,000 × g for 15 min. The postlysosomal supernatant was then centrifuged at 105,000 × g for 30 min. The resulting cytosol contains all the enzymes necessary for the conversion of L-tyrosine to acetoacetic acid[8].

Protein was determined by a slightly modified Lowry technique[9] with bovine serum albumin as a standard. 4-Hydroxyphenylpyruvate dioxygenase assays were carried out at 30 °C. The basic incubation medium contained chemical reagents and different enzyme preparations made up to a total of 2.0 ml by the addition of the appropriate volume of 0.2 M potassium phosphate pH 6.5 (for details see table and figures). Sodium dithionite was used to set zero when calibrating the oxygen electrode. The 'air line' was adjusted to an appropriate value with 2.0 ml of air saturated distilled water. The amount of oxygen in µmoles was determined from the empirical formula of Truesdale and Downing[10].

4-Hydroxyphenylpyruvate dioxygenase was also measured, for comparison, by following the disappearance of the enolborate complex of 4-hydroxyphenylpyruvate at 310 nm.

*Results and discussion.* Preliminary experiments, using the oxygen electrode, showed that homogentisate 1,2-dioxygenase (EC 1.13.11.5) is seven times more active than

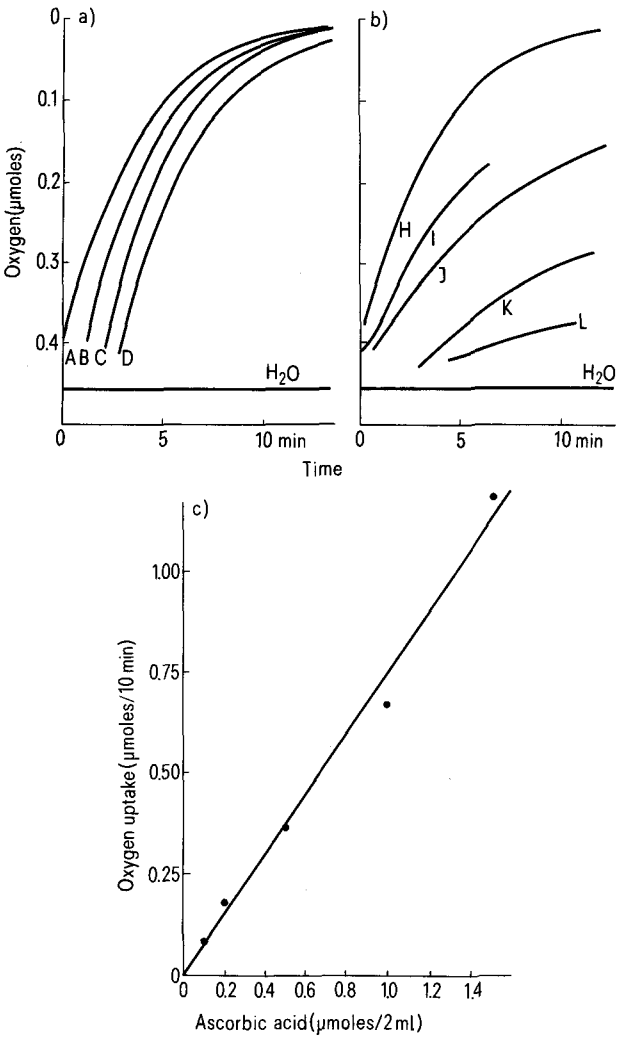


Figure 1. Effects of varying concentrations of *a,a'*-dipyridyl and ascorbic acid on the oxygen uptake in the nonenzymic reaction between these 2 substances. *a* Effects of different concentrations of *a,a'*-dipyridyl (2 µmoles of ascorbic acid). A, 2.0 µmoles *a,a'*-dipyridyl; B, 1.0 µmole *a,a'*-dipyridyl; C, 0.5 µmoles *a,a'*-dipyridyl; D, 0.1 µmoles *a,a'*-dipyridyl. *b* Effects of different concentrations of ascorbic acid (2 µmoles *a,a'*-dipyridyl). H, 2.0 µmoles ascorbic acid; I, 1.5 µmoles ascorbic acid; J, 1.0 µmole ascorbic acid; K, 0.5 µmoles ascorbic acid; L, 0.1 µmoles ascorbic acid. *c* Relationship between the initial rate of the nonenzymic oxygen uptake and the concentration of ascorbic acid (in the presence of 2 µmoles *a,a'*-dipyridyl).

4-hydroxyphenylpyruvate dioxygenase. Thus the former must be inhibited before the activity of the latter can be assayed by the oxygen electrode. Two methods are available, namely acid denaturation[8] and inhibition by chelating agents[11]. The former method resulted in only partial inhibition (94.7%) as measured by both the oxygen electrode and by the Fellman assay[12] while the use of 1 µmole $\alpha,\alpha'$-dipyridyl resulted in complete inhibition of homogentisate 1,2-dioxygenase without reducing the activity of 4-hydroxyphenylpyruvate dioxygenase.

In the course of this work it became evident that a nonenzymic reaction took place between ascorbic acid and $\alpha,\alpha'$-dipyridyl. Both the rate and extent of this reaction became greater as the ascorbic acid concentration increased (fig. 1,a and b). Indeed, there is a direct proportionality relationship (fig. 1,c). The phenomenon does not appear to have been reported elsewhere. The reaction illustrates the strength and weakness of the Clark-type oxygen electrode in studies of this type. Its strength lies in the sensitivity with which the uptake of oxygen can be determined and its weakness is the limited amount of oxygen available in the reaction chamber. The reaction between $\alpha,\alpha'$-dipyridyl and ascorbic acid consumes a large proportion of the total oxygen present in the 2 ml reaction vessel used; however, the reaction quickly reaches a plateau value. $\alpha,\alpha'$-Dipyridyl is chemically unreactive and thus it seems unlikely that it takes a part in anything other than chelating metal ions. A possible explanation for the nonenzymic reactions may be proposed on the basis of the information given in the table and in figure 2. In the presence of reducing agents such as ascorbic acid, reduced glutathione or reduced 2,6-dichlorophenol indophenol the traces of iron in the reagents and buffer will be present as $Fe^{2+}$. Now $Fe^{2+}$ decomposes hydrogen peroxide[13] and $\alpha,\alpha'$-dipyridyl, when present, chelates this $Fe^{2+}$, thus hydrogen peroxide accumulates as it is produced and so an enhanced oxygen uptake is observed. This is consistent with the modulating effect of catalase (table).

Under the conditions used, pig liver 4-hydroxyphenylpyruvate dioxygenase preparations do not require ascorbic acid for their activity until at least 4 days after preparation (fig. 2, 0.25 µmoles $O_2$/10 min). This observation was confirmed by the enol-borate method (0.26 µmoles $O_2$/10 min). Reports such as those of Taniguchi et al.[14] stating that 4-hydroxyphenylpyruvate dioxygenase has an absolute requirement for ascorbic acid may be explained on the basis of the information shown in figure 2. Chloroform, which has been used in many enzyme preparations in the past, makes the reaction dependent on ascorbic acid. The addition of catalase may, therefore, be essential in any attempt to fully purify 4-hydroxyphenylpyruvate dioxygenase.

In conclusion, the oxygen electrode provides an excellent measure of 4-hydroxyphenylpyruvate dioxygenase activity under conditions where maximum enzyme activity is obtainable in the absence of a reducing agent. However, after certain treatment or in different species a reducing agent is required for expression of 4-hydroxyphenylpyruvate dioxygenase activity. In such cases alternative methods of inhibiting homogentisate 1,2-dioxygenase other than $\alpha,\alpha'$-dipyridyl must be used, e.g. lowering in pH to 5[8]. In the present studies (oxygen electrode and the Fellman technique[12]) residual homogentisate 1,2-dioxygenase activity was observed following the latter treatment.

Oxygen utilization in certain nonenzymic reactions in buffer (pH 6.5)

| Reactants | µmoles $O_2$/5 min × $10^2$ |
|---|---|
| 2 µmoles ascorbic acid | 2.0 |
| 20 µmoles reduced neutralized gluthathione | 2.5 |
| 0.25 µmoles reduced 2,6-dichlorophenol indophenol | 2.5 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl | 0.0 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl + 2 µmoles ascorbic acid | 37.5 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl + 2 µmoles ascorbic acid + 1600 µg catalase | 20.0 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl + 2 µmoles ascorbic acid + 20 µmoles reduced neutralized gluthathione | 15.0 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl + 20 µmoles reduced neutralized gluthathione | 13.0 |
| 2 µmoles $\alpha,\alpha'$-dipyridyl + 0.25 µmoles reduced 2,6-dichlorophenol indophenol | 6.5 |

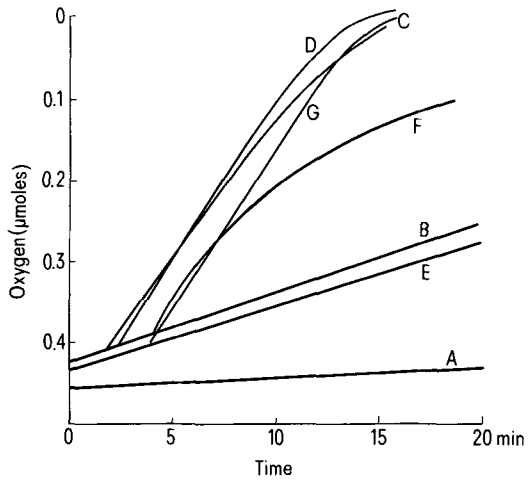


Figure 2. Effect of ascorbic acid on 4-hydroxyphenylpyruvate dioxidase as measured by oxygen uptake. A, Control (buffer pH 6.5); B, 0.5 ml acid treated 105,000 × g supernatant; C, 0.5 ml acid treated 105,000 × g supernatant + 1 µmole 4-hydroxyphenylpyruvate; D, as for C + 2 µmoles ascorbic acid; E, 0.5 ml of chloroform treated 105,000 × g supernatant; F, as E + 1 µmole 4-hydroxyphenylpyruvate; G, as F + 2 µmoles ascorbic acid.

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