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EFFECTS OF DITHIOTHREITOL ON DOPA OXIDASE ACTIVITY FROM POTATO TUBERS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato tuber; *o*-diphenol oxidase; DOPA oxidase; inhibition by dithiothreitol.

Abstract—A filtrate, prepared from potato tuber by grinding in an isotonic medium, has been separated into a particulate and a 'soluble' fraction by ultracentrifugation. Following dialysis and lyophilization, both fractions catalysed the oxidation of L-DOPA, with approximately 30% of the L-DOPA : oxygen-oxidoreductase (EC 1.14.18.1; DOPA oxidase) activity being associated with the particulate fraction. When dithiothreitol (DTT, 10^{-2} M) was included in the grinding medium, much lower yields of DOPA oxidase were obtained and 80% appeared to be associated with the particulate fraction. DTT proved to be a powerful inhibitor of DOPA oxidase. With concentrations of DTT causing only partial inhibition, the kinetics of the inhibited rate of dopachrome formation from L-DOPA were complex. When oxygen consumption was measured inhibition was not transient. The degree of inhibition was inversely related to the DOPA oxidase activity, indicating interaction of a product of this activity with DTT. Direct determination of –SH groups in DTT using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) showed that they were all oxidised during the initial phase of inhibition of dopachrome formation. It is concluded that the first phase of inhibition involves oxidation of DTT by an intermediate between L-DOPA and dopachrome. The second phase of inhibition also appeared to require –SH groups initially, since *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT) caused very little inhibition at all.

INTRODUCTION

Dithiothreitol (DTT) is a highly water-soluble solid with little tendency to be oxidized directly by air [1] and has been used to maintain thiol groups of proteins in the reduced state. It is also used during tissue disruption to prevent enzymic browning resulting from oxidation of phenolic compounds catalysed by phenolase [2, 3]. Using a grinding medium containing 10^{-2} M DTT, Ruis [2] found that 56% of the *o*-diphenol oxidase activity from a potato (*Solanum tuberosum*) tuber filtrate occurred in a particulate fraction which precipitated during centrifugation (100 000 *g* for 2 hr). This was referred to as the particulate fraction, while the activity remaining in the supernatant was referred to as the 'soluble' fraction. McIlroy [3], using the same grinding medium [2] but possibly a more gentle technique, found 80% of *o*-diphenol oxidase activity was associated with the particulate fraction. McIlroy [3] put forward a hypothesis to explain the connection between physical damage to potato tubers and the subsequent development of enzymic browning. It was suggested that *o*-diphenol oxidase was contained in membrane-bound vesicles like peroxisomes, which were impermeable to phenolic substances. Hence no enzymic browning could take place until the membranes surrounding the vesicles were ruptured, when the enzyme would react with its substrate to initiate a series of chemical reactions, possibly of the type proposed by Blois [4].

Experiments in this paper show that DTT is a powerful inhibitor of potato tuber DOPA oxidase. DTT caused a greater inhibition of the enzyme in the 'soluble' phase than in the particulate phase. The nature of the inhibition has been investigated and the results are discussed briefly with respect to current concepts of the biochemistry of bruising in potato tuber tissue.

RESULTS

The inclusion of DTT in the grinding medium was found to result in a significant reduction of the activity of DOPA oxidase measured by dopachrome formation. It can be seen from the data in Table 1 that the total maximum rate of dopachrome formation was decreased from 7.6 and 6.3 A_{475} units min^{-1} g $tissue^{-1}$ (experiments 1 and 2) to 1.41 and 0.86 A_{475} units min^{-1} g $tissue^{-1}$ (experiments 3 and 4), by grinding the tissue in a medium containing 10^{-2} M DTT. Thus DTT caused 80–90% inhibition of the total activity. In addition, the percentage activity appearing in the particulate fraction increased from 30 to 78–85% as a result of DTT inclusion in the grinding medium. The data show that the change in distribution of DOPA oxidase occurred because the 'soluble' component was more inhibited than the particulate fraction.

When the rate of dopachrome formation catalysed by the enzyme prepared in the absence of DTT in the grinding medium (control enzyme) was measured with either DTT or oxidized DTT present at increasing initial concentration in the assay medium, the data presented in Fig. 1 were obtained. It can be seen that only the reduced form caused the major degree of inhibition with a maximum corresponding to 10^{-4} M DTT.

The rates used to plot Fig. 1 were maximum rates measurable when DTT was added before the enzyme.

Table 1. Effect of 10^{-2} M DTT in the grinding medium on the activity and distribution of DOPA oxidase

Experiment No.	Fraction	Weight of lyophylate (g) from 100 g potato tissue	Dopachrome formation K_m (L-DOPA mM)	Dopachrome formation V_{max} (A_{475} units min^{-1} g tissue^{-1})	% Activity in the particulate fraction
1	'Soluble'	2.3	5.9	5.3	30
Control	Particulate	0.36	4.4	2.3	
2	'Soluble'	2.0	6	4.5	29
Control	Particulate	0.36	6.7	1.8	
3	'Soluble'	2.1	ND	0.21*	85
+ DTT	Particulate	0.34	20	1.2	
4	'Soluble'	1.98	ND	0.19*	78
+ DTT	Particulate	0.34	12.5	0.67	

Where possible V_{max} and K_m values were determined from $1/V$ vs $1/S$ plots of V vs S data, where V = A units at 475 nm min^{-1} g potato tissue^{-1} and S = L-DOPA concentration, mM. 0.5 g of lyophylate were used per assay. * = value measured with 30 mM L-DOPA as substrate; ND = not determinable. Experiment numbers 1 and 2 are controls (no DTT in grinding medium) and 3 and 4 had 10^{-2} M DTT in the grinding medium.

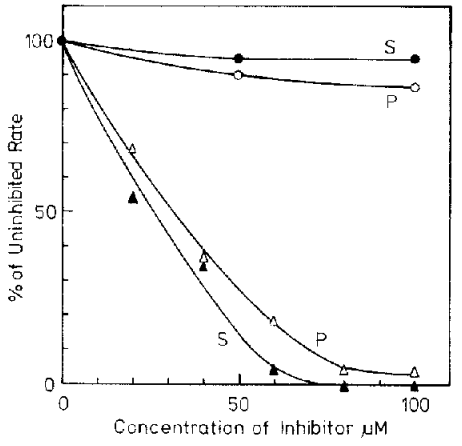

Figure 1. Effect of DTT and *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT) on 'soluble' (S) and particulate (P) DOPA oxidase activity. Dopachrome formation was followed spectrophotometrically at 475 nm, using 1 and 0.5 mg lyophylate per assay for 'soluble' (S) and particulate (P) fractions, respectively. For details see Experimental. DTT (▲, △) or oxidized DTT (●, ○) was added to the reaction cuvette before DOPA oxidase and rates of dopachrome formation were maximum rates measurable. The initial concentration of L-DOPA was 25 mM and the 100% values were 5.3 and 2.9 A_{475} units min^{-1} g potato tissue^{-1} for 'soluble' and particulate fractions, respectively.

In fact, under these conditions, the kinetics of dopachrome formation were not simple as Fig. 2 shows. An initial phase of very great inhibition was followed by recovery to a less inhibited phase of dopachrome formation. The duration of the first phase and the extent of inhibition during the second phase were directly related to the amount of DTT added. The transient nature of the initial response is consistent with an intermediate between L-DOPA oxidation by oxygen and the formation of dopachrome, acting as an oxidant of DTT during the first inhibited phase of dopachrome formation.

Consistent with this hypothesis was the finding that an increase in the initial rate of L-DOPA oxidation resulted in a decrease in the duration of the initial phase of inhibition of dopachrome formation caused by DTT, as the data in Fig. 2 also show. In Fig. 2A, the rate of L-DOPA oxidation was increased by increasing the concentration of L-DOPA initially present in the reaction cuvette, keeping the enzyme concentration constant, and in Fig. 2B, the initial concentration of L-DOPA was kept constant while the enzyme concentration was varied.

Dopachrome itself was unlikely to be acting as an oxidant of DTT, since DTT added before or after establishment of a rate of production of dopachrome resulted in a similar pattern of inhibition, i.e. there was no evidence for the absorbance at 475 nm decreasing indicating that dopachrome formation stopped temporarily following DTT addition, and did not appear to be oxidized under these conditions (Fig. 3).

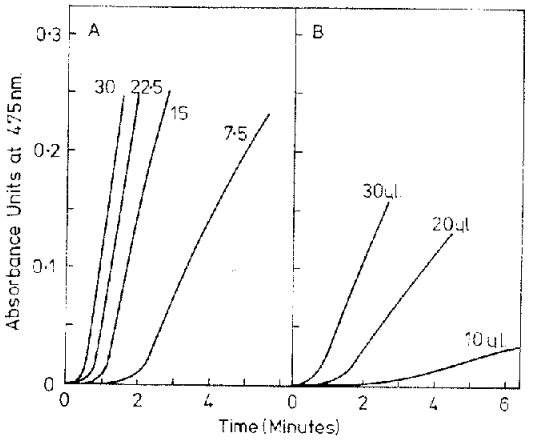

Fig. 2. Effect of DOPA oxidase activity on the degree of inhibition of dopachrome formation by DTT. (A) Constant enzyme concentration (2 mg lyophylate ml^{-1}; 'soluble' fraction) and variable initial concentrations of L-DOPA, as indicated by the numerals adjacent to curves (mM). Control rates of dopachrome formation (minus DTT) were 5.9, 4.6, 3.7 and 2.5 A_{475} units min^{-1} g potato^{-1} for 30, 22.5, 15 and 7.5 mM L-DOPA, respectively. (B) Constant initial L-DOPA concentration (25 mM) and variable enzyme concentration. Volumes adjacent to each curve represent the volumes of enzyme suspension used in each case, taken from a stock suspension containing 0.05 g lyophylate ml^{-1}. Control rates were 4.5, 8.8 and 12.5 A_{475} units min^{-1} with 10, 20 and 30 μl enzyme, respectively.

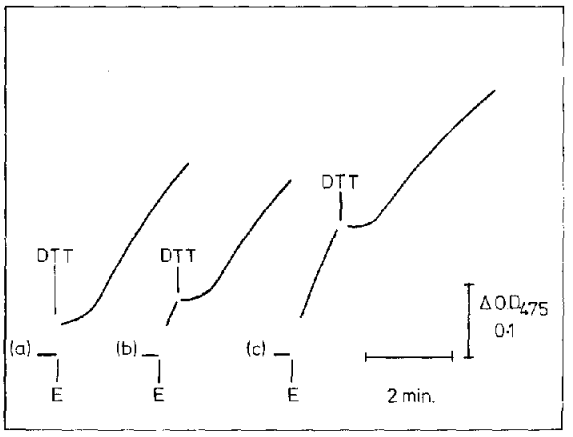

Fig. 3. Effect of time of addition of DTT on the pattern of inhibition of dopachrome formation. Initial L-DOPA concentration was 15 mM. E = enzyme as 1 mg lyophylate ('soluble' fraction) per ml assay volume. Initial DTT concentration was 2.5 μM.

If an intermediate between L-DOPA interacting with oxygen and the subsequent formation of dopachrome were to be acting as an oxidant of the –SH groups of DTT during the initial phase of inhibition caused by this compound, then no inhibition by DTT of the rate of oxygen consumption would be expected. Data in Fig. 4 support this hypothesis, since no transient initial phase of inhibition was observed when oxygen consumption was measured. Instead, inhibition to a constant rate of oxygen consumption occurred.

The data shown in Figs. 2–4 were obtained using 'soluble' fractions of DOPA oxidase and essentially similar data were obtained using the particulate enzyme, with the possibility that inhibition was less powerful compared with that found with the 'soluble' enzyme. Sodium metabisulphite was found to cause a similar pattern of inhibition to DTT, with 2×10^{-4} M causing total inhibition of dopachrome formation under similar experimental conditions to those used to produce the data for Fig. 1. Sodium metabisulphite at a concentration of 2×10^{-3} M has been used to prevent enzymic browning during the isolation of mitochondria from plant tissues [5], which it does very well, without apparently adversely affecting mitochondrial enzyme activity or membrane integrity.

To confirm that oxidation of DTT was taking place during the initial phase of inhibition of dopachrome

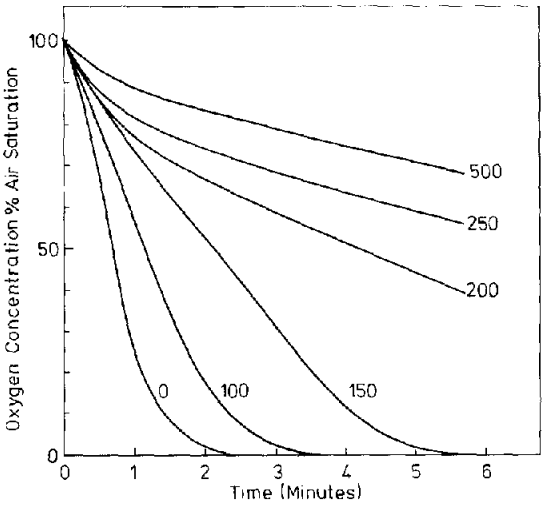

Fig. 4. Effect of DTT on oxygen consumption resulting from DOPA oxidase activity. Oxygen electrode traces produced using 8 mg lyophylate ('soluble' fraction) ml^{-1} assay volume. The numerals adjacent to the curves represent initial concentrations of DTT (μM) added 30 seconds before t_0. At t_0 the enzyme was added. The control rate (0 μM DTT) was 0.67 μmol oxygen min^{-1} g potato $^{-1}$.

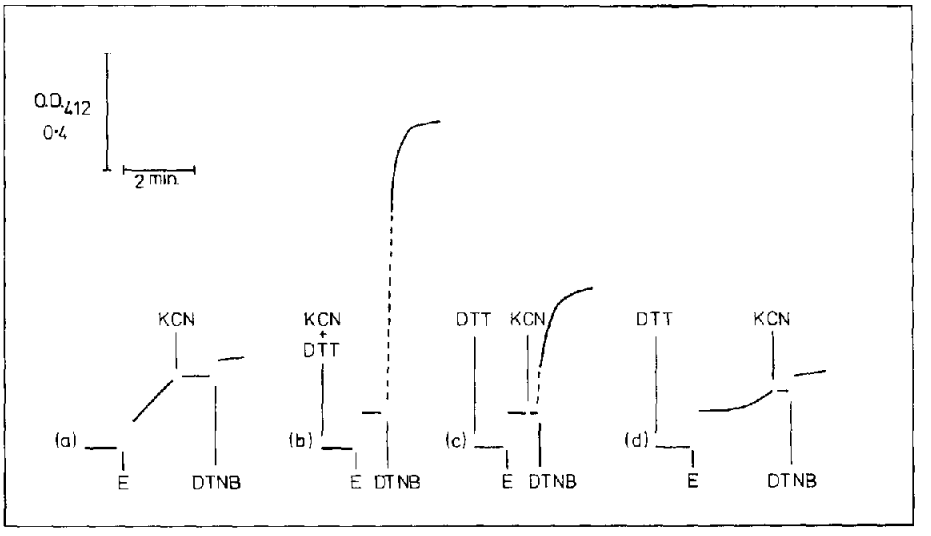

Fig. 5. Estimation of –SH groups of DTT before, during and after the first phase of inhibition of dopachrome formation. Additions to reaction cuvette were as follows: E, DOPA oxidase as 1.5 mg lyophylate, 'soluble' fraction; KCN (potassium cyanide), 5 μmol; DTNB, 0.2 μmol; DTT, 30 nmol. The assay medium was 0.1 M phosphate buffer, pH 6, containing L-DOPA at a concentration of 12.5 mM. For other details see Experimental.

formation, DTNB was used to estimate -SH groups in DTT before, during and after the initial inhibited phase. First, it was confirmed that only DTT and not oxidized DTT gave a coloured product with DTNB by measuring the absorbance at 412 nm as a function of DTT and oxidized DTT concentrations, respectively. Within the concentration range 0–50 μM, DTT produced a coloured complex with DTNB whose absorbance at 412 nm was directly related to the DTT concentration. Then DTNB was added to the reaction cuvette in which DOPA oxidase activity was being followed at 412 nm, in the presence of DTT, and Fig. 5 shows that once the rate of dopachrome formation had recovered from DTT (Fig. 5d), no coloured product was formed with DTNB, indicating complete oxidation of the –SH groups in the DTT during the initial phase of inhibition. It is of interest to note that the second phase of inhibition persisted after oxidation of the –SH groups of DTT.

DISCUSSION

Dithiothreitol prevents enzymic browning when it is added to aqueous media in which potato tuber tissue has been ground. Results presented here suggest, however, that it does so by a mechanism which renders a large proportion of the enzyme catalysing the process, phenolase, permanently inhibited. A temporary inhibition had been expected while the enzyme was in contact with the dithiothreitol, as appears to be the case with 2-mercaptobenzothiazole for example [6], but the finding that the inhibition persisted after centrifugation and dialysis was not expected. In addition, there was evidence for the enzyme in the 'soluble' fraction of the potato tuber filtrate being more sensitive to dithiothreitol than the particulate enzyme fraction. This differential sensitivity between the two fractions resulted in a change in apparent distribution of DOPA oxidase activity between the fractions which favoured the particulate rather than the 'soluble' fraction. Thus, the activity remaining in the particulate fraction, prepared with 10^{-2} M dithiothretiol present in the grinding medium, represented approximately 80% of the total activity compared with only 30% in the control experiment, where enzymic browning was not prevented (Table 1). The total DOPA oxidase activity had, however, been inhibited from approximately 7 to 1 A_{475} units min^{-1} g potato $tissue^{-1}$ (Table 1) by the presence of 10^{-2} M dithiothreitol in the grinding medium. While enzymic browning may be considered detrimental to enzyme activity [7], more DOPA oxidase activity was retained when browning was not prevented. It is concluded therefore that dithiothreitol is probably more detrimental than enzymic browning to the yield of DOPA oxidase from potato tissue.

Ruis [2] found 56% of *o*-diphenol oxidase activity from fresh potato slices was in the particulate fraction and McIlroy [3], using a similar method, found 80% particulate. McIlroy [3] used a tissue grinding technique which may have been more gentle than that used by Ruis [2], i.e. a Serval Omnimixer at half-speed for 10 sec compared with 10 min with an onion chopper. However, both authors had 10^{-2} M dithiothreitol in their grinding medium, and there was evidence for a low total yield of enzyme in the case of Ruis [2], who reported a total activity of 28.2 U *o*-diphenol oxidase from 50 g of fresh slices of potato tuber cv 'Allerfrüheste Gelbe (Böhm)'. This compares with 95 U from 50 g tubers found in this study when prepared without dithiothreitol and using L-DOPA as the *o*-diphenol substrate and cv 'King Edward'. It should be noted that Ruis [2] was using the method of Balasingham and Ferdinand [7] to assay for *o*-diphenol oxidase, in which 4-methylcatechol was used as the *o*-diphenol substrate. Potato *o*-diphenol oxidase can catalyse the oxidation of 4-methylcatechol 50–100 times faster than L-DOPA [7, 8], so it is possible that much of the *o*-diphenol oxidase activity released from the potato tissue had also been permanently inhibited by dithiothreitol in the data presented by Ruis [2].

From the foregoing discussion, it will be clear that it is not possible to confidently say how much of the *o*-diphenol oxidase in potato tissue is particulate and how much 'soluble'. From the effects of dithiothreitol on the activity and apparent distribution between the two fractions described here, it is considered that the value of 30% particulate is a better guide than the 80% reported by McIlroy [3] and reported here when dithiothreitol was present in the grinding medium.

In relation to the mechanism of inhibition of DOPA oxidase caused by DTT, the data presented here are consistent with there being two modes of inhibition, one mode which is temporary and probably involves the oxidation of DTT by a product of L-DOPA oxidation, and a second mode which results in permanent inhibition. There is strong evidence from spectroscopic studies for the production of a series of quinone products following the initial oxidiation of *o*-diphenols catalysed by *o*-diphenol oxidase [4, 6, 9, 10]. In addition, thiol containing compounds have been shown to react with such quinone oxidation products by a non-enzymatic reaction to produce *o*-diphenol–thiol conjugates [10]. Cysteine formed the conjugate, *S*-(2,3-dihydroxyphenyl)cysteine, with a product of pyrocatechol oxidation, and both cysteine and the conjugate caused a permanent inhibition of *o*-diphenol oxidase activity, while only cysteine resulted in a marked initial temporary phase of inhibition preceeding the permanently inhibited state. The similarity between the data presented here for dithiothreitol to that reported for cysteine and mushroom *o*-diphenol oxidase [10] probably means that dithiothreitol forms a conjugate with an *o*-quinone product of L-DOPA oxidation, which in turn is inhibitory to the enzyme which catalysed its production.

Deinum *et al.* [11] have reported that reaction of *Neurospora* tyrosinase with β-mercaptoethanol resulted in a green, enzymatically completely inactive complex displaying unusual EPR characteristics. The formation of similar, though not obviously coloured complexes may be involved in the inactivation of potato *o*-diphenol oxidase caused by the presence of dithiothreitol in the tissue grinding medium used in this study.

The doubt raised by the present study concerning the precise subcellular location of *o*-diphenol oxidase, and the finding of Matheis and Belitz [12] that a sample of 'soluble' *o*-diphenol oxidase from potato tuber could be separated into seventeen active bands by polyacrylamide gel electrophoresis, suggests that the mechanism of prevention of enzymic browning *in vivo* could be difficult to elucidate. Reducing agents have been shown to inhibit DOPA oxidase, which may indicate their involvement in retarding *o*-diphenol oxidase activity and hence enzymic browning *in vivo*. Clearly, considerably

more work is required in order to approach an understanding of the mechanism and sequence of events occurring during enzymic browning in potato tuber tissue.

EXPERIMENTAL

Extraction and preparation of fractions. Potato tubers cv 'King Edward' were purchased from a local market and stored in polyethylene film bags in the dark at 12–15° until required. Particulate and 'soluble' fractions were obtained in the following manner. Tubers were scrubbed under cold H_2O to remove soil and chilled to 4° by immersion in a mixture of ice and distilled H_2O for *ca* 10 min before grinding. 100 g tuber were gently ground by hand in 100 ml of the medium described by Ruis [2], using a flat cheese grater with holes of 2 mm dia. The grinding medium contained 0.4 M sucrose, 0.165 M Tricine, 10^{-2} M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M EDTA, 10^{-2} M DTT and 0.2% (w/v) bovine serum albumin, adjusted to pH 7.5 with KOH, and was kept at 1–4° on ice. For control preparations of enzyme, DTT was omitted. In each case, the resultant slurry was filtered through 2 layers of muslin and the filtrate vol. recorded to facilitate the calculation of enzyme activity per g fr. wt of potato tuber tissue ground. The filtrate was then centrifuged at 1000 *g* for 5 sec in a Sorvall RC2-B refrigerated centrifuge to precipitate starch grains and large cell debris. A known vol. of the supernatant was then centrifuged at 100 000 *g* for 2 hr in a Beckman L-50 ultracentrifuge at *ca* 10°. The resulting supernatant contained the 'soluble' enzyme fraction and the pellets contained the particulate fraction. The pellets were resuspended in the grinding medium minus DTT, combined, homogenized with a teflon-glass homogenizer and made to a vol. of 10 ml with the same medium. The control and plus-DTT fractions were then dialysed separately (to avoid cross contamination by DTT) against 5 l. of 5 mM phosphate buffer, pH 7 at *ca* 5°, with continuous stirring for 20 hr. The dialysis soln was changed once after 8 hr. The dialysates were then lyophilized separately using an Edwards Modulyo freeze drier, and the lyophylate weighed and stored in a desiccator at *ca* −15°. The samples were reweighed before use to prevent errors introduced by the lyophylate absorbing atmospheric moisture. Aliquots of the lyophylates were weighed out and suspended in 0.1 M phosphate buffer, pH 6 at 1–4°, before assay.

Assay of DOPA oxidase activity. The method described by ref. [12] was used to assay for dopachrome formation as catalysed by each fraction of enzyme. The assay medium was 0.1 M phosphate buffer, pH 6, and dopachrome formation was followed as increased absorbance measured at 475 nm. Stock 30 mM solutions of L-DOPA were prepared immediately before a given set of assays. The solid was dissolved in phosphate buffer, 0.1 M at pH 6, by warming. O_2 consumption resulting from the oxidation of L-DOPA was measured continuously using a Rank oxygen electrode having a 1 ml capacity reaction chamber and connected to a Servoscribe chart recorder. All reactions were carried out at room temp. (22–23°) in a reaction vol. of 1 ml. O_2 concn was taken as 270 μM [13].

Assay of -SH *groups in DTT.* An aq. soln of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [14] was prepared at a concn of 10 mM and 20 μl used to react with -SH groups in a 1 ml reaction vol. Absorbance increase at 412 nm was measured.

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