Biochemical Pharmacology, Vol. 34, No. 17, pp. 3147–3152, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

TRYPANOSOMATID IRON-SUPEROXIDE DISMUTASE INHIBITORS

SELECTIVITY AND MECHANISM OF N^1, N^6-BIS (2,3-DIHYDROXYBENZOYL)-1,6-DIAMINOHEXANE

STEVEN R. MESHNICK*†, KARL R. KITCHENER* and NGUYEN LE TRANG‡

* Division of International Medicine, Department of Medicine, Cornell University Medical College, New York, NY 10021; and ‡ Laboratory of Medical Biochemistry, Rockefeller University, New York, NY 10021, U.S.A.

(*Received* 17 *September* 1984; *accepted* 24 *January* 1985)

Abstract—Crithidia, like trypanosomes and leishmania, has an iron-containing superoxide dismutase. The iron chelator N^1,N^6-bis (dihydroxybenzoyl)-1,6-diaminohexane proved to be a potent inhibitor of this enzyme. Inhibition of the crithidial superoxide dismutase by this compound was dependent on the presence of oxygen and associated with the formation of a complex which could not be dissociated by gel-filtration chromatography. We propose that this biscatecholic inhibitor is first oxidized to a quinone which then covalently modifies a nucleophilic residue on the enzyme. This compound was less effective as an inhibitor of a mammalian copper- and zinc-containing superoxide dismutase. Thus, this inhibitor could serve as a prototype for the design of antiparasitic agents.

Leishmania and trypanosomes are parasitic protozoa which cause widespread disease in underdeveloped countries. Leishmaniasis affects over one million persons per year. Twenty-four million people in Latin America suffer from Chagas' disease, caused by *Trypanosoma cruzi*, whereas perhaps one million people in Africa have sleeping sickness, caused by *Trypanosoma gambiense* and *Trypanosoma rhodesiense* [1]. Leishmaniasis and sleeping sickness can only be treated with relatively toxic drugs; there is no effective treatment for chronic Chagas' disease [2].

We have been looking for biochemical differences between parasites [3–7] and their hosts which might serve as targets for the rational design of chemotherapeutic agents. One such difference was noted when we observed that the superoxide dismutases (SODs) of trypanosomes and leishmania are structurally different than SODs of mammals; the parasite SODs are iron-containing [3–5], whereas mammalian SODs are either copper, zinc- or manganese-containing [8]. SODs are nearly-ubiquitous enzymes that catalyze the conversion of superoxide anions (O_2^-) to hydrogen peroxide [8]. Subsequently, we isolated an iron-containing SOD, SOD-2, from *Crithidia fasciculata*, a non-parasitic trypanosomatid related to trypanosomes and leishmania [4].

In this paper, we report our initial results of studies on inhibitors of the iron-SOD from *C. fasciculata*. The iron-chelator N^1N^6-bis(dihydroxybenzoyl)-1,6-diaminohexane (bis-C6-DHB), proved to be a selective and irreversible inhibitor of this SOD. This compound may serve as a model compound and lead to the development of new antiparasitic drugs.

† Address all correspondence to: Dr Steven R. Meshnick, Division of International Medicine, Department of Medicine, Cornell University Medical College, 525 East 68th St., New York, NY 10021.

EXPERIMENTAL PROCEDURES

SOD-2 was purified from *C. fasciculata* as previously described [4]. Bovine erythrocyte copper, zinc-SOD, bovine serum albumin (BSA) and desferrioxamine were obtained from the Sigma Chemical Co. (St. Louis, MO) and *p*-quinone was obtained from the Aldrich Chemical Co. (Cedar Knolls, NJ). Bis-C6-DHB was the gift of Dr. Kuldeep Bhargava (Albert Einstein Medical College, Bronx, NY) [9]. Inhibitors were first dissolved in dimethyl sulfoxide (DMSO) and then added to solutions of SOD-2 or bovine erythrocyte SOD in 50 mM potassium phosphate buffer, pH 7.8 (KP buffer). The final concentration of DMSO was in all cases 1%. Control incubations consisted of enzyme in KP buffer with 1% DMSO.

To determine the dose dependence of inhibition by bis-C6-DHB, various concentrations of this compound were incubated with SOD-2 (0.42 μM) or bovine erythrocyte SOD (0.69 μM) for 24 hr at 25°. Enzyme was then separated from free inhibitor by gel-filtration chromatography on G-25-80 Sephadex columns (0.45 cm × 1.4 cm with a loading volume of 50 μl). Elution volumes for these columns had been determined previously, using solutions of BSA and methylene blue. The time dependence of inhibition was determined by periodically removing aliquots (50 μl) from solutions of SOD-2 (0.36 μM) or bovine erythrocyte SOD (1.0 μM) with or without bis-C6-DHB (100 μM) at 25°. These aliquots were then subjected to gel-filtration chromatography as described above and assayed for SOD activity by the method of McCord and Fridovich [10]. For both the

dose–response and time–course studies, duplicate aliquots were assayed and averaged. In all cases, duplicates differed from averages by < 15%. Percent inhibitions were calculated for each incubation as follows:

$$\% \text{ Inhibition} = [(\text{control} - \text{inhibited})/\text{control}] \times 100$$

Attempts were made to reactivate bis-C6-DHB-inhibited enzyme with exogenous iron. SOD-2 was inhibited by 90% by incubation in 100 μM bis-C6-DHB for 24 h, separated from inhibitor by G-25 Sephadex chromatography as described above, and incubated at 25° in aqueous solutions of 1 mM $FeSO_4$, 1 mM $FeCl_3$, or in the absence of exogenous metal. After 24 h, the enzyme was again subjected to gel-filtration chromatography as described above and assayed for SOD activity.

The effects of desferrioxamine on SOD-2 were assessed by incubation of the enzyme in the presence of desferrioxamine (1 mM) for 4 hr. Free desferrioxamine was then separated from enzyme by dialysis against 2000 vol. of KP buffer at 4° for 24 hr with two changes of buffer. Studies were also made on the effects of various concentrations of *p*-quinone on both SOD-2 (0.34 μM) and bovine erythrocyte SOD (0.75 μM). Enzyme and inhibitor were incubated for 4 hr at 25° and then separated by gel-filtration chromatography as described above.

To determine the oxygen dependence of bis-C6-DHB inhibition, incubations were made under reduced oxygen tension. Solutions were degassed with vacuum for 15 min prior to addition of inhibitor and then incubated under a continuous stream of water-saturated prepurified nitrogen. To evaluate the role of H_2O_2 in this inhibition, SOD-2 (0.33 μM) was incubated with bis-C6-DHB (1 mM) in the presence and absence of catalase (80 nM) for 4 hr and then separated from the inhibitor by dialysis as described above.

To measure bis-C6-DHB-induced changes in absorption spectra, BSA (15 μM) or SOD-2 (11 μM) was incubated in KP buffer and 1% DMSO in the presence and absence of bis-C6-DHB (100 μM). In one experiment, $FeCl_3$ was added to a final concentration of 100 μM. Free inhibitor was separated from protein using PD-10 gel-filtration columns (Pharmacia, Piscataway, NJ). Absorption spectra were measured on a Hewlett–Packard 8450A spectrophotometer.

RESULTS

Bis-C6-DHB inhibited crithidial SOD-2 in an irreversible manner. Enzyme was incubated with different concentrations of bis-C6-DHB for 24 hr and then separated from free inhibitor by G-25 Sephadex chromatography (Fig. 1). In this experiment, there was 81% inhibition by 100 μM bis-C6-DHB and 100% with 1 mM. As expected, inhibition by bis-C6-DHB (100 μM) was time dependent, with 50% inhibition obtained after approximately 9 hr (Fig. 2). To show that the measured inhibition was due to enzyme inactivation rather than to the presence of tightly-bound bis-C6-DHB which might interfere with the SOD assay, the following experiment was performed. One unit of SOD-2 was incubated in 100 μM bis-C6-DHB for 18 hr and then subjected to G-25 Sephadex chromatography. The eluted protein (0.25 μg), which had no SOD activity, was mixed with an aliquot (0.4 μg) of untreated SOD-2. This aliquot contained the same total activity (1.5 units) before and after mixing.

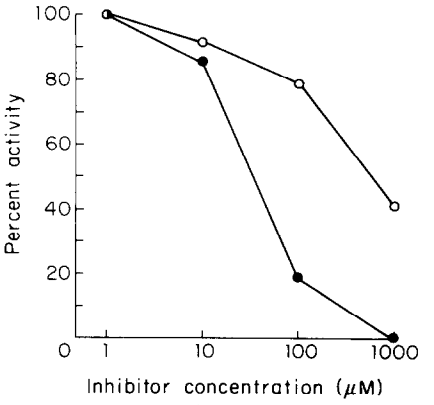

Fig. 1. Inhibition of SOD-2 (—●—●—) and bovine erythrocyte SOD (—○—○—) by different concentrations of bis-C6-DHB after 24 hr at 25°. Percent activity was determined by the following equation: [(activity of treated sample)/(activity of control sample)] × 100.

Bis-C6-DHB inhibited the crithidial iron-SOD better than the mammalian copper, zinc-SOD. In the dose–response experiment, the crithidial SOD was inhibited to a greater extent than the mammalian SOD at both 100 μM and 1 mM (Fig. 1). Furthermore, bis-C6-DHB inhibited SOD-2 at a faster rate than it did bovine erythrocyte copper, zinc-SOD (Fig. 2).

The inhibition of SOD-2 by bis-C6-DHB did not appear to involve the chelation and removal of iron. Activity was not restored to inhibitor-treated enzyme by incubation with $FeSO_4$ or $FeCl_3$. (In fact, these

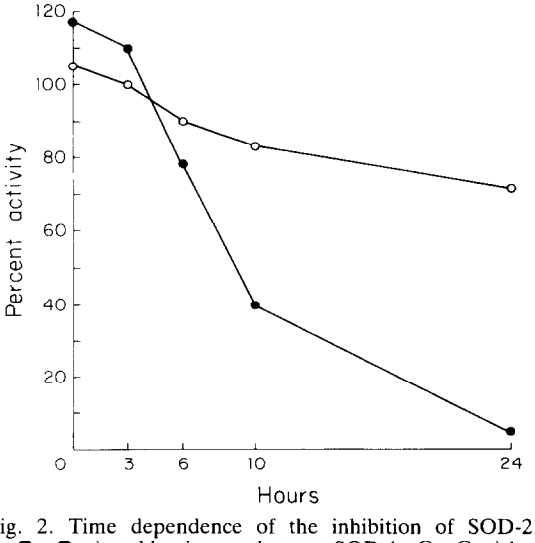

Fig. 2. Time dependence of the inhibition of SOD-2 (—●—●—) and bovine erythrocyte SOD (—○—○—) by bis-C6-DHB. Percent activity was determined by the following equation: [(activity of treated sample)/(activity of control sample)] × 100.

Table 1. Effects of desferrioxamine and bis-C6-DHB on SOD-2

Incubation	Percent inhibition ± S.D.	N
Desferrioxamine*	0 ± 0	3
Bis-C6-DHB in air†	87.5 ± 7	4
Bis-C6-DHB under nitrogen†	24 ± 16	4

* SOD-2 was incubated in KP buffer containing 1% DMSO for 4 hr with and without desferrioxamine (1 mM) at 25°. Unihibited SOD activity after gel filtration was 12 units/ml.

† SOD-2 was incubated in KP buffer containing 1% DMSO for 72 hr with and without bis-C6-DHB (100 μM) at 25°. Nitrogen gassing was performed as described in Experimental Procedures. Uninhibited SOD activity after gel filtration was 433 units/ml.

incubations caused 16 ± 13 and 14 ± 23% decreases in activity of inhibited enzyme respectively). In addition, the incubation of SOD-2 in the presence of 1 mM desferrioxamine, a very powerful iron-chelator, did not result in loss of activity (Table 1).

The inhibition of SOD-2 by bis-C6-DHB proved to be dependent on the presence of oxygen (Table 1). Enzyme incubated with 100 μM bis-C6-DHB for 72 hr in the presence of air lost 87.5% of its activity, whereas only 24% was lost after incubation under reduced oxygen tension. These data suggest that bis-C6-DHB may be oxidized to a quinone before it inhibits the enzyme.

The oxidation of bis-C6-DHB to a quinone may be associated with the production of H_2O_2, a substance which has been shown to inactivate trypanosomatid SODs [3–5]. To determine whether H_2O_2 production was responsible for the inhibitory activity of bis-C6-DHB, SOD-2 was incubated with bis-C6-DHB (1 mM) in the presence and absence of catalase. After 4 hr, there was little difference in the degree of inhibition (78% in the absence of catalase vs 76% in the presence of catalase).

Since the quinone form of bis-C6-DHB might serve as an alkylating agent [11–15], we next attempted to determine whether bis-C6-DHB could form covalent bonds with proteins. A mixture of bis-C6-DHB and BSA was incubated for 72 hr and then applied to a gel-filtration column. Even though free bis-C6-DHB was separated from BSA, the eluted protein had a prominent absorption peak at 325 nm (Fig. 3B) which is characteristic of bis-C6-DHB (Fig. 3A). The association of bis-C6-DHB and BSA increased as incubation times increased (Fig. 4), suggesting the time-dependent formation of covalent bonds. This association appeared to be accelerated by iron and inhibited by incubation under reduced oxygen tension (Figs. 3B and 4).

Bis-C6-DHB also appeared to associate tightly with SOD-2. The enzyme was incubated in the presence of inhibitor (100 μM) for 72 hr both aerobically and anaerobically. Free inhibitor was then removed by gel-filtration chromatography. Under these incubation conditions, SOD-2 was inhibited markedly in air (85%) and only slightly inhibited in the presence of reduced oxygen tension (5%). A prominent absorbance at 330 nm was seen associated with the eluted protein after incubation of the enzyme and inhibitor in air. As expected, none was seen after anaerobic incubation (Fig. 5).

We next attempted to determine if the quinone form of bis-C6-DHB could also inhibit SOD-2 and whether this quinone could also inhibit the crithidial enzyme better than the mammalian enzyme. Unfortunately, the quinone form of bis-C6-DHB proved too unstable to isolate (R. W. Grady, personal communication). As an alternate, we studied the effects of *p*-quinone on both SODs (Table 2). *p*-Quinone proved to be a potent inhibitor of SOD-2, causing 45 and 90% inhibition at 10 and 100 μM respectively. In contrast, bovine erythrocyte SOD was unaffected by these concentrations. Thus, this quinone, like bis-C6-DHB, also appears to inhibit crithidial SOD-2 to a greater extent than it does mammalian copper, zinc-SOD.

DISCUSSION

Bis-C6-DHB appears to irreversibly inhibit SOD-2 by the following mechanism:

$$\text{Bis-C6-DHB(catechol)} + O_2 \rightarrow \text{Bis-C6-DHB(quinone)} + H_2O_2$$

$$\text{Bis-C6-DHB(quinone)} + \text{SOD-2} \rightarrow \text{Bis-C6-DHB–SOD-2 complex}$$

Bis-C6-DHB, which contains two catechol moieties, may first be oxidized to a quinone and then alkylate a nucleophilic site on SOD-2. A similar mechanism may account for the reaction between proteins and urushiols, toxins found in poison ivy and poison oak. These toxins are lipophilic *o*-catechols which bind serum albumin, oxidize in air, and then add via 1,4-nucleophilic addition [15].

The reaction sequence described above is likely to be the mechanism of SOD-2 inhibition by bis-C6-DHB on the basis of several of our experimental results. First, the ineffectiveness of desferrioxamine, one of the strongest known chelators [17], and the inability of exogenous iron to reactivate bis-C6-DHB-inhibited enzyme suggest that this inhibitor acts by a mechanism other than chelation. Second, bis-C6-DHB inhibition was found to be oxygen dependent, consistent with a requirement for the catechol to be oxidized to a quinone. Third, the H_2O_2 which may be produced is not important in the mechanism, since catalase did not reverse the effects

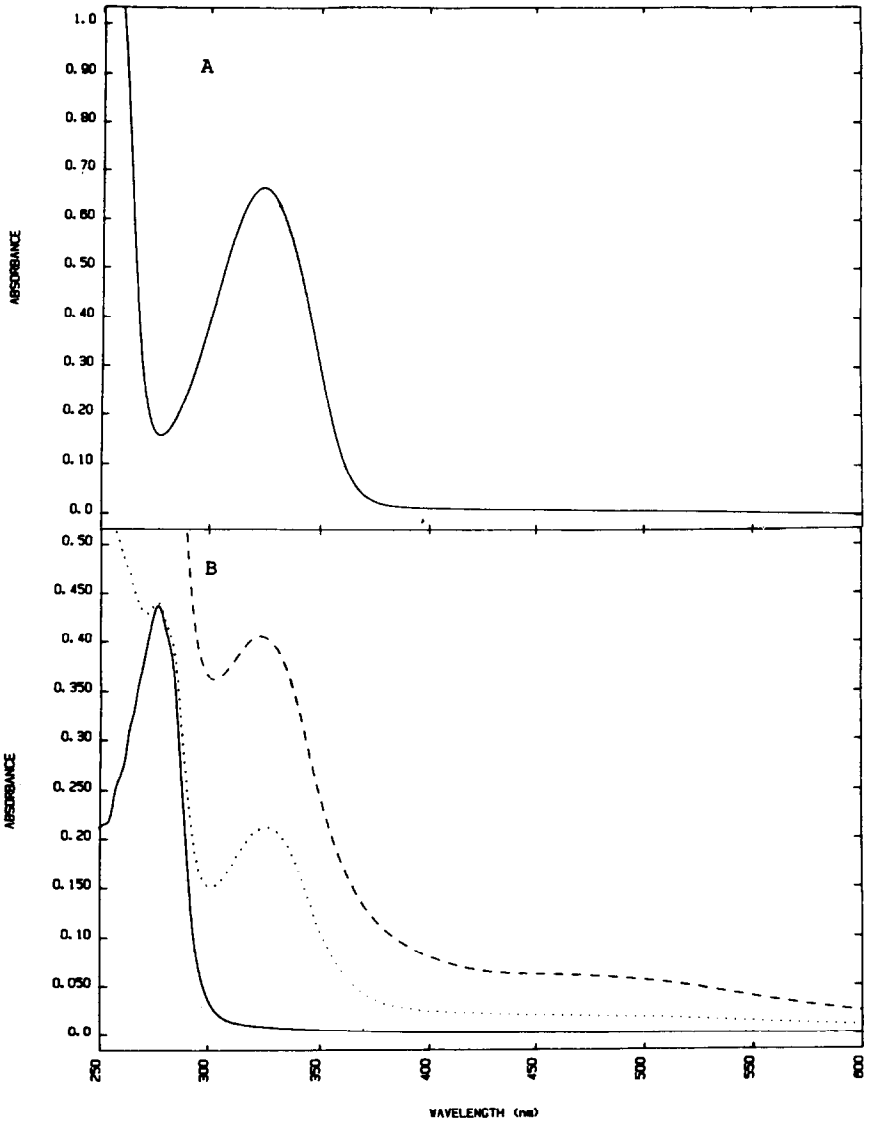

Fig. 3. Absorption spectra of bis-C6-DHB and bis-C6-DHB-treated BSA after 72 hr at 25° in KP buffer. (A) Bis-C6-DHB alone (0.1 mM). (B) BSA alone (———); BSA + bis-C6-DHB under nitrogen (· · · ·); and BSA + bis-C6-DHB in air (– – –).

of this inhibitor. Fourth, inhibition was probably associated with the formation of a covalent bond because a complex between bis-C6-DHB and SOD-2 formed which could not be separated by gel-filtration chromatography. However, the possibility remains that bis-C6-DHB forms a very tight noncovalent complex with SOD-2 which blocks its catalytic activity.

The oxygen dependence of bis-C6-DHB inhibition (Table 1) and binding (Figs. 3–5) is particularly striking in light of the fact that only modest attempts were made to remove oxygen. Often, much more stringent conditions are needed in order to demonstrate an oxygen effect. Also striking is the effect of iron on bis-C6-DHB binding to BSA (Fig. 4). This effect was probably so rapid that measurable binding occurred in the t = 0 sample in the time it took to run the gel-filtration column. Further studies on the kinetics of this reaction are now in progress.

In this publication, we measured only irreversible inhibition. An earlier attempt to identify reversible inhibitors of an iron-SOD [16] was impeded by the fact that many compounds interfere with the SOD assay. Indeed, bis-C6-DHB interferes with the SOD assay (unpublished data).

The selectivity of bis-C6-DHB for SOD-2 is important because it may have implications for the development of chemotherapeutic agents. Bis-C6-DHB may preferentially inhibit SOD-2 because this enzyme contains iron in its active sites. These iron atoms might bind the inhibitor and orient it in a way that facilitates reaction with a nucleophilic moiety. In addition, iron may, through its electron-withdrawing effect, make the quinone a better target for nucleophilic attack. The latter may explain why iron promotes the association of the catechol with BSA.

Other catechol and quinone derivatives, like

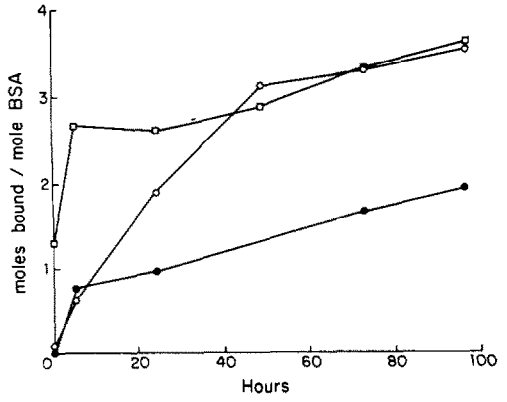

Fig. 4. Time dependence of binding of bis-C6-DHB to BSA. Key: BSA (16 μM) + bis-C6-DHB (0.1 mM) under nitrogen (—●—●—), in air (—○—○—) and in the presence of $FeCl_3$ (0.1 mM) (—□—□—). The concentration of bound bis-C6-DHB was calculated using an extinction coefficient at 325 nm of 6600 $m^{-1} cm^{-1}$ for both free and iron-complexed bis-C6-DHB.

doxorubicin [18], menoctone [19] and 2,3-dihydroxybenzoic acid [20] have been found to be useful or potentially useful pharmacological agents. We are now attempting to assess the antiparasitic activities of bis-C6-DHB and related compounds.

Table 2. Inhibition of SOD-2 and bovine erythrocyte SOD by *p*-quinone*

Enzyme	% Inhibition 10 μM	100 μM	1 mM
SOD-2	45	90	100
Bovine copper, zinc-SOD	0	0	48

* SOD-2 and bovine copper, zinc-SOD were incubated for 4 hr at 25° in KP buffer containing 1% DMSO and various concentrations of *p*-quinone. SOD activities after gel filtration were 0.42 and 1.13 units/ml for uninhibited SOD-2 and bovine copper, zinc-SOD respectively.

Acknowledgements—We thank Drs. Anthony Cerami, John Eaton, Harry Dougherty, Irwin Fridovich, Robert Grady and Kuldeep Bhargava for their helpful discussions and for the provision of compounds. This work was supported by National Institutes of Health Grant AI 20197, Rockefeller Foundation Grant RF 82043, and by a grant from Merck & Co. S.R.M. is a recipient of an Irma T. Hirschl Career Scientist Award.

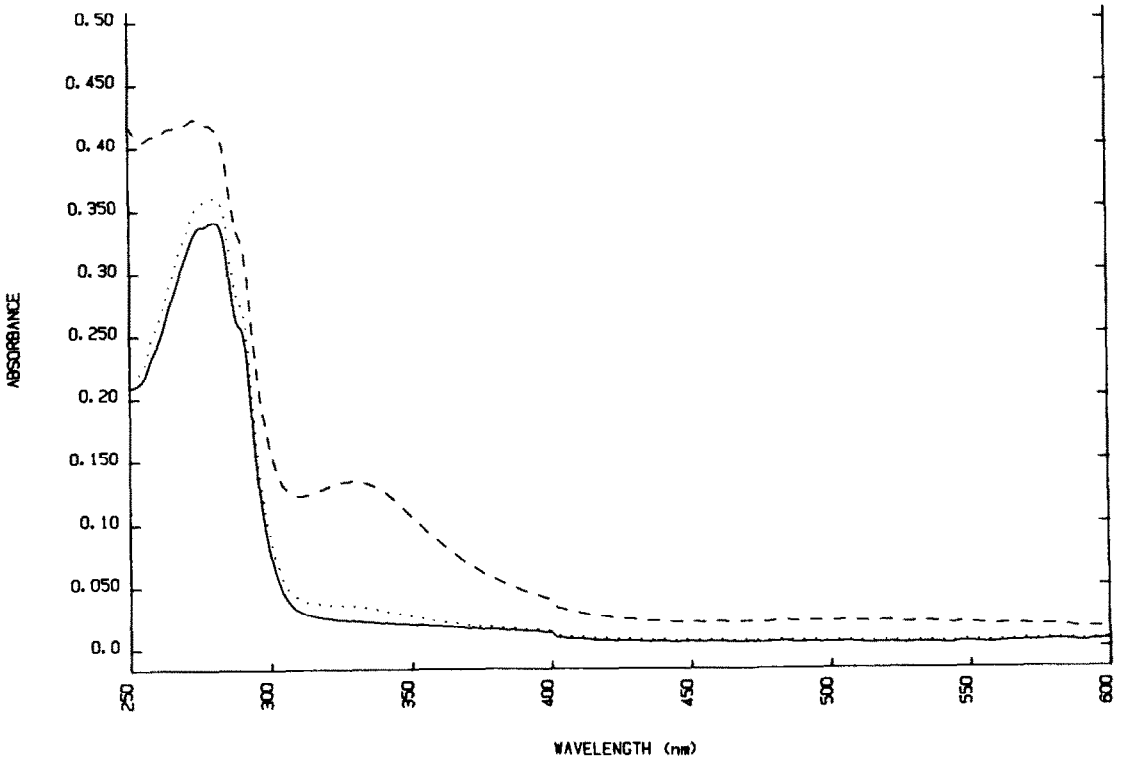

Fig. 5. Absorption spectra of bis-C6-DHB-treated SOD-2 after 72 hr at 25° in KP buffer. Key: SOD-2 alone (———); SOD-2 + bis-C6-DHB under nitrogen (· · · ·); and SOD-2 + bis-C6-DHB in air (– – –).

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