Does superoxide anion participate in 2-oxoglutarate-dependent hydroxylation?

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The possible role of superoxide anion in 2-oxoglutarate-coupled dioxygenase reactions has been investigated. γ -Butyrobetaine hydroxylase (EC 1.14.11.1) was inhibited by human erythrocyte superoxide dismutase (EC 1.15.1.1), probably due to release of Cu^{2+} or Zn^{2+} , as the inhibition was more pronounced after heat-inactivation of the dismutase and as Cu^{2+} was a potent inhibitor. Bovine superoxide dismutase and the Mn^{2+} -containing superoxide dismutase from *Escherichia coli* were not inhibitory. Superoxide anion generated from xanthine/xanthine oxidase was not stimulatory and could not replace ascorbate. Thymine 7-hydroxylase (EC 1.14.11.6) and thymidine 2'-hydroxylase (EC 1.14.11.3) were not inhibited by erythrocyte superoxide dismutase or stimulated by superoxide anion. γ -Butyrobetaine hydroxylase was inhibited by a number of low-molecular-weight compounds, such as tetranitromethane, Nitro Blue Tetrazolium, adrenaline and Tiron, which may act as scavengers of superoxide anion. Involvement of this radical in other oxygenase reactions has been inferred from the findings that they were inhibitory for the respective enzymes. Several of these compounds also inhibited γ -butyrobetaine hydroxylase. It could be concluded from these experiments, however, that mechanisms other than disposal of superoxide anion might equally well be operative, such as hydrophobic interaction with the enzyme protein and interaction with compounds required for full enzymic activity, e.g. iron and ascorbate. The results appear to rule out a requirement for superoxide anion generated in free solution, and have not yielded evidence for participation of enzyme-bound superoxide anion in 2-oxoglutarate-dependent hydroxylations.

The intermediates in the reduction of O_2 during oxygenase reactions are still largely unknown. Superoxide anion is involved in the reactions catalysed by indolamine 2,3-dioxygenase and 2-nitropropane dioxygenase (Ohnishi *et al.*, 1977, and references cited therein; Kide & Soda, 1978); in the first reaction, molecular O_2 or superoxide anion is used, depending on the oxidation state of the iron associated with the enzyme (Taniguchi *et al.*, 1979). Several other oxygenases are inhibited by superoxide dismutase or low-molecular-weight superoxide scavengers, which has suggested involvement of superoxide anion (for references, see Michelson *et al.*, 1977). In the present study we find no evidence for the participation of $\text{O}_2^{\cdot-}$ in the 2-oxoglutarate-dependent hydroxylation and suggest that present and previous results (Bhatnagar & Liu, 1972; Tuderman *et al.*, 1977; Myllylä *et al.*, 1979), which could be used as arguments for participation of $\text{O}_2^{\cdot-}$, are more likely due to interference in the enzyme reaction not involving $\text{O}_2^{\cdot-}$.

Materials and methods

Sources of chemicals and enzymes

γ -Butyrobetaine chloride was obtained from E. Merck, A.G., Darmstadt, Germany. 2-Oxo[1- ^{14}C]-glutarate was obtained from New England Nuclear Corp., Frankfurt/Main, Germany. [$\text{Me-}^{14}\text{C}$] γ -Butyrobetaine was synthesized as described previously (Lindstedt, 1967). Cu(II) -salicylate was prepared as described by de Alvare *et al.* (1976). Other chemicals were from standard suppliers.

Thymine 7-hydroxylase (thymine, 2-oxoglutarate dioxygenase; EC 1.14.11.6) and thymidine 2'-hydroxylase (thymidine, 2-oxoglutarate dioxygenase; EC 1.14.11.3) were prepared from *Neurospora crassa* strain STA 4 (FGSC 262A) (Holme *et al.*, 1971; Bankel *et al.*, 1972) and γ -butyrobetaine hydroxylase (γ -butyrobetaine, 2-oxoglutarate dioxygenase; EC 1.14.11.1) from *Pseudomonas* sp. AK 1 (Lindstedt *et al.*, 1977). Superoxide dismutase (EC 1.15.1.1) from human erythrocytes was prepared

and assayed by the method of McCord & Fridovich (1969). The enzymic activity was expressed in the units defined by these authors. The specific activity was approx. 3000 units/mg of protein and 50×10^9 units/mol of Cu^{2+} . Superoxide dismutase from *Escherichia coli* was prepared by the method of Keele *et al.* (1970). The specific activity was approx. 3200 units/mg of protein. Bovine superoxide dismutase from Sigma Chemical Co., St. Louis, MO, U.S.A.

Enzyme assays

Oxygenase assays were based on the oxidative decarboxylation of 2-oxoglutarate (Lindstedt *et al.*, 1968; Holme *et al.*, 1970; Bankel *et al.*, 1972). The incubation mixtures had the following composition ('complete system'). For γ -butyrobetaine hydroxylase assay, the mixture contained enzyme (0.3–0.9 μg , 0.08–0.25 nkat), γ -butyrobetaine (14 mM), 2-oxo[1- ^{14}C]glutarate (1.5 mM, 0.14 mCi/litre), FeSO_4 (0.6 mM), sodium ascorbate (14 mM), catalase (1.4 g/litre) and potassium phosphate buffer (14 mM), pH 7.0, in a volume of 0.35 ml. When indicated, phosphate buffer was replaced by Tris/HCl (14 mM) at pH 7.0 and the FeSO_4 concentration was decreased to 0.06 mM. For thymine 7-hydroxylase assay; the mixture contained enzyme (6 μg , 20 pkat), thymine (0.5 mM), 2-oxo[1- ^{14}C]glutarate (0.25 mM, 0.25 mCi/litre), FeSO_4 (1 mM), sodium ascorbate (5 mM), catalase (2 g/litre) and potassium phosphate buffer at pH 7.5 (100 mM) in a total volume of 0.20 ml. For thymidine 2'-hydroxylase assay, the mixture contained enzyme (30 μg , 20 pkat), thymidine (0.5 mM), 2-oxo[1- ^{14}C]glutarate (0.25 mM, 0.25 mCi/litre), FeSO_4 (5 mM), sodium ascorbate (5 mM), catalase (2 g/litre), dithiothreitol (1 mM) and potassium phosphate buffer at pH 7.5 (50 mM) in a total volume of 0.20 ml.

Production of superoxide anion from xanthine/xanthine oxidase was measured by cytochrome *c* reduction in an incubation mixture similar to that used in the hydroxylase assay, but without Fe^{2+} , ascorbate and catalase, because of their interference with the cytochrome *c* measurements.

Incubations with low-molecular-weight scavengers

The compounds were dissolved in water except for tetranitromethane and adrenochrome, which were dissolved in 0.2 M-ethanol. In experiments with these compounds appropriate controls with 0.2 M-ethanol were run.

The compounds were either added to the incubations immediately before assay, which was started by the addition of enzyme or first incubated with enzyme ('preincubation') before assay. In some experiments part of the pre-incubation mixture was fractionated on a Sephadex G-25 column (1 ml). The enzyme was collected in about 200 μl around V_0 . Protein was determined in the eluate, which was then

suitably diluted before assay. The remaining part of the pre-incubation mixture, not passed through Sephadex, was diluted to approximately the same protein concentration before determination of enzyme activity.

Results

Effect of a superoxide-anion-generating system

Table 1 shows that a combination of xanthine and xanthine oxidase could not replace ascorbate in the assay for γ -butyrobetaine hydroxylase; in incubations with the 'complete' assay system, there was, however, an inhibition by xanthine/xanthine oxidase. Xanthine alone was inhibitory. In other experiments with xanthine/xanthine oxidase, it was shown that Fe^{3+} could not replace Fe^{2+} in the absence of ascorbate. Similar results were obtained when xanthine/xanthine oxidase was added to incubations of thymine 7-hydroxylase and thymidine 2'-hydroxylase.

Superoxide dismutase and Cu^{2+}

We first tested superoxide dismutase from human erythrocytes and from *E. coli*. The human erythrocyte enzyme was inhibitory. This inhibitory effect was more pronounced when γ -butyrobetaine hydroxylase had first been incubated with the dismutase before enzyme assay. Heat-denatured erythrocyte dismutase was a better inhibitor than native dismutase (Table 2). This suggested that the inhibition observed was due to the liberation of metal ions from the dismutase. Cu^{2+} was then added to the incubation mixture under the same conditions as those used for erythrocyte superoxide dismutase and pronounced inhibition was found (Table 2). Superoxide dismutase from *E. coli*, an enzyme that

Table 1. *Effect of a superoxide-anion generating system (xanthine/xanthine oxidase) on γ -butyrobetaine hydroxylation*

The basal incubating medium contained γ -butyrobetaine hydroxylase (0.1 nkat), γ -butyrobetaine (0.14 mM), 2-oxo[1- ^{14}C]glutarate (0.15 mM, 0.14 mCi/litre), catalase (1.4 g/litre) and phosphate buffer (14 mM) at pH 7.0. Additions were made at the following final concentrations: Fe^{2+} , 0.6 mM; ascorbate, 14 mM; xanthine, 1 mM; xanthine oxidase, 0.1 g/litre.

Addition to the basal medium	Relative activity (%)
Fe^{2+} , ascorbate	100
Fe^{2+} , ascorbate, xanthine	84
Fe^{2+} , ascorbate, xanthine, xanthine oxidase	36
Fe^{2+}	61
Fe^{2+} , xanthine, xanthine oxidase	14
Ascorbate	17
Ascorbate, xanthine, xanthine oxidase	14
No addition	<0.1
Xanthine, xanthine oxidase	<0.1

Table 2. Effect of superoxide dismutase, Cu(II)-salicylate and Cu²⁺ on the activity of γ -butyrobetaine hydroxylase

In experiment I Tris/HCl buffer was used. The enzyme was pre-incubated with the dismutase and Cu²⁺ at 4°C for 10 min. In experiment II phosphate buffer was used. No pre-incubation was performed.

Incubation conditions	Relative activity (%)
Experiment I	
Complete system	100
+ Human erythrocyte superoxide dismutase (160 units; 9 μ M-Cu ²⁺)	52
+ Boiled human erythrocyte superoxide dismutase (160 units; 9 μ M-Cu ²⁺)	28
+ Cu ²⁺ (9 μ M)	<1
+ <i>E. coli</i> superoxide dismutase (170 units)	96
+ Boiled <i>E. coli</i> superoxide dismutase (170 units)	81
Experiment II	
Complete system	100
+ Bovine erythrocyte superoxide dismutase (300 units; 18 μ M-Cu ²⁺)	106
+ Cu(II)-salicylate (10 μ M)	56
+ Cu(II)-salicylate (20 μ M)	5
+ Cu ²⁺ (10 μ M)	69
+ Cu ²⁺ (20 μ M)	6

contains Mn²⁺, caused no significant inhibition in similar experiments with up to 190 units. Slight inhibition was, however, observed with heat-inactivated *E. coli* dismutase (Table 2). No inhibitory effect was observed when erythrocyte superoxide dismutase was added to the incubation mixtures of thymine 7-hydroxylase or thymidine 2'-hydroxylase under similar conditions. Bovine erythrocyte superoxide dismutase did not inhibit γ -butyrobetaine hydroxylase (Table 2; Fig. 1d).

In experiments with Cu(II)-salicylate and CuSO₄ the compounds (0–80 μ M) were added to the incubation medium before the enzyme. Cu(II)-salicylate and CuSO₄ inhibited by 44 and 31% respectively at 10 μ M concentration (Table 2). Concentrations above 20 μ M resulted in about 95% inhibition.

Tetranitromethane and Nitro Blue Tetrazolium

Tetranitromethane inhibited γ -butyrobetaine hydroxylase by 50% at 1.6 mM concentration (Fig. 1a). Pre-incubation of enzyme with 0.6 mM or higher concentrations of tetranitromethane in buffer at 4°C for 60 min resulted in about 90% inhibition.

Nitro Blue Tetrazolium in the presence of ascorbate caused almost complete inhibition at concentrations of 0.2 mM and above; the inhibition was less without ascorbate (Fig. 1b). Pre-incubation of enzyme at 4°C for 60 min with Nitro Blue Tetrazolium (about 0.02 mM) resulted in 19% inhibition. In other experiments, larger amounts of Nitro Blue Tetrazolium (1.5 μ mol) were incubated with enzyme (1.3 nmol) in 0.03 M-phosphate buffer (0.15 ml), pH 7.0, for 30 min at 4°C. The same inhibition was

observed in samples that had been subjected to gel filtration as in samples that had not been passed through Sephadex (71% compared with 67% inhibition). Addition immediately before assay of Nitro Blue Tetrazolium to the same final concentration as in these experiments resulted in an inhibition of 35%. No inhibition was observed with nitrobenzene at 6 mM.

Quinols and quinones

Several quinols and quinones in the concentration range 0.03–6 mM were tested as inhibitors in the reaction catalysed by γ -butyrobetaine hydroxylase. Inhibition at 50% was obtained with 0.9 mM-DL-adrenaline, 0.7 mM-dopamine, 0.03 mM-3,4-dihydroxybenzoate, 0.6 mM-catechol and 1.6 mM-quinol. The inhibition with DL-adrenaline was not affected by the addition of 10 mM-dithiothreitol to the incubations. The inhibition by DL-adrenaline was less pronounced when higher concentrations of Fe²⁺ were used (0.6–3 mM); it was only 15% with 3 mM-Fe²⁺. Adrenaline was more inhibitory in the absence of ascorbate; 50% inhibition was then obtained with 0.3 mM-DL-adrenaline (Fig. 1c). Two quinones were more effective as inhibitors than the parent catechols; 50% inhibition was noted with 0.9 mM-1,4-benzoquinone and with 0.09 mM-adrenochrome. In an experiment with a low concentration of adrenochrome (about 0.01 mM) there was no detectable inhibition but the inhibition was 71% when the same amount of adrenochrome had been pre-incubated with enzyme in buffer 4°C for 60 min. When a larger amount (1.4 nmol) of enzyme had been incubated with adrenochrome (1.5 μ mol) in 0.03 M-phosphate buffer (0.15 ml), pH 7.0, for 30 min at 4°C, the inhibition was 76% when the enzyme had been passed through a column of Sephadex G-25 and 72% when this step had been omitted. Addition immediately before assay of adrenochrome to the same final concentration resulted in 28% inhibition. No inhibition was obtained with tyramine in the concentration range 0.03–6 mM.

The presence of 1.8 mM-Tiron resulted in 50% inhibition. As shown in Fig. 1(d) the magnitude of the inhibition varied with the concentration of Fe²⁺ in the assay medium, but Fe²⁺ could not abolish the inhibition completely. The rate of 2-oxoglutarate decarboxylation was constant in the presence of 2 mM-Tiron when Tiron was first incubated with the assay mixture without enzyme for 5 min at 37°C. The addition of bovine erythrocyte superoxide dismutase to the incubations with Tiron did not abolish the inhibition. There was no further inhibitory effect by Tiron as a result of 30 min pre-incubation with enzyme in buffer at 4°C. In one series of experiments, the reaction mixtures after 10 min of incubation with Tiron and the complete system were fractionated by gel filtration. A second

Fig. 1. Effect of different low-molecular-weight compounds on γ -butyrobetaine-dependent decarboxylation of 2-oxoglutarate, catalysed by γ -butyrobetaine hydroxylase from *Pseudomonas* sp. AK 1 (a) Tetranitromethane added immediately before assay (●), or incubated with enzyme in buffer at 4°C for 60 min before assay (○). (b) Nitro Blue Tetrazolium without ascorbate (●), or in the presence of 14 mM-ascorbate (○). (c) Adrenaline without ascorbate (○) or in the presence of 14 mM-ascorbate (●), and adrenochrome in the presence of 14 mM-ascorbate (□). (d) Fe^{2+} (○), Fe^{2+} in the presence of 100 μg of bovine superoxide dismutase (●), Fe^{2+} in the presence of 1.7 mM-Tiron (□), and Fe^{2+} in the presence of 100 μg of bovine superoxide dismutase and 1.7 mM-Tiron (■).

Table 3. Effects of Tiron on the γ -butyrobetaine-dependent decarboxylation of 2-oxoglutarate, catalysed by γ -butyrobetaine hydroxylase

Conditions	Enzymic activity (% of control)
(a) Complete system + Tiron (1.4 mM)	50
(b) After pre-incubation of enzyme + Tiron (1.4 mM) at 4°C for 30 min	56
(c) After incubation as in (a) and subsequent fractionation by gel filtration	99

incubation with the enzyme fraction of the eluate was then carried out after the addition of the remaining components of the complete system. The decarboxylation of 2-oxoglutarate was then the same as in the control without Tiron (Table 3).

Paraquat

Paraquat (1,1'-dimethyl-4,4'-dipyrimidinium) (0.1–2 mM) inhibited the enzyme activity. The inhibition was 77% when paraquat had been added to the enzyme solution about 2 min before addition of the other components of the assay system, but only 13% when paraquat was added to the enzyme solution with the other components. When the enzyme was incubated with the same concentration of paraquat at 4°C for 1 h before assay the inhibition was 98%.

8-Anilino-naphthalene-1-sulphonate, 6-(4-N-toluidinyl)naphthalene-1-sulphonate and aliphatic alcohols

8-Anilino-naphthalene-1-sulphonate and 6-(4-N-toluidinyl)naphthalene-1-sulphonate were inhibitory.

After 15 min of pre-incubation with enzyme, about 50% inhibition was observed at 0.8 and 3 mM final concentration. Aliphatic alcohols were inhibitory after pre-incubation at 4°C for 15 min; n-butanol at 0.2 M concentration inhibited by 39%, whereas the same concentration of t-butanol, n-propanol, propan-2-ol, and ethanol inhibited by 30, 23, 19 and 23% respectively. With ethanol, there was a nearly linear relationship between degree of inhibition and concentration in the range 0–1 M.

Stoichiometry between 2-oxoglutarate decarboxylation and γ -butyrobetaine hydroxylation

No formation of $^{14}\text{CO}_2$ from 2-oxo[1- ^{14}C]-glutarate was observed in incubations of γ -butyrobetaine hydroxylase in the absence of γ -butyrobetaine, i.e. less than 5% compared with the reference incubation. These experiments were performed with tetranitromethane (3 mM), Nitro Blue Tetrazolium (0.3 mM), DL-adrenaline (1.3 mM) and Tiron (3 mM). Identical amounts of carnitine and $^{14}\text{CO}_2$ were obtained in experiments without and with DL-adrenaline (0.7, 1.4 and 2.4 mM), using [Me- ^{14}C]- γ -butyrobetaine and 2-oxo[1- ^{14}C]-glutarate as substrates. In these experiments carnitine formation was determined (Lindstedt, 1967).

Discussion

We used the oxidative decarboxylation of 2-oxoglutarate to assay for oxygenase activities, which was justified since the stoichiometry between 2-oxoglutarate decarboxylation and γ -butyrobetaine hydroxylation was 1:1 also in the presence of inhibitory compounds.

The inhibition of γ -butyrobetaine hydroxylase by the human erythrocyte superoxide dismutase may be explained by release of copper or zinc ion from this enzyme. The results obtained with the other two superoxide dismutases seem to exclude a requirement for free superoxide anion in the reactions catalysed by γ -butyrobetaine, thymine and thymidine hydroxylases.

A reductant is needed for full activity in the 2-oxoglutarate-dependent dioxygenase reactions; ascorbate has been found to be the most efficient (for review, see Hayaishi *et al.*, 1975). The need for ascorbate is quite specific, although not absolute for pure γ -butyrobetaine hydroxylase (Lindstedt *et al.*, 1980) and prolyl 4-hydroxylase (Myllylä *et al.*, 1978). A role for ascorbate would be to reduce enzyme-bound Fe^{3+} to Fe^{2+} (Myllylä *et al.*, 1978). For indolamine 2,3-dioxygenase it has been shown that the ferric enzyme utilizes $\text{O}_2^{\cdot -}$ and the ferrous enzyme utilizes O_2 (Taniguchi *et al.*, 1979). If the same mechanism is operating in the 2-oxoglutarate-dependent dioxygenase reactions a superoxide-generating system would be able to replace ascorbate in the reaction. The xanthine/xanthine oxidase system inhibited the hydroxylase

reaction, which complicated the interpretation of the results, but it is clear that xanthine plus xanthine oxidase could not replace ascorbate in the reaction when added in amounts that generate superoxide anion at a rate exceeding the rate of hydroxylation about ten times.

In attempts to demonstrate involvement of enzyme-bound $\text{O}_2^{\cdot -}$, scavengers which may be either oxidized or reduced by $\text{O}_2^{\cdot -}$ were used. γ -Butyrobetaine hydroxylase requires ferrous ion and is normally assayed in the presence of ascorbate and the incubation mixture thus contains several redox couples as well as intermediates in the reduction of O_2 catalysed by the metal ion. The scavengers may therefore be reduced or oxidized during the hydroxylase assays in other ways than by reaction with superoxide anion.

Adrenaline was more effective when ascorbate was absent, i.e. when DL-adrenaline is autooxidized to a greater extent than when ascorbate is present. The inhibition caused by Nitro Blue Tetrazolium was more pronounced in the presence of ascorbate than in its absence, whereas the opposite would have been expected if it inhibited the enzymic reaction by oxidizing $\text{O}_2^{\cdot -}$. Bhatnagar & Liu (1972) found that prolyl hydroxylase, which is also stimulated by Fe^{2+} and ascorbate, may be inhibited by Nitro Blue Tetrazolium. These authors suggest that a free radical, possibly superoxide radical, may be generated at a 'reducing site' and serve to reduce Fe^{3+} , which was presumed to be formed from Fe^{2+} during incorporation of oxygen atoms into the substrates. However, since electrons for the reduction of oxygen are provided by 2-oxoglutarate, there is no need to postulate an extraneous reductant in the catalytic cycle. The kinetic arguments presented by the authors implying that the enzyme and dye compete for the radical are not convincing, and the results may have been due to effects of Nitro Blue Tetrazolium other than that of a radical scavenger. From a kinetic study Tuderman *et al.* (1977) concluded that Nitro Blue Tetrazolium reacts with an active form of O_2 . It is not possible from their experiments, carried out with high concentrations of ascorbate, to conclude whether Nitro Blue Tetrazolium or its reduced form acts competitively with O_2 or if they consume enzyme-bound 'activated' O_2 or a reduced form of O_2 .

The compounds used probably affect enzyme activity in other ways than by reaction with $\text{O}_2^{\cdot -}$. Tuderman *et al.* (1977) reported that DL-adrenaline inhibits prolyl hydroxylase; the inhibition was competitive with respect to Fe^{2+} , indicating that this catecholamine acts mainly by way of Fe^{2+} binding. Inhibition of γ -butyrobetaine hydroxylase by DL-adrenaline decreased with increasing concentration of Fe^{2+} . Considering the results obtained with adrenochrome, we did not further study the inter-

action between Fe^{2+} and DL-adrenaline in our system. Since the oxidation products of DL-adrenaline and other catechols may react with proteins we tested adrenochrome and 1,4-benzoquinone, which proved to be more inhibitory than the corresponding catechols, and probably affect the enzyme by mechanisms other than by interference in catalysis. Murray *et al.* (1977) reported inhibition of lysyl hydroxylase by DL-adrenaline but, like Tuderman *et al.* (1977), they did not test the effect of its oxidation products.

Apparently adrenochrome and Nitro Blue Tetrazolium interact with the enzyme protein. The enzyme might be susceptible to hydrophobic interaction as it was also inhibited by short-chain alcohols and by two different naphthalene sulphonates, commonly used as hydrophobic probes. Quinones may react with thiol groups. The inability of dithiothreitol to affect the inhibition caused by DL-adrenaline argues, however, against the idea that catechols would inhibit the enzyme as a result of an addition of a quinone to thiol groups. Tetranitromethane may also affect proteins, chiefly by way of nitration of tyrosine residues. The inhibition by tetranitromethane cannot therefore be ascribed solely to disposal of superoxide anion.

With Tiron there was no evidence of damage to the enzyme after its exposure to the inhibitor, and the inhibition by Tiron, which could not be relieved by Fe^{2+} , could possibly be taken as evidence for involvement of superoxide anion. However, superoxide ion, possibly toxic for the enzyme, could be generated by autoxidation of this catechol, but since the inhibition was not affected by superoxide dismutase this seems unlikely. Tiron has a high affinity for Fe^{3+} , and it could react with enzyme-bound Fe^{3+} generated in the catalytic cycle or as a side reaction.

Nitrones, which have been used as spin-trapping agents, are not suited for the present assay system as ascorbate may reduce *N*-oxides. The results from the pre-incubation experiments indicate that paraquat is also unsuitable to test the involvement of free radicals in γ -butyrobetaine hydroxylation.

Both Cu(II) -salicylate and Cu^{2+} have $\text{O}_2^{\cdot-}$ dismutating activity (Brigelius *et al.*, 1974; de Alvare *et al.*, 1976). Cu^{2+} is a potent inhibitor for the 2-oxoglutarate dioxygenases, and has been proposed to inhibit the prolyl hydroxylase reaction by competition with Fe^{2+} (Ryhänen, 1976). Myllylä *et al.* (1979) have reported inhibition of prolyl hydroxylase and lysyl hydroxylase reactions by four copper chelates. From such data and from kinetic studies these authors concluded that superoxide anion is the active form of oxygen in the prolyl hydroxylase and lysyl hydroxylase reactions. In our experiments with Cu(II) -salicylate and CuSO_4 in the γ -butyrobetaine hydroxylation we obtained essentially

the same degree of inhibition with both free and chelated copper ion, i.e. 50% inhibition at a concentration of about $10\text{ }\mu\text{M}$. Even if our enzyme is more sensitive to Cu^{2+} inhibition, neither our data nor those of Myllylä *et al.* (1979) can be taken as evidence for the involvement of superoxide anion in 2-oxoglutarate-dependent oxygenation.

The findings in the present study of 2-oxoglutarate-dependent oxygenases show that the results from experiments with superoxide scavengers of the types tested may be difficult to interpret and that adequate controls are mandatory.

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