neurones in this brain area. However, (−) bicuculline methiodide stimulates neuronal firing without antagonizing the action of GABA in several neural systems[13,14]. Therefore, the action of (+) bicuculline methiodide on masculine sexual behavior seems to be specifically associated with its property of antagonizing a GABAergic inhibitory input on the neural substrates of this behavior.

The antagonizing effects of (+) bicuculline methiodide appear to be GABA specific; this compound, for example, does not interfere with the action of noradrenaline and 5-HT on neuronal activity[16]. However, since monoamines have been implicated as neurotransmitters in mediating masculine sexual behavior[7,8], and GABAergic neurons are known to interact with monoaminergic neurons[17], the possibility that (+) bicuculline methiodide stimulates sexual behavior by interfering with the interaction between GABA and brain monoamines cannot be excluded from the present findings.

Comparing the present findings with those following treatment with drugs affecting the central monoamine transmission[7,18-20], (+) bicuculline methiodide influences both the copulatory series and the postejaculatory intervals. Recently, McIntosh and Barfield[18] reported a shortening of the postejaculatory interval following either electrolytic or chemical lesions selectively affecting the serotoninergic system and suggested the involvement of this system in the control of the length of the postejaculatory interval. However, the effect of (+) bicuculline methiodide on the postejaculatory interval is far more pronounced than that observed after interference with the central monoaminergic system.

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The effect of sulfhydryl compounds on the catalytic activity of Cu, Zn-superoxide dismutase purified from rat liver[1]

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Summary. Sulfhydryl compounds such as reduced glutathione, cysteine and 2-mercaptopropionylglycine, a hepato-protective agent, activated Cu, Zn-superoxide dismutase purified from rat liver at low concentrations (below 10 μM). Furthermore we found evidence indicating that this activation is achieved by reducing Cu^{2+} present in the catalytic site of the dismutase, and thereby promoting the dismutation of superoxide anions.
Key words. Reduced glutathione; cysteine; 2-mercaptopropionylglycine; Cu, Zn-superoxide dismutase (rat liver).

Superoxide dismutase (SOD) (EC 1.15.1.1) is a metalloprotein and catalyzes the dismutation of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen[3]. It has been shown that in mammalian tissue cells, SOD is distributed in the mitochondria and cytosol and exists in two forms, i.e., the Cu- and Zn-containing enzyme in both the intermembrane space of mitochondria and the cytosol and the Mn-containing enzyme in the matrix space of mitochondria[4]. It has been demonstrated that Cu, Zn- and Mn-SODs operate a redox cycle in which the active site metal, the Cu in the Cu, Zn-enzyme, is alternately reduced and reoxidized by O_2^-[5,6]. SOD is also widely recognized to play an important role in protecting the cell against oxygen toxicity[7].

Besides SOD, reduced glutathione (GSH) and cysteine, which are thiols present in nature, have been demonstrated to react with O_2^- nonenzymatically in vitro, and it has been thus suggested that these SH-compounds might play a major role in controlling O_2^- concentrations together with SOD in vivo[8,9]. 2-Mercaptopropionylglycine (MPG), which is a synthetic amino acid containing one SH-group that is used clinically as a hepato-protective agent, is reported to have chemical, biochemical and pharmacological properties similar to GSH[10]. This synthetic SH-compound has also been found to function as a scavenger of O_2^- in vitro[11,12]. However, although it is well known that SH-compounds form complexes with metal ions such as Cu^{2+} and Zn^{2+} and that they transfer one electron to metal ions such as Cu^{2+}[13,14], there has been no available information on the direct interaction between these SH-compounds and Cu, Zn- and Mn-SODs.

In the present study, we examined how SH-compounds such as GSH, cysteine and MPG affect the catalytic activity of Cu, Zn-SOD purified from rat liver. These SH-compounds were found to activate the dismutase at low concentrations. The mechanism for this activation was further investigated.

Materials and methods. Electrophoretically homogeneous Cu, Zn-SOD was prepared from rat liver according to the method described previously[15]. SOD was assayed in terms of its ability to inhibit the autoxidation of pyrogallol[16]. The reaction mixture contained 40 mM Tris-cacodylic acid buffer (pH 8.2) containing 0.08 mM diethylenetriamine-N,N′,N″,N″-pentaacetic acid, 0.2 mM pyrogallol and an appropriate amount of enzyme with or without GSH, cysteine or MPG, of which concentrations were all 5 or 10 μM, in a final volume of 1.0 ml. The reaction was initated by addition of pyrogallol after preincubation at 37 °C for 3 min and monitored by measuring the increase in absorbance at 420 nm. The rate of pyrogallol autoxidation was taken from the linear increase in the absorbance which is seen for a number of minutes after an induction period. GSH and MPG at the concentrations used here inhibited the rate of pyrogallol autoxidation (below 15%) but cysteine did not. Therefore, SOD activity in the presence of GSH and MPG was corrected by determination of the autoxidation rate in their presence only. One unit of SOD activity is defined as the amount of enzyme causing a 50% inhibition of the rate of pyrogallol autoxidation under the conditions described above.

The inactivation of Cu, Zn-SOD by diethyldithiocarbamate (DDC) was performed by incubating 10 μg/ml of pure Cu, Zn-SOD with 1.0 mM DDC in 50 mM potassium phosphate buffer (pH 7.4) at 37 °C for 30 min. The incubated mixture (2.5 ml) was immediately cooled in ice and then applied to a PD-10 column (1.5 × 5 cm) (obtained from Pharmacia Fine Chemicals), which was packed with Sephadex G-25 gel, equilibrated in 10 mM Tris-HCl buffer (pH 7.5) at 4 °C to remove an excess of DDC, since DDC inhibited the rate of pyrogallol autoxidation in the above-described SOD assay by 15%, even at the concentration of 0.1 μM. The enzyme applied to the column was eluted with 3.5 ml of the equilibrating buffer and the recovery was 95%. The complex of DDC and Cu, Zn-SOD was prepared by incubating 1.0 mg/ml of pure Cu, Zn-SOD with 1.0 mM DDC at 37 °C for 60 min and its formation was checked by measuring the increase in absorbance at 450 nm as described by Misra[17]. The formation of the DDC-SOD complex reached the maximum within this incubation time. An excess of DDC was removed by passing the incubated mixture through a PD-10 column equilibrated in 10 mM Tris-HCl buffer (pH 7.5). The dissociation of the DDC-enzyme complex at pH 8.2 was monitored by measuring the decrease in absorbance at 450 nm. The optical spectra of pure Cu, Zn-SOD in the presence of DDC, SH-compounds and H_2O_2 were recorded on a Beckman model 25 spectrophtometer. Protein was measured by the method of Lowry et al.[18], using bovine serum albumin as a standard.

MPG was kindly provided by Santen Pharmaceutical Co., Ltd. GSH, L-cysteine (monohydrochloride) and DDC (sodium salt) were purchased from Wako Pure Chemical Ind., Ltd. All other chemicals used were of the highest grade available. When necessary, the data were analyzed statistically using Student's t-test.

Results and discussion. We established in a preliminary experiment that the activity of SOD in the soluble fraction of rat liver cells was increased by adding SH-compounds such as GSH, cysteine and MPG. In order to clarify further the interaction between these SH-compounds and Cu, Zn-SOD, we studied the effect of the SH-compounds on the catalytic activity of pure Cu, Zn-SOD isolated from rat liver.

As shown in the table, the activity of the pure enzyme was significantly increased by addition of GSH, cysteine and MPG

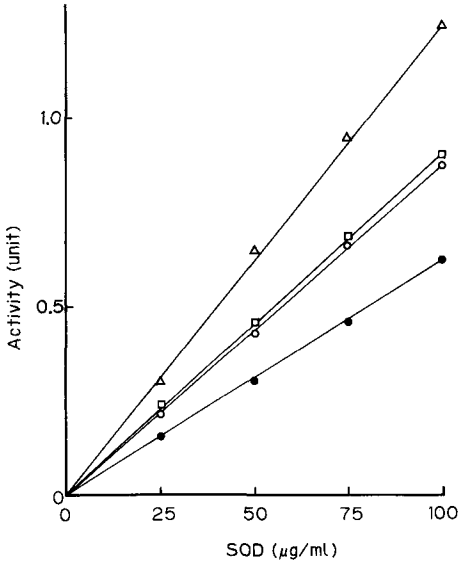

Figure 1. Effects of GSH, cysteine and MPG on the activity of various concentrations of pure rat liver Cu, Zn-SOD. The concentration of each added SH-compound was 10 μM. Assay conditions were described in 'Materials and methods'. Values given are means of three experiments. Symbols are: ●, no addition; ○, GSH; △, cysteine; □, MPG.

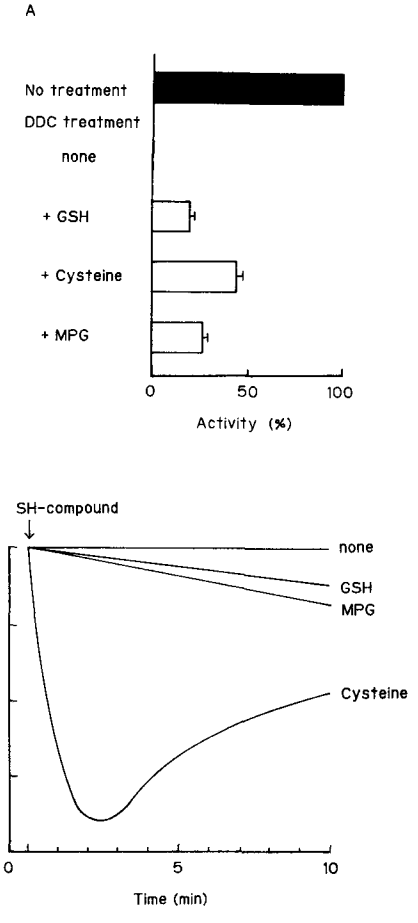

Figure 2. Effects of GSH, cysteine and MPG on the activity of pure rat liver Cu, Zn-SOD treated with DDC (A) and on the dissociation of the DDC-SOD complex (B). *A* The enzyme solution (10 μg/ml) was passed through a PD-10 column after treatment with or without 1.0 mM DDC. The eluate without DDC treatment had an activity of 42.4 units/ml. Concentration of each added SH-compound was 10 μM. Values given are means ± SE ($n = 3$). *B* The DDC-SOD complex was prepared as described in 'Materials and methods' and the concentration used was 0.5 mg of enzyme/ml. The dissociation of the complex was traced in 50 mM Tris-HCl buffer (pH 8.2) at 37 °C after addition of 0.5 mM SH-compounds.

at a concentration of 5 or 10 μM. Cysteine was the most effective activator among these SH-compounds and its addition at the concentration of 5 or 10 μM caused a 1.48- or 2.08-fold increase in SOD activity, respectively. On the other hand, there was little difference between GSH and MPG in ability to increase the enzyme activity. In addition, these SH-compounds had no effect on the heat-denatured enzyme which had lost its activity completeley (data not shown), suggesting that GSH, cysteine and MPG affect the catalytic activity of Cu, Zn-SOD directly.

Next, SOD activity was measured in reaction mixtures consisting of various concentrations of pure SOD and a constant concentration of GSH, cysteine or MPG, to investigate how these SH-compounds work as activators in the enzymatic reaction. The results are shown in figure 1. When the amount of enzyme was varied from 25 to 100 ng/ml in the presence of 10 μM GSH, cysteine or MPG, SOD activity in the presence of these SH-compounds increased in proportion to the enzyme concentrations, as was found in the absence of SH-compounds. Thus GSH, cysteine and MPG enhanced the activity of Cu, Zn-SOD at a constant ratio, suggesting that these SH-compounds activate the enzyme by acting on a specific site of the enzyme.

Furthermore, we examined whether or not GSH, cysteine and MPG activated Cu, Zn-SOD irreversibly, since it is well known that SH-compounds form complexes with metal ions such as Cu^{2+} and Zn^{2+} [13,14]. The experiment was carried out as follows: Pure Cu, Zn-SOD (10 μg/ml) was incubated with 1.0 mM GSH, cysteine or MPG in 40 mM Tris-HCl buffer (pH 8.2) at 37 °C for 30 min and then the incubated mixture was passed through a PD-10 column to remove an excess of these SH-compounds. The activity of these SH-compound-treated SODs was measured. These SODs were found to cause no increase in activity (data not shown), indicating that the activation of Cu, Zn-SOD due to SH-compounds such as GSH, cysteine and MPG is reversible. The interaction between these SH-compunds and Cu present in pure Cu, Zn-SOD from rat liver was further investigated with the enzyme treated with DDC, since it is known that DDC inactivates Cu, Zn-SOD by forming a complex with Cu^{2+} present in the catalytic site of the enzyme[17,19]. The results are shown in figure 2A. Although the pure enzyme which had been treated with 1.0 mM DDC for 30 min lost activity completely, activity was observed when this DDC-treated enzyme was incubated with 10 μM GSH, cysteine or MPG. The order of this reactivation was as follows: Cysteine > MPG > GSH. This phenomenon was very surprising. We therefore studied how the SH-compound-induced reactivation occurred. The reactivation was assumed to be partly due to the dissociation of a DDC-enzyme complex by SH-compounds. Hence the dissociation of the DDC-SOD complex (which was prepared by incubating pure Cu, Zn-SOD (1.0 mg/ml) with 1.0 mM DDC for 60 min) by GSH, cysteine and MPG, was examined by checking the decrease in absorbance at 450 nm. As shown in figure 2B, an apparent decrease in the absorbance occurred on the addition of these SH-compounds. Cysteine caused the most rapid decrease in absorbance. This suggests that the SH-compound-induced reactivation might be partly due to the dissociation of the DDC-enzyme complex by SH-compounds. In the case of cysteine, however, a slow reincrease in the absorbance was observed (fig. 2B), which might be due to the rebinding between the dissociated DDC and SOD. From these findings, it appeared that SH-compounds such as GSH, cysteine and MPG activate Cu, Zn-SOD by acting on the Cu^{2+} present in the catalytic site of the enzyme.

It is well known that SH-compounds transfer one electron to metal ions such as Cu^{2+} [13,14]. It has been demonstrated that Cu present at the active site of Cu, Zn-SOD takes part in its enzymatic reaction by cycling between divalent and monovalent states during the catalytic reaction[5]. It has also been shown that H_2O_2 at high concentrations (mM) causes a rapid reduction of Cu^{2+} present in Cu, Zn-SOD, which is associated with a bleaching of its absorption in the visible range[20]. Hence the effects of GSH, cysteine and MPG on the reduction of Cu^{2+} present in pure Cu, Zn-SOD were compared with that of H_2O_2 by observing changes in the visible absorption spectrum of the enzyme (400–750 nm). As shown in figure 3, the addition of these SH-compounds at 5.0 mM per 5.0 mg/ml of the enzyme caused a slow bleaching of the visible absorption due to Cu^{2+} in the enzyme as compared with that of the same concentration of H_2O_2. In addition, cysteine had the highest ability to bleach the absorption among these SH-compounds (fig. 3), indicating that cysteine can reduce Cu^{2+} present in Cu, Zn-SOD more rapidly than GSH and MPG, which have a similar ability to reduce the Cu^{2+}. The order of this reduction was quite consistent with that of the activation on the enzyme (table), suggesting that the reduction of Cu^{2+} present in Cu, Zn-SOD by SH-compounds is involved in the activation of the enzyme due to the SH-compounds.

From the present results we propose the following mechanism for the activation of Cu, Zn-SOD by SH-compounds such as GSH, cysteine and MPG: The catalytic reaction of the enzyme is based on alternate reduction and reoxidation of the Cu^{2+} at the active site during successive interaction with O_2^-. In the presence

Effects of GSH, cysteine and MPG on the activity of Cu, Zn-SOD purified from rat liver

Addition	Concentration (μM)	Activity (units/mg protein)	Ratio
None	–	6030 ± 160[a]	1.00
GSH	5	7530 ± 260[b]	1.25
	10	8930 ± 120[b]	1.48
Cysteine	5	8950 ± 280[b]	1.48
	10	12110 ± 250[b]	2.08
MPG	5	7680 ± 290[b]	1.27
	10	9250 ± 220[b]	1.53

[a] Values given are means ± SE (n = 5). [b] Significance of the difference between no addition and SH-compound: $p < 0.001$.

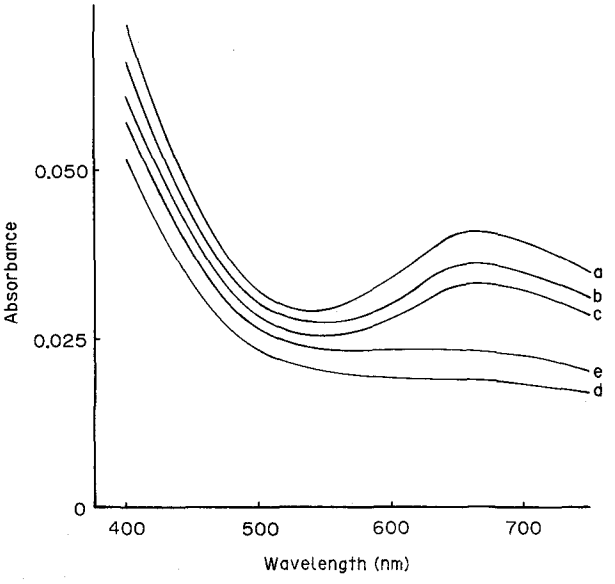

Figure 3. Effects of GSH, cysteine, MPG and H_2O_2 on the absorption spectrum of pure rat liver Cu, Zn-SOD. Enzyme (5.0 mg/ml), in 50 mM Tris-HCl buffer (pH 7.7)–0.1 mM EDTA, was treated with 5.0 mM SH-compounds or 5.0 mM H_2O_2 at 25 °C and optical spectra were recorded 30 min after addition of SH-compounds and 3 min after addition of H_2O_2. Line a, no addition; line b, GSH; line c, MPG; line d, cysteine; line e, H_2O_2.

of these SH-compounds, the Cu^{2+} is reduced by the SH-compounds as well as by O_2^- and then reoxidized by O_2^-, by which the redox cycle between Cu^{2+} and Cu^+ is accelerated. Consequently, the dismutation of O_2^- by the enzyme is enhanced. The present results also suggest that these SH-compounds might participate in protecting the cell against oxygen toxicity in vivo by activating Cu, Zn-SOD, in addition to their function of directly scavenging O_2^- which has been found in vitro[8,9,11,12].

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3[H]-Sulpiride labels mesolimbic non-dopaminergic sites that bind antidepressant drugs[1]

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Summary. 3[H]-(−)-Sulpiride and 3[H]-spiperone binding was compared in rat amygdala, nucleus accumbens and striatum, using (+/−)-sulpiride to define specific binding. 3[H]-(−)-Sulpiride bound to twice as many sites in amygdala and nucleus accumbens as 3[H]-spiperone. 3[H]-(−)-Sulpiride binding was directed to these additional sites by using 1 μM spiperone to mask dopaminergic binding. The binding of 3[H]-(−)-sulpiride to these sites was high affinity, reversible, Na^+-dependent, but not stereospecific. Metoclopramide, tiapride and antidepressant medications, but not other neuroleptics, ADTN, or serotonin displaced 3[H]-(−)-sulpiride binding to these sites. These data suggest that 3[H]-(−)-sulpiride labels mesolimbic sites other than dopamine receptors which may mediate antidepressant effects.
Key words. Sulpiride; spiperone; antidepressants; substituted benzamide; alprazolam.

Dopamine (DA) receptors are present in several brain areas. Because of efforts to measure DA receptor binding in mesolimbic brain areas as well as striatum, we compared the binding of 3[H]-(−)-sulpiride and 3[H]-spiperone in amygdala, nucleus accumbens, and striatum of rat brain. Both of these ligands bind with high affinity to post-synaptic DA D2 (i.e. non-adenyl cyclase coupled) receptors. In addition, 3[H]-(−)-sulpiride as well as 3[H]-spiperone binds pre-synaptic DA receptors on corticostriatal glutamate neurons in rat[3–5]. Other investigators have shown that in striatum these two ligands bind the same number of sites when sulpiride is used as the counterligand to define specific binding[6]. However, we now report that in amygdala and nucleus accumbens, 3[H]-(−)-sulpiride binds additional sites distinct from DA receptors bound by 3[H]-spiperone. Antidepressants and other substituted benzamides, but not other neuroleptics, ADTN, or serotonin demonstrated high affinity for these sites.

Methods. Male Sprague-Dawley rats (150 g, Simonsen Laboratories, Gilroy, CA) were sacrificed by decapitation and their brains were removed for receptor binding assays[7]. Frontal cortex, amygdala, nucleus accumbens, and striatum were dissected on ice and homogenized in ice-cold 50 mM Tris buffer (pH 7.6), containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM tranylcypromine, and 0.1% ascorbate, using a Brinkmann Polytron homogenizer (setting 5, 10 s). The homogenates were centrifuged twice at 50,000 × g with resuspension in fresh buffer. The total assay volume of 1.0 ml contained 0.3–0.8 mg of protein, measured using the Biorad protein assay[8], depending on the brain area assayed. 3[H]-(−)-Sulpiride (78.2 Ci/mmole) and 3[H]-spiperone (22.8 Ci/mmole) were obtained from New England Nuclear (Cambridge, MA). Bound and unbound ligand were separated by rapid filtration through Whatman GF-B filters followed by two 5-ml washes of buffer. The filters were counted after the addition of 10 ml Aquasol-2 scintillation cocktail. For experiments where 3[H]-(−)-sulpiride binding was directed to non-dopaminergic sites 1.0 μM spiperone was added to the assay mixture. The reversibility of this component of 3[H]-(−)-sulpiride binding was assessed by measuring the residual binding of 10 nM 3[H]-(−)-sulpiride in triplicate after the addition of 10 μM (+/−)-sulpiride at various time points (0.5–20 min), and its Na^+ dependence by comparing binding in the presence or absence of 140 mM NaCl.

Results. Table 1 summarizes the binding characteristics of 3[H]-spiperone, 3[H]-(−)-sulpiride, and 3[H]-(−)-sulpiride in the presence of 1.0 μM spiperone, in three brain areas. No specific binding was detected in frontal cortex for any of the three binding conditions. 3[H]-(−)-sulpiride binding was performed in the presence of 1 μM spiperone in the amygdala and nucleus accum-