

Bibliotheksexemplar

TITLE:

Effect of sulfite on human neutrophils.

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Subdivision: Membranes and bioenergetics

Abbreviations:

CL, chemiluminescence; PMA, phorbol myristate acetate; PBS, phosphate buffered saline.

Enzymes:

Catalase EC 1.11.1.6; NADPH oxidase EC 1.6.-; sulfite oxidase EC 1.8.3.1; superoxide dismutase EC 1.15.1.1; xanthine oxidase EC 1.1.3.22.

SUMMARY:

The addition of sulfite in concentrations of 0.01 mM to 1 mM results in a 6 fold increase in chemiluminescence of non-stimulated human neutrophils at 37°C and pH 7. Neutrophils stimulated with zymosan or phorbol myristate acetate have an additional 2 fold stimulation when sulfite is added. Higher sulfite concentrations (2 mM to 10 mM) decrease the chemiluminescence of both resting and stimulated cells. When sulfate is added, no changes in chemiluminescence of resting and stimulated neutrophils are seen, indicating that the effect is specific for sulfite. Chemiluminescence of resting cells in the presence of low sulfite concentrations is significantly inhibited by superoxide dismutase, but not by catalase, mannitol, or azide. These findings suggest that sulfite in low concentrations stimulates neutrophils by activating oxidative metabolism to produce superoxide anions. Ultrastructural studies confirm the stimulating effect of sulfite on neutrophils with sulfite-treated cells exhibiting increased ruffled surface membranes and degranulation changes similar to those seen in PMA-stimulated cells.

INTRODUCTION:

Sulfur dioxide and sulfates are well known air pollutants [1, 2]. The role of sulfite forms of sulfur oxides in the ambient air is gaining increasing attention [3, 4]. Sulfite is also frequently used as a preservative in foods, beverages, and many drug preparations [5]. Both intravenous infusion solutions and peritoneal dialysis solutions contain sulfite in concentrations up to 15 mM [5, 6]. We have reported previously that sulfite can adversely affect energy metabolism and that target organ sensitivity is related to the activity of sulfite oxidase [7] which detoxifies sulfite by oxidation to sulfate [8, 9]. Tissues and cells with very low activities of sulfite oxidase have significant decreases in ATP levels when exposed to sulfite [7]. Human neutrophils and other phagocytic cells are among those exhibiting very low sulfite oxidase activity [7].

The primary physiological function of neutrophils is to kill bacteria by phagocytosis and production of highly reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxyl-radicals and hypochlorous acid [10]. These radicals are involved in oxidation reactions in which chemically excited compounds are produced. The resulting phenomenon of

chemiluminescence (CL) can be used as a measurement of oxidative metabolism during phagocytosis as well as a parameter for oxidative defense capacity [11-13].

In the study reported here, we examine the effect of sulfite on the oxidative metabolism of resting, phagocytosis-associated, and phorbol myristate acetate (PMA)-stimulated human neutrophils by using measurements of lucigenin-dependent CL and morphological methods. Sulfite in low concentrations stimulates both resting and activated neutrophils as measured by CL and as observed in the ultrastructure of the cells.

MATERIALS AND METHODS:

Materials:

Zymosan, PMA, and lucigenin were purchased from Sigma (Deisenhofen, FRG); PBS-buffer from Serva (Heidelberg, FRG); superoxide dismutase, catalase, and xanthine oxidase from Boehringer (Mannheim, FRG); neutrophil isolation medium from Los Alamos Diagnostics (Utrecht, Netherlands); all other chemicals (analytical grade) were from Merck (Darmstadt, FRG).

Isolation of neutrophils:

Human neutrophils were isolated from citrate-anticoagulated freshly drawn human venous blood with neutrophil isolation medium according to Beck-Speier et al. [14]. The purified cells were resuspended in PBS-buffer (without Ca^{2+} and Mg^{2+}), pH 7, containing 0.1 % glucose, and diluted immediately before use in the CL-assay with PBS-buffer, pH 7, containing Ca^{2+} , Mg^{2+} , and 0.1 % glucose. The viability of the cells was always over 95 % by trypan blue exclusion.

Preparations of sulfite solutions:

Sodium sulfite was dissolved in PBS-buffer, pH 7, containing 0.1 % glucose. The pH-value was readjusted to pH 7 by adding 1M HCl. Sulfite solutions were always prepared just prior to use.

Chemiluminescence of the neutrophils:

The lucigenin-dependent CL of neutrophils was measured at 37°C in a six-channel Biolumat LB 9505 (Berthold, Wildbad, FRG). Neutrophils (3×10^4 cells) were resuspended in 0.5 ml PBS-buffer, pH 7, containing 0.1 % glucose and 0.8 mM lucigenin, and preincubated for 10 min in the biolumat. The cells were maintained at 37°C in the CL-assay from which measurements were made continuously for 20 min. Immediately after the beginning of the assay, sulfite, in concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1.0 mM, 2.0 mM, 5.0 mM and 10.0 mM, was added to the resting cells. The CL was measured as counts integrated over 18 min for 3×10^4 cells. The same cells were measured again for 20 min after the addition of the stimuli, zymosan (500 µg) or PMA (100 ng). A stock solution of PMA in dimethylsulfoxide at a concentration of 1 mg/ml was prepared, stored in small aliquots at -20°C, and diluted with PBS, pH 7, containing 0.1% glucose immediately before use. Superoxide dismutase (100 µg), catalase (200 µg), azide (0.1 mM) or mannitol (10 mM) were added to the CL-assay when the maximal CL of resting cells, incubated in the presence of 0.5 mM sulfite, was reached. For studies with stimulated cells these substances were added to the CL-assay before zymosan or PMA.

Chemiluminescence of the xanthine oxidase reaction:

The CL of xanthine oxidase (10 µg) was used as a cell free control system to assess sulfite effects on oxidative metabolism. It was measured in a total volume of 0.5 ml 0.1 M potassium phosphate buffer, pH 7.6, containing 0.1 mM xanthine and 0.8 mM lucigenin, and saturated with oxygen. The various sulfite concentrations were added immediately before the beginning of the xanthine oxidase reaction. The CL was measured with a one-channel biolumat (Berthold, Wildbad, FRG) and estimated as counts of a 10 sec integral after 1 min reaction. 100% corresponds to 610×10^3 cpm.

Incubation and preparation of neutrophils for electron microscopy:

Resting and PMA-stimulated neutrophils (1×10^6 cells/ml) were incubated with 1 mM sulfite at pH 7 and 37°C for 20 min.

Resting and PMA-stimulated control cells were incubated simultaneously. After incubation, the cells were centrifuged at 400 g for 10 min and fixed with 1.5 % glutaraldehyde in phosphate buffer for 1 hour. They were then washed in buffer and post-fixed with 2% osmium tetroxide in sodium cacodylate buffer. The cells were embedded in araldite and 30 nm sections were prepared for study with a Zeiss CEM902 electron microscope (Zeiss, Oberkochen, FRG). The 30 nm sections are

necessary for elemental analysis of these cells which will be reported separately. These 30 nm sections are used in this study to evaluate the morphology of the neutrophils using standard ultrastructural criteria for resting and stimulated cells [15, 16]. At least 4 grids were prepared from different levels within the blocks for each treatment group in 2 repetitions of the experiments. All cells in suitably thin grid spaces were observed and photographed at 3000 - 7000x. All neutrophils, for which the entire sectioned surface was visible within the grid space, and for which the nucleus and sufficient cytoplasm were visible to be certain that they were neutrophils, were scored as resting (no evidence of activation), 1+ activation (minimal degranulation and/or shape changes), 2+ activation (obvious degranulation or shape changes), and 3+ activation (degranulation and marked shape changes). More than 400 neutrophils were observed and photographed. Since these very thin sections are quite delicate and often have holes, relatively few good grid spaces are found. Any grid space with a hole cannot be used because long exposure times are needed for photography. (Holes result in movement of the membrane and loss of focus). Therefore, all suitable neutrophils were studied, rather than random sampling. There is no evidence of a reproducible error resulting from ignoring grid spaces with holes. Holes were not

associated with cells or a particular cell type. Hence there was no bias in the selection of cells studied ultrastructurally.

RESULTS:

Sulfite in concentrations from 0.01 mM to 1.0 mM resulted in 6 fold increase in lucigenin-dependent CL of resting neutrophils as seen in Fig. 1. With higher concentrations of sulfite, CL did not further increase, but instead diminished. Fig. 2 shows that neutrophils stimulated with zymosan (Fig. 2a) or PMA (Fig. 2b) exhibited a two-fold higher CL in the presence of sulfite concentrations up to 1.0 mM than stimulated control cells. With higher sulfite concentrations in PMA-stimulated cells (Fig. 2b), the CL of stimulated neutrophils reduced to control values or to less than control values in zymosan-stimulated cells (Fig. 2a). With opsonized and unopsonized zymosan findings were the same. When neutrophils were stimulated simultaneously with two stimuli, zymosan and PMA, the increase in CL (from 100 % to about 900 %) was the total of the increases in CL of zymosan-stimulated cells (from 100 % to about 600 %) and PMA-stimulated cells (from 100 % to about 300 %). A similar additive effect was seen when neutrophils were stimulated with zymosan or PMA in the presence of low sulfite concentrations. It appears that sulfite induces stimulated cells to their maximal capacity for CL. In the absence of cells, sulfite did not increase CL of lucigenin

Fig. 1

Fig. 2

alone (data not shown). The viability of resting and stimulated cells after incubation with sulfite was nearly 100 %, as measured by trypan blue exclusion.

The CL of stimulated neutrophils measured in the presence of lucigenin is due to the reactions of O_2^- with lucigenin [17]. A control experiment was therefore performed to study the effect of sulfite on the CL of lucigenin with O_2^- produced without cells by xanthine oxidase (18). Sulfite did not change the enzymatic activity nor the CL produced by xanthine oxidase using xanthine as substrate. The lucigenin-dependent CL of the xanthine oxidase reaction changed in the presence of 0.1 mM, 1.0 mM or 10.0 mM sulfite by a factor of 1.16, 1.33, and 0.99, respectively. Since these changes are not the magnitude seen with cells, this indicates that sulfite is not acting simply by interfering with the reaction of lucigenin and superoxide anion. The increasing effect of sulfite on the lucigenin-dependent CL is only seen in the presence of the cells.

To determine whether the effect of sulfite on the CL of neutrophils is specific or whether other anions are also able to produce a similar result, experiments were performed with sulfate and nitrite. Table 1 shows that sulfate has no influence on the CL of resting or stimulated neutrophils. The

same result was obtained for nitrite (data not shown). This indicates that the elevated CL of neutrophils is specific for sulfite.

Tab. 2

To elucidate which oxygen species is involved in this sulfite-dependent process, enzymatic and chemical scavengers of oxygen metabolites including superoxide dismutase, catalase, mannitol or azide [19], were added to sulfite-treated cells. As shown in Table 2 only superoxide dismutase, which is a catalyst in the dismutation of superoxide anions to hydrogenperoxide and oxygen, inhibits the increased CL of resting neutrophils in the presence of 0.5 mM sulfite. For neutrophils stimulated with zymosan or PMA in the presence of 0.5 mM sulfite, a similar result was obtained: superoxide dismutase inhibited by more than 90 % the CL of stimulated control cells as well as the CL of cells stimulated in the presence of sulfite. The other oxygen scavengers did not change the CL. The inhibition of the sulfite-induced CL of resting and stimulated cells by superoxide dismutase indicates that superoxide anions are involved in this process.

These data suggest that sulfite activates the oxidative metabolism of neutrophils to produce superoxide anions. To

Fig. 3

correlate CL measurements with morphologic changes in the cells, resting and PMA-stimulated cells were incubated with 1 mM sulfite and studied ultrastructurally. Fig. 3 illustrates representative morphologic changes seen within each treatment group. In Fig. 3a, resting control cells are round with relatively smooth surface membranes, and no degranulation is seen. Cells incubated with sulfite (Fig. 3b) have an irregular shape and a membrane with pseudopodia-like ruffles. Figures 3c and 3d are PMA-stimulated control cells and cells stimulated with PMA in the presence of sulfite. The sulfite-treated neutrophils (Fig. 3b) have an appearance similar to the PMA-stimulated control cells. Stimulation of cells with PMA in the presence of sulfite results in degranulation and marked ruffling. Table 3 enumerates the cell changes seen in each treatment group.

Tab. 3

DISCUSSION:

Our findings suggest that human neutrophils are affected by sulfite in phagocytosis-connected processes as shown by CL-measurements and morphological studies. Low sulfite concentrations (0.01 mM to 1 mM) increase the CL of resting and stimulated neutrophils, whereas higher sulfite concentrations diminish the CL of the cells. Our previous studies revealed that sulfite in higher concentrations significantly decreased the ATP content and oxygen consumption of tissues and cells exhibiting only very low activities of sulfite oxidase [7]. Therefore, higher sulfite concentrations not only affect energy metabolism, but also inhibit processes associated with phagocytosis. However, sulfite in low concentrations shows an activating effect on resting neutrophils as well as after stimulation in vitro. The inhibition of this activating effect of sulfite on the cells by superoxide dismutase indicates that superoxide anions are involved in this process. The use of lucigenin for measuring CL of neutrophils also shows that superoxide anions participate in the effect of sulfite on neutrophils, because CL is due to the reaction of superoxide anions with lucigenin [17, 20]. The activating effect of low sulfite concentrations is also seen morphologically. Cells incubated with sulfite

exhibit a similar membrane appearance as cells stimulated with PMA. These results indicate that sulfite is a stimulating agent for human neutrophils by inducing changes in the plasma membrane and by activating oxidative metabolism to produce superoxide anions. Our findings are specific for sulfite. The increase of CL of neutrophils is therefore not a general phenomenon for anions.

Sulfite is known to react with numerous biomolecules [21]. Because sulfite induces the neutrophils to produce superoxide anions, it is likely that sulfite interacts with the membrane-bound enzyme NADPH oxidase which represents the first step of the respiratory burst by reducing oxygen to superoxide anions. NADPH oxidase is a complex, made up of membrane-associated catalytic components including cytochrome b_{558} . This complex functions as an electron transport chain [10, 22, 23], and includes cytosolic components possibly involved in oxidase activation [24-26]. Sulfite is oxidized by the enzyme sulfite oxidase [8, 9] which contains two cofactors, a molybdenum and a cytochrome of the b type [27, 28]. During oxidation, the electrons are transported from sulfite to molybdenum and then to cytochrome b_5 which is required for the electron transfer to the physiological acceptor [28]. Possible mechanisms for our findings include: (1) sulfite is oxidized by the electron

transport system of the NADPH oxidase complex containing cytochrome b_{558} ; (2) sulfite interacts with the cytosolic components of NADPH oxidase involved in oxidase activation; (3) sulfite interferes with components of the different activation pathways for the NADPH oxidase as has been proposed by Bellavite [29]; (4) sulfite interacts with receptors on the cell surface which transfer the signal for the stimulation inside the cell. Preliminary studies of the effect of sulfite on the NADPH oxidase activity of crude neutrophil membranes showed two-fold enhancement of the NADPH oxidase activity by sulfite (unpublished results). This supports the hypothesis that sulfite is oxidized by the electron transport system of NADPH oxidase in which the electron flow from sulfite to the system could be used for the reduction of oxygen to superoxide anion. The findings of Aviram and Sharabani [30] in regard to the reduction of NADPH oxidase cytochrome b_{558} by dithionite support this mechanism. Nevertheless, in view of the number of possible mechanisms this system needs to be studied in greater detail.

It is known that sulfite, added to various injectable and inhalable drug preparations at concentrations up to 15 mM, can cause hypersensitive reactions in asthmatic and non-asthmatic patients [5]. The mechanism of sulfite hypersensitivity is not

known but there is evidence that sulfite may stimulate degranulation and mediator release [5]. The level of sulfite oxidase also seems to play an essential role in the sulfite hypersensitivity syndrome, because chronic asthmatic patients contain lower levels of this enzyme in skin fibroblasts than normal subjects [5, 31]. Our observation is that sulfite in very low concentrations is able to activate neutrophils, which contain a very low level of sulfite oxidase, to produce superoxide anions. This may lead to harmful effects and the use of sulfite-preserved drug preparations should be carefully considered.

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TABLE 1: Effect of sulfate on the CL of resting and zymosan-stimulated neutrophils at 37°C and pH 7.

Human neutrophils were incubated with sulfate in the same way as with sulfite and then stimulated with zymosan as described in "Materials and Methods". The CL of resting control cells is set to 100 %.

Sulfate concentration (mM)	% Chemiluminescence	
	resting cells	stimulated cells

-	100	380
0.5	129	403
1.0	132	314

TABLE 2: Influence of superoxide dismutase, catalase, mannitol or azide on the enhanced CL of resting human neutrophils by 0.5 mM sulfite at 37°C and pH 7.

Resting human neutrophils were incubated in the absence and presence of 0.5 mM sulfite at 37°C and pH 7. After about 10 min the maximum of CL-production was reached. At this time superoxide dismutase (100 µg), catalase (200 µg), mannitol (10 mM) or azide (0.1 mM) were added and the CL was measured for further 10 min. The CL of resting control cells integrated over 10 min after addition of the oxygen scavengers is set to 100 %. The other values are given in percentage increase of the CL of resting control cells and are expressed as means with n=3.

Additions	% Chemiluminescence of resting cells	
	without sulfite	with sulfite
None	100	625
Superoxide dismutase	32	148
Catalase	100	787
Mannitol	126	722
Azide	287	682

TABLE 3: Effect of sulfite on the morphologic activation patterns of human neutrophils.

Resting and PMA-stimulated neutrophils were incubated in the absence and presence of 1 mM sulfite for 20 min at pH 7 and 37°C, prepared for electron microscopy studies, and classified in morphologic patterns of activation (no, 1+, 2+, and 3+) as described in "Materials and Methods".

Treatment group	% Cells with activation			
	No	1+	2+	3+
Resting control cells	88	10	2	< 1
Cells + 1 mM sulfite	43	39	16	2
PMA-stimulated control cells	< 1	19	73	8
PMA-stimulated cells + 1mM sulfite	< 1	2	30	68

LEGENDS TO FIGURES:

FIG. 1: Effect of sulfite on the CL of resting human neutrophils at 37°C and pH 7.

Human neutrophils were incubated with sulfite as described in "Materials and Methods". The CL of resting control cells was set to 100 %. The other values are given in percentage increase of the CL of resting control cells and expressed as means $\pm$ SEM with n representing the number of experiments. The mean value of resting control cells $\pm$ SEM: $(2128 \pm 187) \times 10^3$ counts per 3×10^4 cells integrated over 18 min (n=22).

FIG. 2: Effect of sulfite on the CL of (a) zymosan- and (b) PMA-stimulated human neutrophils at 37°C and pH 7.

Human neutrophils were incubated with sulfite and stimulated with (a) zymosan and (b) PMA as described in "Materials and Methods". The values are given as percentage increase of the CL of resting control cells and expressed as means $\pm$ SEM with n representing the number of experiments.

FIG. 3: Effect of sulfite on the ultrastructure of human neutrophils at 37°C and pH 7.

Resting and PMA-stimulated human neutrophils were incubated in the absence and presence of 1 mM sulfite for 20 min at pH 7 and 37°C, and prepared for electron microscopy studies as described in "Materials and Methods". (a) Resting control cells, (b) cells incubated with 1 mM sulfite, (c) PMA-stimulated control cells, (d) cells stimulated with PMA in the presence of 1 mM sulfite.

FIG. 1

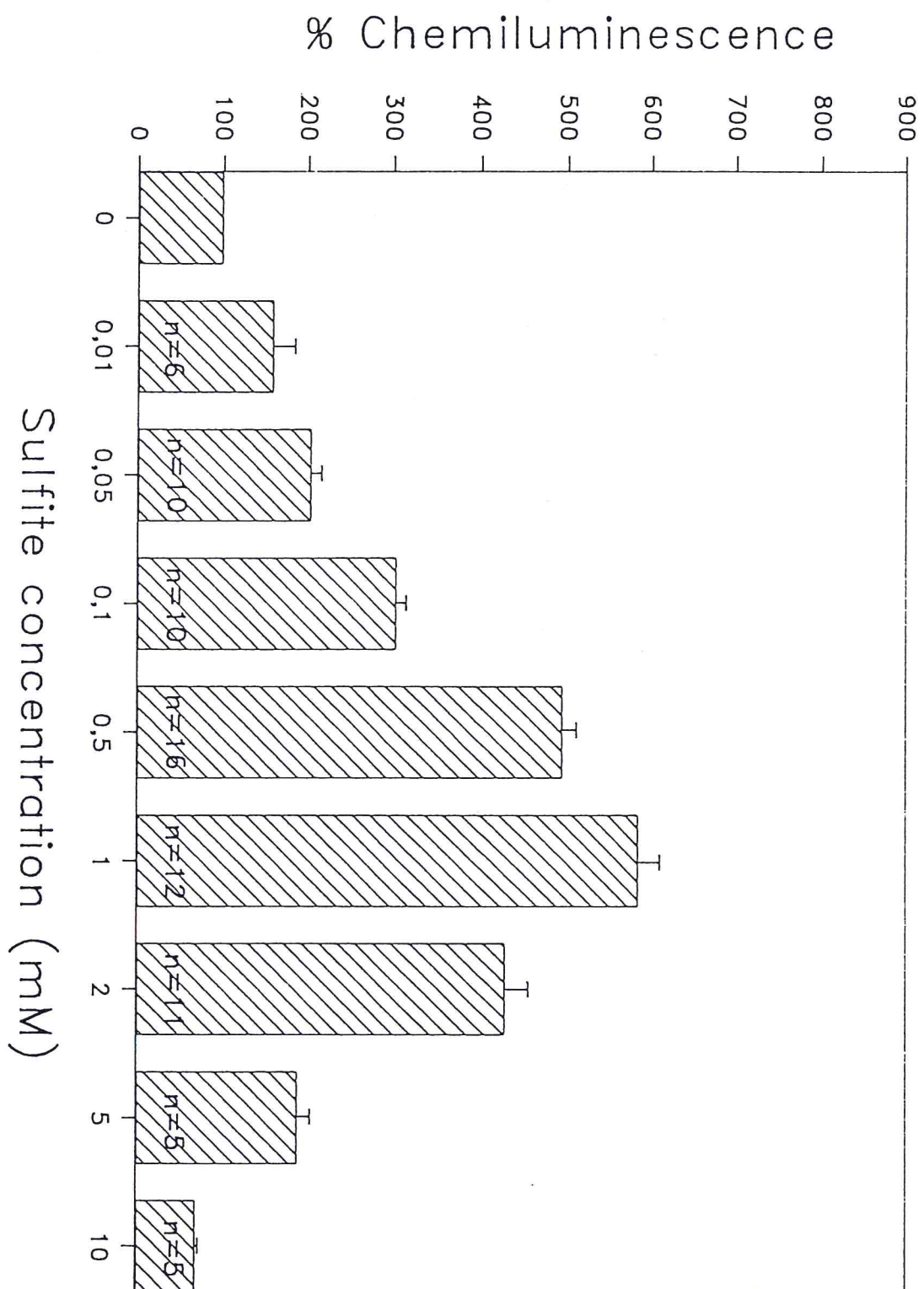


FIG. 2a

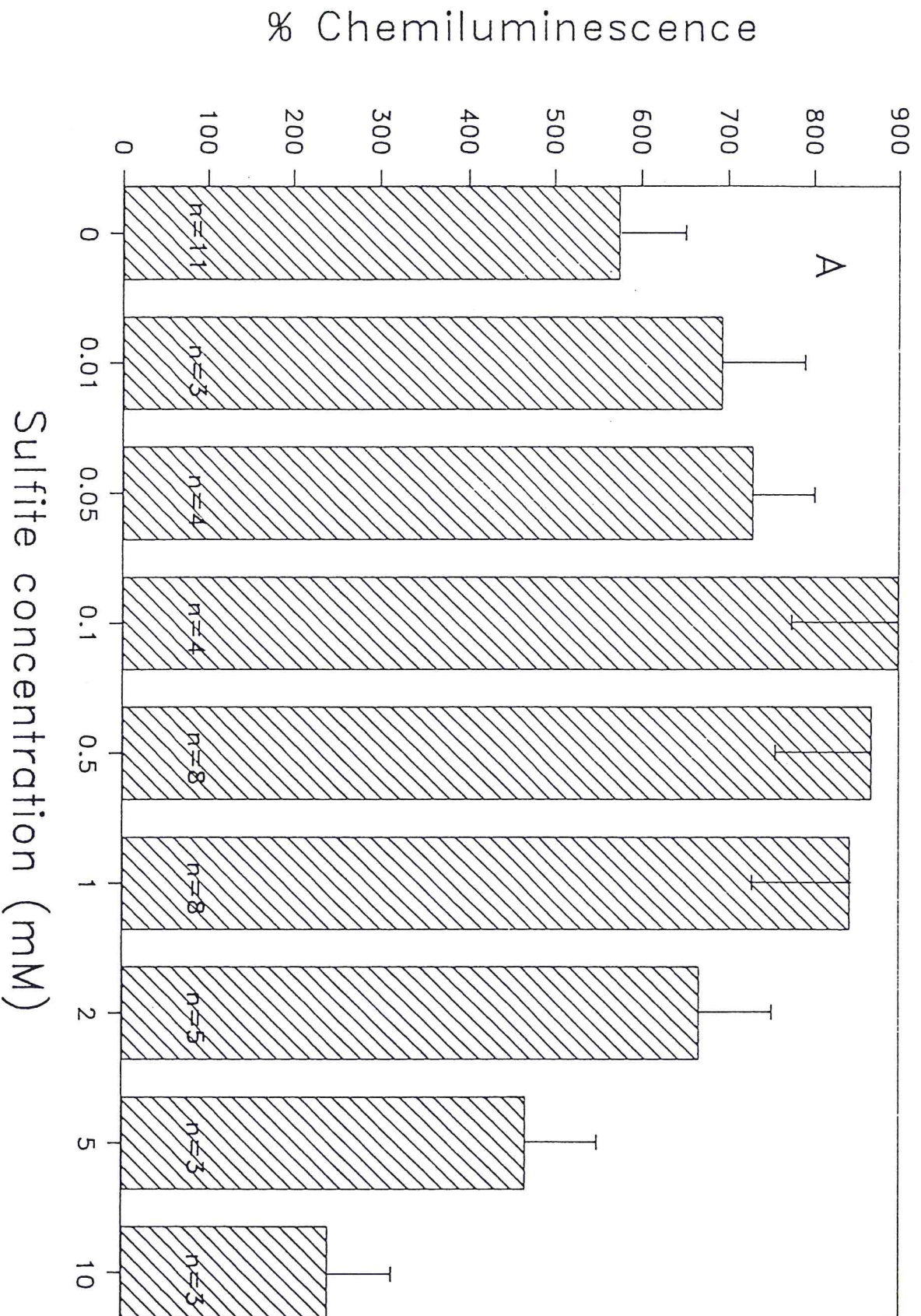


FIG. 2b

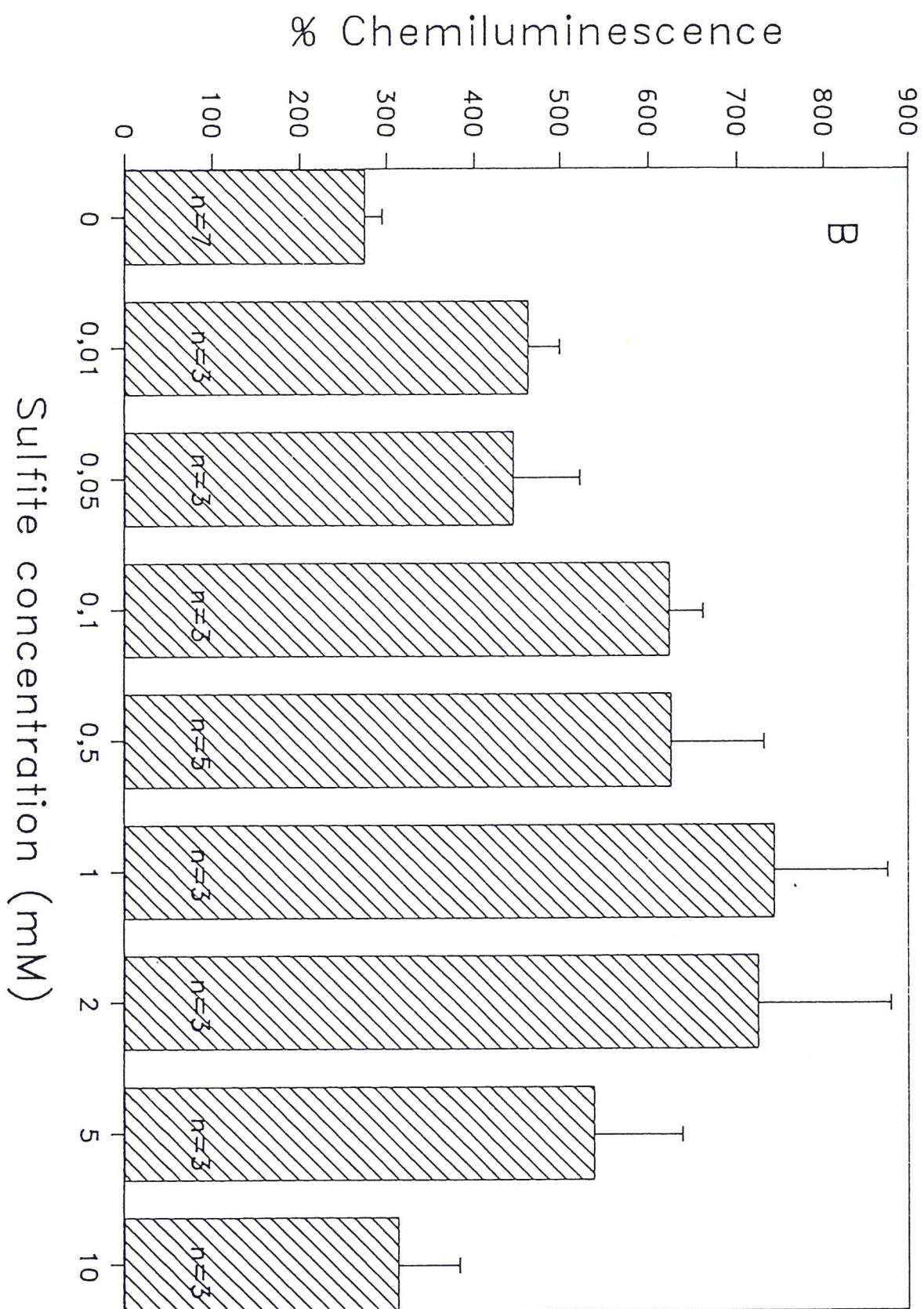


FIG. 3

