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Mechanisms of Nitroso Compound-Induced Inhibition of Superoxide Generation in Neutrophils: Fluorescence Quenching of Perylene by Nitroso-Compounds in the Membrane Fractions of Neutrophils[1]

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To investigate the mechanism of nitroso compound-induced inhibition of the respiratory burst in neutrophils, we studied fluorescence quenching of perylene by nitroso-compounds in the membrane fractions of neutrophils at 17, 27, and 37°C and the reagent-induced inhibition of superoxide generation at 28 and 37°C. With increasing temperature, the quenching of perylene fluorescence and inhibition of superoxide generation by nitrosobenzene (NB) were both diminished, while those by 2-nitrosotoluene (NT) were both enhanced. The temperature dependence of the inhibition constants and the quenching constants indicates that the binding of NB is exothermic ($\Delta H = -27$ kJ/mol for inhibition and $\Delta H = -29$ kJ/mol for quenching) and essentially enthalpy-driven. On the other hand, that of NT is endothermic ($\Delta H = +16$ kJ/mol for inhibition and quenching) and essentially entropy-driven. Quenching studies of perylene fluorescence in synthetic vesicles made of endogenous polar lipids of neutrophils showed that the enthalpy changes of NB- and NT-binding with perylene in lipids were similar to each other. Moreover, their values were in good agreement with that of NT, but not of NB, in the membrane fractions, an assembly of proteins and lipids, of neutrophils. These results suggest that NB inhibits the activity by binding to proteins in the membrane, whereas inhibition by NT occurs through hydrophobic interaction with lipids and/or proteins.

Key words: fluorescence quenching, inhibition, nitroso-compounds, porcine neutrophils, respiratory burst.

Neutrophils, a main component of leukocytes, utilize toxic oxidants, including hypochlorous acid and hydroxyl radicals, for microbicidal activity (for reviews, see Refs. *1-4*). These active oxygen species originate from the superoxide anion, the primary product generated by a membrane-bound NADPH-oxidase system during the metabolic response known as the "respiratory burst." This enzyme system consists of a membrane-bound *b*-type cytochrome (cytochrome b_{558}), which seems to have FAD as well as the heme as a prosthetic group (*5, 6*), and soluble regulatory proteins adsorbed on the cytochrome upon activation of oxidase (*7-11*). Through the stimulation, electrons are transferred from NADPH to molecular oxygen *via* FAD and the heme in cytochrome b_{558}.

In the preceding paper (*12*), we showed that nitroso-compounds were more potent inhibitors than pyridine and imidazole (*13*) against the respiratory burst of neutrophils in intact cells and in cell-free preparations. We also noted a clear correlation between the inhibition constant and the hydrophobic parameter of nitroso-compounds. This indicates that the inhibitors interact with hydrophobic sites of the cell membrane to exert the inhibitory effect either directly on NADPH-oxidase activity or indirectly as a secondary effect *via* lipid bilayers.

In fact, the decrease of plasma membrane microviscosity monitored by fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene paralleled the extent of phagocytosis induced by oil emulsions or polystyrene beads (*14*). Furthermore, Badwey and coworkers reported that *cis*-unsaturated fatty acids, which stimulate superoxide generation, decreased the fluorescence polarization of parinaric acids used as membrane-microviscosity probes, whereas saturated fatty acids, which did not stimulate superoxide release, increased the polarization of the dye (*15*). These observations indicate that the activation of the superoxide-generating system was accompanied by an increase of membrane fluidity.

In considering the mechanism of inhibition, therefore, the interaction between inhibitors and lipid bilayers should be taken into account as well as the interaction between inhibitors and a protein component(s) of NADPH oxidase. A fluorescent probe, perylene, is adequate to investigate such interaction, because it is partitioned in the hydrophobic regions both of lipids and proteins including cytochrome b_{558}. In this work, we studied the fluorescence

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Abbreviations: HBSS, Hanks' balanced salt solution; NB, nitrosobenzene; NT, 2-nitrosotoluene; PMA, phorbol 12-myristate 13-acetate.

quenching of perylene by nitroso-compounds in the membrane fractions of neutrophils and estimated the thermodynamic parameters of binding of inhibitors with perylene in the hydrophobic regions both of lipids and cytochrome b_{558}. We also studied the fluorescence quenching in the vesicles reconstituted with polar lipids of neutrophils in order to separate the reagent effects on perylene in lipids from those in protein parts of the membrane fractions. The results provide an insight into the mechanisms of nitroso-compound-induced inhibition of the respiratory burst.

MATERIALS AND METHODS

Phorbol 12-myristate 13-acetate (PMA) and perylene were purchased from Sigma. The proteinase inhibitor was obtained from Boehringer Mannheim. Nitroso-compounds, nitrosobenzene (NB), and 2-nitrosotoluene (NT), were from Aldrich Chemical. All other reagents were commercially available and of reagent grade.

Porcine neutrophils were isolated according to the established procedure (*16*). The cell suspension [1×10^8 cells/ml in a Hanks' balanced salt solution without Ca^{2+} buffered with 25 mM HEPES at pH 7.4 (Ca^{2+}-free HBSS)] was frozen in liquid nitrogen and stored at −80°C (*17*). The membrane fractions were prepared by sonication in the presence of 1 mM phenylmethylsulfonyl fluoride and by differential centrifugation according to the procedure of Kakinuma *et al.* (*18*). Polar lipids were extracted form porcine neutrophils following Lind *et al.* (*19*), and lipid vesicles were prepared by reverse-phase evaporation (*20*). In the vesicle preparation procedures, Ca^{2+}-free HBSS without glucose was used.

The quantities of nitroso-compounds incorporated into neutrophils were measured as follows. Three sets of two neutrophil suspensions of 3×10^7 cells/ml were preincubated in HBSS at 28°C. The desired volume of 60 mM nitroso-compound in ethanol was added into one of each set of cell suspensions and the same volume of ethanol was added into the other as a control. After incubation at 28°C for 2 min, cells were removed by centrifugation at 800 rpm for 10 min. The optical density at a maximum wavelength in the UV absorption band (line A in Fig. 1) of the supernatant of nitroso compound-treated cell suspension *vs.* that of ethanol-treated was measured and gave the quantity of nitroso-compound remaining in the suspension without being incorporated into neutrophils.

Measurement of oxygen consumption by intact neutrophils was performed as described in the preceding paper (*12*). For the activation of superoxide generation, PMA was added at a final concentration of 0.2 μg/ml. The effect of inhibitors at each concentration was expressed as residual activity (%) relative to the superoxide-generating activity in the absence of inhibitors. For comparison of different inhibitors or different temperatures, the potency of each inhibitor was expressed as its inhibition constant (IC_{50}), the concentration causing 50% reduction of activity.

Perylene was chosen because its fluorescence spectrum (line C in Fig. 1) did not overlap the absorption spectrum of nitroso-compounds (line B in Fig. 1). The fluorophore incorporation was achieved by diluting a solution of 0.06 mM perylene in ethanol 1,200-fold with a stirred aqueous suspension of the membrane fractions of neutrophils, and by incubation for ~60 min at 37°C. Fluorescence spectra of dyes were measured at 17, 27, or 37°C with a Hitachi 650-60 fluorescence spectrophotometer. The filters were set to cut off the excitation light below 380 nm and the emission light below 420 nm, respectively. The fluorescence intensities at the peak of the emission spectrum, 475±5 nm, excited by 439±2 nm light, the peak of the excitation spectrum, were measured to determine the effect of inhibitors.

RESULTS

The Quantity of Nitroso-Compounds Incorporated into Neutrophils—We measured the absorption spectra of the supernatant of the cell suspensions after spinning off the nitroso compound-treated neutrophils in order to estimate the quantity of reagents incorporated into cells. As shown in Fig. 2, no significant difference was found between the quantities of NB and NT incorporated into neutrophils under the experimental conditions. This indicates that a difference in the quantity of inhibitors incorporated could be excluded as a possible cause for the correlation between the hydrophobicity and the inhibitory effect.

Nitroso Compound-Induced Inhibition of Superoxide Generation in Neutrophils at 28 and 37°C—The inhibitory effect of nitroso-compounds, NB and NT, on the respiratory burst by PMA-stimulated neutrophils was observed at 28 and 37°C. With increasing temperature, NB became less effective: the inhibition constant, IC_{50}, of NB rose from 0.11 mM at 28°C to 0.15 mM at 37°C (Fig. 3). On the contrary, NT became a more effective inhibitor: the IC_{50} value of NT fell from 0.094 mM at 28°C to 0.079 mM at

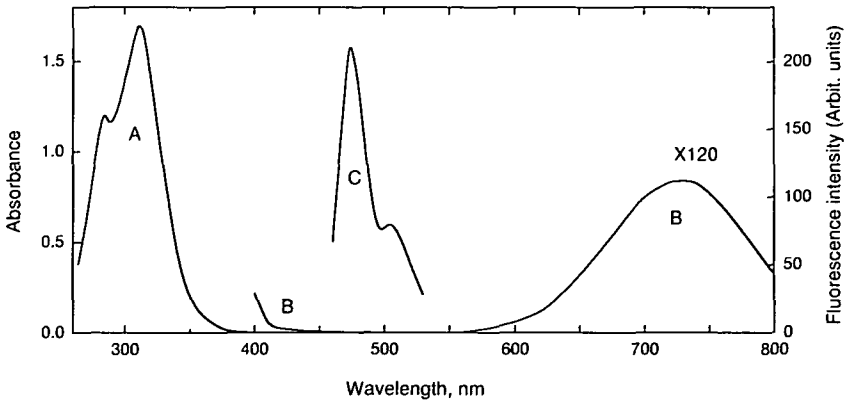

Fig. 1. **Absorption spectra of nitrosobenzene (NB) in the 1-cm path-length cuvette and the fluorescence emission spectrum of perylene in the membrane fractions of neutrophils.** NB was dissolved in Ca^{2+}-free HBSS at a concentration of 0.2 mM for the UV region (line A) and 1.2 mM for the visible light region (line B), and its absorption spectra were recorded at room temperature. The latter spectrum (line B) is shown at a magnification of 120. The fluorescence spectrum of 50 nM perylene (line C) in the membrane fractions (90 μg/ml of protein), which were dissolved in Ca^{2+}-free HBSS without glucose, was recorded at 27°C.

37°C (Fig. 3). Namely, the increase of temperature had opposing effects on the inhibition by nitroso-compounds: reduction for NB and enhancement for NT. This result suggests that the mechanisms of inhibition by these nitroso-compounds differ from each other.

The stability of nitroso-compounds was checked by their absorption spectra, which did not change during the incubation of the reagent-containing solution with lysed cells (data not shown).

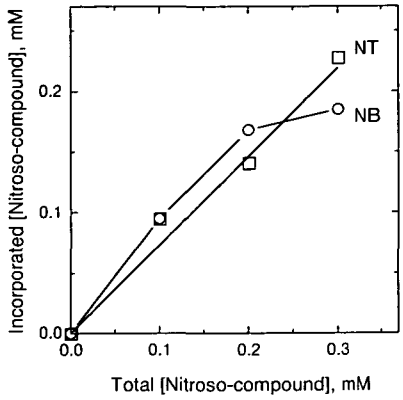

Fig. 2. **Quantity of nitroso-compounds incorporated into neutrophils.** From the optical densities at the maximum wavelength, 310 nm, of the supernatant of nitroso compound-treated cells *vs.* that of ethanol-treated, the concentration of nitroso-compound remaining in the cell suspension was calculated. The quantity incorporated was obtained as the difference between the concentration added and the concentration remaining.

Fluorescence Spectra of Perylene and Its Quenching by Nitroso-Compounds—The line C in Fig. 1 shows the emission spectrum of perylene fluorescence in the membrane fractions of neutrophils at 27°C. The addition of NB quenched the fluorescence of perylene without changing the spectral profile (data not shown). The fluorescence spectrum of perylene (line C in Fig. 1) does not overlap the absorption spectrum of NB (line B in Fig. 1), indicating that the quenching by NB is not due to the energy transfer from perylene to NB. The negligible contribution of scatter was also checked by the fluorescence spectrum of the membrane fractions prior to dye incorporation (data not shown).

The temperature dependence of the quenching of perylene fluorescence by nitroso-compounds in the membrane fractions was observed at 17, 27, and 37°C. Stern-Volmer plots of relative fluorescence intensities, F_0/F, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, are shown as a linear function of the concentration of nitroso-compounds (Fig. 4). The broken lines represent the results obtained for free perylene in ethanol at 27°C. The temperature effect on the quenching profile was similar to that on inhibition: the increase of temperature made NB a less effective and NT a more effective quencher. This result suggests the similarity of the mechanisms of quenching and inhibition. The slopes in Fig. 4, the quenching constants, K_{SV}, represent the association constants of nitroso-compounds with perylene. They are listed in Table I together with those in the synthetic vesicles made of endogenous polar lipids of neutrophils.

Temperature Dependence of Nitroso Compound-Induced Inhibition of Superoxide Generation and Reagent-Induced

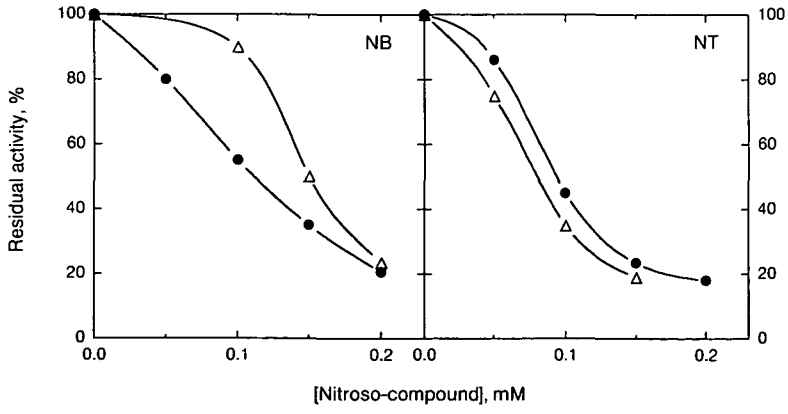

Fig. 3. **Inhibitory effect of nitroso-compounds on the respiratory burst.** The reaction mixture (0.59 ml) contained neutrophils (3×10^7 cells/ml) in HBSS (pH 7.4) at 28 (●) and 37°C (△). After preincubation for 2 min in the presence of various concentrations of nitroso-compound, the reaction was started by the addition of PMA (0.2 μg/ml). The residual activity at each concentration of inhibitor was expressed as the percentage of the oxygen-consumption rate relative to that in the absence of inhibitor. The 100% values typically ranged from 15 to 23 nmol O_2/min/10^7 cells.

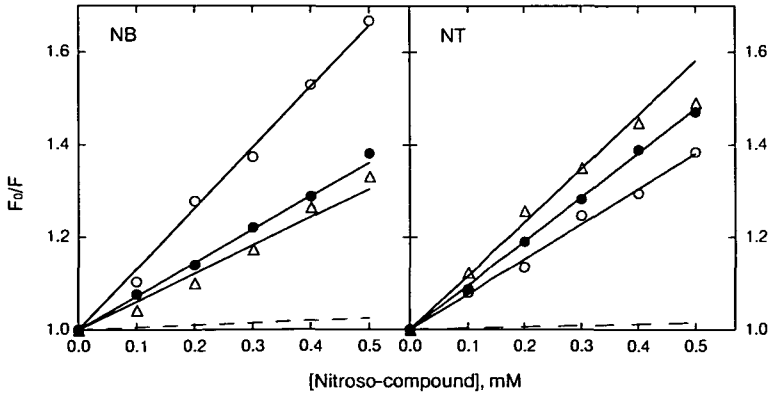

Fig. 4. **Stern-Volmer plots of relative fluorescence intensities of perylene in the membrane fractions of neutrophils.** The fluorescence intensities at 475 nm excited by 439 nm light diminished with increasing concentration of nitroso-compounds at 17 (○), 27 (●), and 37°C (△). The broken lines show the control experiments: reagent-induced quenching of perylene fluorescence in ethanol at 27°C. The slopes represent the quenching constants, K_{SV}, which correspond to the association constants of inhibitors with perylene in the membrane fractions.

Quenching of Perylene Fluorescence—To examine the similarity in the mechanisms of inhibition and quenching by nitroso-compounds, the logarithms of quenching constant, $\log(K_{SV})$, were plotted against $1/T$, where T is the absolute temperature (Fig. 5). The thermodynamic parameters obtained from this plot were compared with those of the inhibition (Table II).

In case of NB, $\log(K_{SV})$ increased linearly with increase of $1/T$. From its slope, the enthalpy change, ΔH_{NB}, of the association of NB with perylene in the membrane fractions of neutrophils was determined to be -29 kJ/mol. This value is similar to the enthalpy change for the inhibition by NB of NADPH-oxidase activity, -27 kJ/mol, which was obtained from the IC_{50} values at 28 and 37°C (Table II). The entropy changes, ΔS_{NB}, were estimated at -13 J/mol/K for inhibition of superoxide generation and -39 J/mol/K for fluorescence quenching (Table II). The difference between the value of ΔS_{NB} for inhibition and that for quenching comes from the fact that the entropy change observed for a specific phenomenon, inhibition, may reflect a part of the reagent-induced change in the order, whereas the entropy change observed for a non-specific phenomenon, quenching, may reflect its overall change.

On the other hand, the plots of $\log(K_{SV})$ of the binding of NT with perylene in the membrane fractions of neutrophils ran straight with a negative slope (Fig. 5). This indicates that the energetically unfavorable reaction, which is characteristic of the positive enthalpy change ($\Delta H_{NT} = +16$ kJ/mol), is compensated by the increase of entropy ($\Delta S_{NT} = +110$ J/mol/K), and the resultant decrease in Gibbs free energy makes the reaction proceed. The changes in enthalpy and entropy for inhibition by NT were estimated at $+16$ kJ/mol and $+130$ J/mol/K, respectively (Table II).

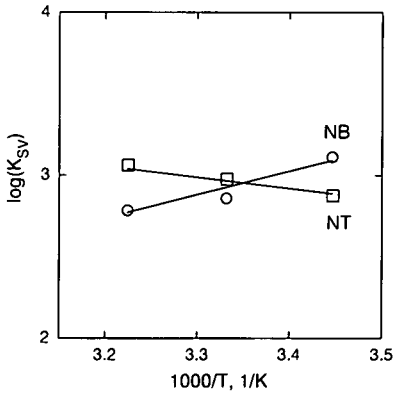

Fig. 5. **Temperature dependence of the quenching constants, K_{SV}.** From the slope of $\log(K_{SV})$, the enthalpy change, ΔH, for the binding of nitroso-compounds with perylene in the membrane fractions of neutrophils was calculated by using the relation $K_{SV} = \exp(-\Delta G/RT)$, where ΔG and R are Gibbs free energy change and gas constant, respectively.

Effect of Nitroso-Compounds on Perylene Fluorescence in Vesicles Reconstituted with Endogenous Polar Lipids—Because of their hydrophobicity, nearly all dyes are distributed in lipids, which compose the membrane fractions of neutrophils together with proteins. Therefore, it is necessary to observe the effects of nitroso-compounds on lipids and proteins separately. For this purpose, we reconstituted vesicles with endogenous lipids and observed fluorescence quenching of perylene by nitroso-compounds at 27 and 37°C.

The Stern-Volmer plots of perylene in the vesicles indicate that the increase of temperature enhanced quenching both by NB and NT of perylene fluorescence in lipids (Fig. 6). Namely, the temperature effect on quenching by NB in lipids was contrary to that in the membrane fractions of neutrophils. This indicates that the interaction between NB and perylene differs depending on whether they are distributed in proteins or in lipids of the membrane fractions of neutrophils. However, the interaction between NT and perylene, which is similar to that between NB and perylene in lipids, is independent of their location in the cell membrane, whether in proteins or in lipids. Thus, the interaction between NB and perylene in proteins differs both from the interaction between NB and perylene in lipids and from the interactions between NT and perylene in lipids and proteins. The quenching constants are summarized in Table I.

TABLE I. **Temperature dependence of quenching constants, K_{SV}, of nitroso-compounds with perylene in the membrane fractions and in the vesicles made of endogenous polar lipids.**

Compound	$K_{SV}(M^{-1})$		
	17°C	27°C	37°C
NB in membranes[a]	1,300	720	610
NB in lipids[b]	—	450	590
NT in membranes[a]	760	950	1,200
NT in lipids[b]	—	920	1,400

[a] Obtained from the slopes of Stern-Volmer plots in Fig. 4. [b] Obtained from the slopes of Stern-Volmer plots in Fig. 6.

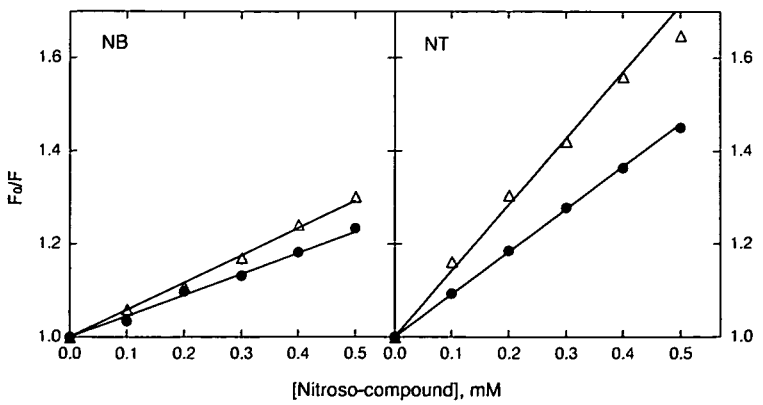

Fig. 6. **Stern-Volmer plots of relative fluorescence intensities of perylene in vesicles reconstituted with polar lipids of neutrophils.** Fluorescence quenching by nitroso-compounds was observed at 27 (●) and 37°C (△). The concentration of vesicles was controlled by adjusting the optical density at 439 nm to 0.05. Other conditions were the same as in Fig. 4.

TABLE II. **Thermodynamic parameters of nitroso compound-induced inhibition of superoxide generation and reagent-induced quenching of perylene fluorescence.** Thermodynamic parameters were obtained by using the relation, $1/IC_{50} = \exp(-\Delta G/RT)$ or $K_{SV} = \exp(-\Delta G/RT)$.

Compound	ΔH (kJ/mol)	ΔS (J/mol/K)
NB inhibition[a]	−27	−13
NB quenching in membranes[b]	−29	−39
NB quenching in lipids[c]	+20	+120
NT inhibition[a]	+16	+130
NT quenching in membranes[b]	+16	+110
NT quenching in lipids[c]	+34	+170

[a]Calculated from inhibition constants, IC_{50} (see details in Fig. 3). [b]Calculated from quenching constants, K_{SV}, of perylene fluorescence in membrane fractions of neutrophils (see details in Figs. 4 and 5). [c]Calculated from quenching constants, K_{SV}, of perylene fluorescence in vesicles made of endogenous polar lipids of neutrophils (see details in Fig. 6).

The thermodynamic parameters, ΔH and ΔS, calculated for the binding of NB and NT with perylene in lipids are summarized in Table II with those for other systems. The fact that the thermodynamic parameters fall into two groups (upper two lines and others) leads us to following explanation. Although NB is distributed in both proteins and lipids, the NB involved in inhibition binds mainly to a protein of the NADPH-oxidase system by exothermic reaction. On the other hand, NT causes inhibition by endothermic interaction with lipids and/or proteins.

DISCUSSION

We studied fluorescence quenching of perylene by nitroso-compounds in order to investigate the mechanism of the reagent-induced inhibition of superoxide generation. Generally, quenching arises from radiationless deactivation by collision of a quencher with an excited fluorophore (dynamic quenching) and/or by formation of a non-fluorescent complex between fluorophore and quencher prior to the excitation (static quenching). In the former case, increase of temperature enhances quenching through the diffusion-controlled mechanism of collision of the quencher with the excited fluorophore (*21*). In the latter, the temperature dependence of quenching reflects whether the binding of the quencher with the fluorophore in the ground state is endothermic (enhancement of quenching) or exothermic (reduction of quenching). Therefore, we must take account of both dynamic and static quenching for NT, because increase of temperature enhanced quenching by NT. However, since quenching by NB can be concluded from its temperature dependence to be essentially static, it is plausible to consider that quenching by NT, whose structural formula is like that of NB (see Fig. 1 in Ref. *12*), arises from the static phenomenon (static quenching) due to the formation of a non-fluorescent ground-state complex.

We have shown that NB was bound to the NADPH-oxidase system by exothermic reaction; $\Delta H_{NB} = -27$ kJ/mol for inhibition and $\Delta H_{NB} = -29$ kJ/mol for quenching (Table II). Since membrane fractions were prepared from neutrophils in the dormant state, NB is considered to react with the membrane component in the NADPH-oxidase system, cytochrome b_{558}, but not with the soluble components which are assembled on the cytochrome upon the activation of oxidase (*7-11*). Therefore, the enthalpy change for the binding of NB, which is about twice that observed when a dimer of *N*-methylacetoamide was formed in benzene [$\Delta H = -15$ kJ/mol, $\Delta S = -37$ J/mol/K (*22*)], indicates that NB may form a stable complex *via* a few hydrogen-bonds with cytochrome b_{558}. Such newly formed hydrogen bonds may result in the inhibition of superoxide generation.

On the other hand, we have concluded that NT inhibited superoxide generation by endothermic reaction with lipids and/or proteins; $\Delta H_{NT} = +16$ kJ/mol for inhibition and quenching (Table II). These thermodynamic parameters are similar to those observed when a hydrocarbon, such as an ethane molecule, is transferred from water to organic solvent; $\Delta H = +11$ kJ/mol and $\Delta S = +79$ J/mol/K (*23*). This suggests that a hydrophobic interaction is responsible for the binding of NT. This interaction may induce conformational changes of lipid bilayers and/or cytochrome b_{558}, with the resultant inhibition.

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