TEMPERATURE DEPENDENCE OF ANESTHETIC EFFECTS ON SUCCINATE OXIDASE ACTIVITY IN UNCOUPLED SUBMITOCHONDRIAL PARTICLES

G. VANDERKOOI, B. CHAZOTTE and R. BIETHMAN
Department of Chemistry, Northern Illinois University, DeKalb, IL 60115, USA

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1. Introduction

It is well known that anesthetics affect the physical and enzymological properties of biological membranes, but the molecular mechanisms by which these effects are induced are poorly understood. Reports from several laboratories have shown that anesthetics increase membrane fluidity [1–3], decrease membrane density and increase surface area [4], and lower the phase transition temperature of purified lipids [3]. Many examples of enzyme inhibition by anesthetics are available [4–7], as well as some cases in which enzyme activation was observed [7,8]. Anesthetics can thus be viewed as membrane perturbants, and one may anticipate that a deeper insight into membrane structure will be obtained in the course of seeking to understand anesthetic action.

We selected the succinate oxidase activity of uncoupled rat liver submitochondrial particles as a model system in which to study the effects of anesthetics on a complex but yet fairly well understood membrane-bound multienzyme system. Our interest was to see how (or if) anesthetic-type molecules would affect the rate of electron transport through this system. If lateral diffusion of the components is involved in the rate limiting step, one should expect anesthetics to increase the rate by virtue of their ability to decrease lipid bilayer viscosity [1–3]. Inhibition by anesthetics, on the other hand, must evidently involve a protein related effect. This could be any of several kinds, viz., changes in tertiary structure; quaternary changes within the multisubunit electron-transport complexes; or disruption of a supermolecular association among the complexes. By using uncoupled submitochondrial particles, we avoided the complications involved with energy transduction as well as with membrane permeability, since succinate dehydrogenase is on the exterior surface of these particles [9].

We found that low concentrations of *n*-butanol or benzyl alcohol gave a slight increase in succinate oxidase activity at low temperatures, but both of these agents, as well as tetracaine, gave enzyme inhibition at higher temperatures or higher concentrations. Arrhenius plots of the data showed 4 slopes or regions in the absence of anesthetic, over the temperature range of 15–45°C. The nature of the anesthetic effect differed in each of these regions. This shows that an interrelationship exists between the effects of heat and anesthetic. We attribute the anesthetic induced inhibition of enzyme activity to a disruption of protein structure or supermolecular organization by anesthetic molecules dissolved in the hydrophobic parts of the membrane. Only the *n*-butanol results are presented in detail in this paper.

2. Materials and methods

Rat liver mitochondria were prepared according to [10], and submitochondrial particles were made by Procedure II in [11]. Protein was determined by the method in [12]. Succinate oxidase activity was measured using a Clark electrode in a water jacketed cell, controlled to within ±0.05°C. The assay medium contained 100 mM KCl, 20 mM Tris–HCl, 5 mM KH_2PO_4, 3 mM $MgCl_2$ and 0.5 mM EDTA, at pH 7.4. To 2.8 ml of this solution was added 100 μl submito-

chondrial particle suspension (6.7 mg protein/ml); 25 μl carbonyl cyanide *m*-chlorophenyl hydrazone (10 mM); and 5 μl horse heart cytochrome *c* (Sigma Type VI, 1.6 mM). Succinate, 20 μl (0.5 M) was added to start the reaction after temperature equilibrium was achieved. Sequential 20 μl additions of *n*-butanol (Fisher, Spectro grade) were made after an initial rate of oxygen consumption was recorded.

3. Results and discussion

The relative rate of succinate oxidation is given as a function of the *n*-butanol concentration in fig.1 for several temperatures. A small (<10%) but reproducible enhancement of activity was given by 75 mM *n*-butanol at temperatures below about 24°C. Increasing either the temperature or the *n*-butanol concentration resulted in a decrease of relative activity. The inhibition curves are distinctly sigmoidal, suggesting that

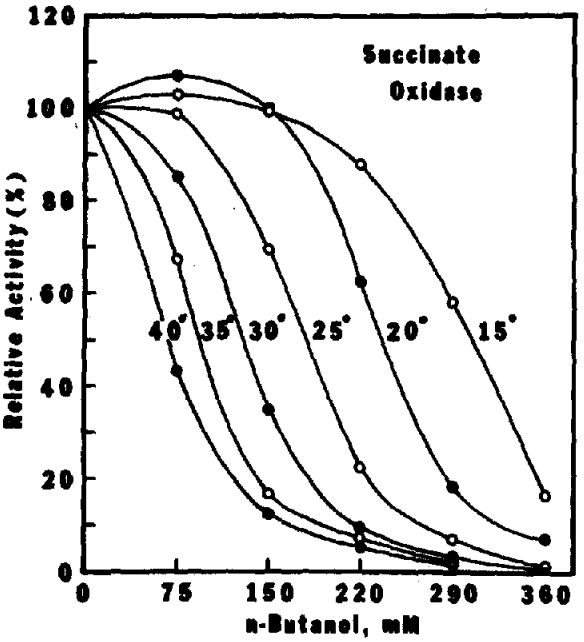

Fig.1. Relative rate of succinate oxidation by uncoupled rat liver submitochondrial particles, as a function of the *n*-butanol concentration. The temperatures are given on the curves. Each point is the average of 2 or 3 measurements. The 100% rate is taken as that in the absence of *n*-butanol, for the given temperature.

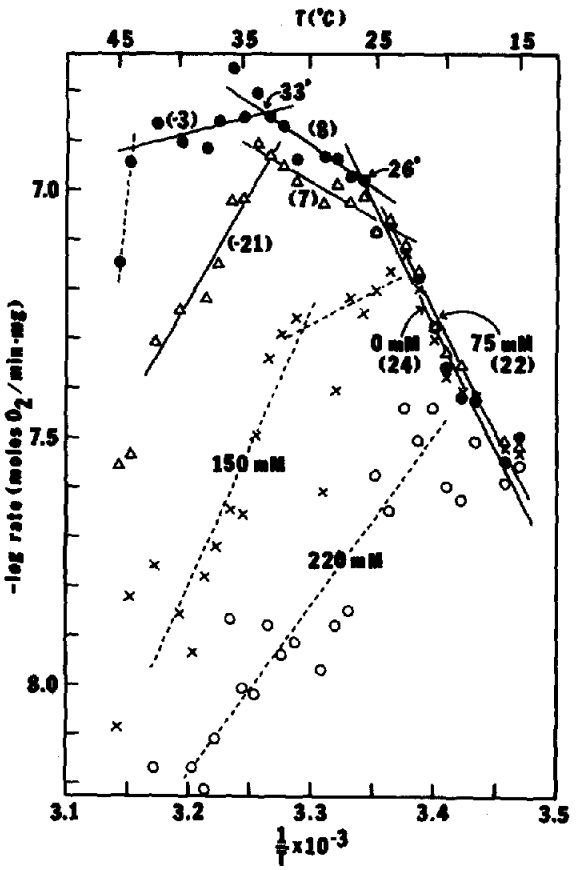

Fig.2. Arrhenius plots of the rate of succinate oxidation at several *n*-butanol concentrations. The slopes are given in parentheses as apparent activation energies, in kcal/mol. The solid lines were determined by least squares, but the dashed lines were drawn by eye through regions of few points or considerable scatter. Symbols: (•) no *n*-butanol; (△) 75 mM *n*-butanol; (x) 150 mM *n*-butanol; (○) 220 mM *n*-butanol.

they represent a transition from an active to an inactive state.

Arrhenius plots of the rate data are given in fig.2. The plot for no added *n*-butanol can be devided into 4 regions: I (<26°C), of high activation energy; II (26–33°C), of lower activation energy; III (33–44°C), of weakly decreasing activity; IV (>44°C), in which irreversible, time-dependent inactivation occurred. The enzyme activity was stable over the time course of the experiments below ~44°C. The 'break' in slope at 26°C is in agreement with report [13,14] on this system; these papers did not give data above 35°C.

n-Butanol, 75 mM, increased the activity in region I without significantly affecting the activation energy. In region II, this concentration gave moderate enzyme inhibition and possibly a decrease of apparent activation energy, but in region III a dramatic change in slope and decrease of activity was observed. The major effect of higher concentrations of *n*-butanol was to further decrease the activity in region III, causing the temperature at which this region commenced to shift to progressively lower values. The negative temperature coefficient in region III in the absence and presence of anesthetics is suggestive of a thermal denaturation, but the heat change associated with the process, as computed from the change in slope of the Arrhenius plot (about 11 kcal/mol with no anesthetic), is much less than usually accompanies protein unfolding [15]. It appears, however, that the alteration which is responsible for the decrease of activity in region III can be induced either by heat or by anesthetics, and that these act in a cooperative manner.

Experiments with benzyl alcohol and tetracaine gave similar results to those reported here for *n*-butanol. The approximate concentrations which caused 50% inhibition at 25°C were: 180 mM (*n*-butanol); 65 mM (benzyl alcohol); 0.3 mM (tetracaine). These values are each about 3-times the concentrations of the same substances required to give nerve blocking [4]. Since the relative concentrations are the same in the 2 cases, and since the nerve blocking concentrations are known to be nearly proportional to the lipid–water partition coefficients [4], we conclude that the inhibition of succinate oxidase is also related to the concentration of anesthetic dissolved in the lipid bilayer of the membrane.

The anesthetic induced activation observed at low concentration and temperature may be explanable in terms of membrane viscosity changes (although this is not proven by the data in hand), but the inhibition is evidently a reflection of anesthetic effects on membrane proteins. Anesthetic molecules dissolved in the lipid bilayer may directly affect the proteins by interacting with the portions of the proteins embedded in the lipid, or else they may have an indirect effect. We have shown [16] that agents which influence the properties of boundary lipid may alter the conformations of membrane proteins as well as their distribution in the plane of the membrane. Since heat and anesthetics appear to yield the same inhibitory effect, it seems safe to say that there is a loss of organization at some level. If it is true that the electron-transport complexes are organized into a supermolecular structure [17], then it is conceivable that the inhibition results from a disruption of this structure. Experiments in progress are designed to test whether the inhibition is caused at the supermolecular level or at the level of protein structure in the individual complexes.

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