EFFECT OF NATURAL INHIBITORS OF FREE RADICAL REACTIONS ON ADRENALIN AUTOXIDATION

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Water-soluble antioxidants (1 μM reduced glutathione, 3 μM cysteine, 1 μM ascorbate), in the presence of EDTA, inhibit the adrenalin autoxidation reaction at pH 10.2, whereas their oxidized forms, and also α-tocopherol (40 μM), have no such effect. The inhibiting power of superoxide dismutase is many times greater than that of the substances tested. Adrenochrome formation during free-radical oxidation of adrenalin takes place without the participation of the hydroxyl radical.

KEY WORDS: antioxidants; autoxidation of adrenalin; superoxide dismutase; adrenochrome.

Evidence has now been obtained that free radicals participate in the reaction of adrenalin oxidation [1-4]. The formation of free radicals has been proved during adrenalin oxidation catalyzed by cerium, permanganate, and ferricyanide and during its autoxidation at high pH values [2]. With the discovery of superoxide dismutase (SOD), an enzyme removing superoxide anions (O_2^-), as a result of catalysts of their dismutation reaction [4]

$$O_2^- + O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$$

a convenient and very sensitive method became available for demonstrating the role of superoxide anions in a wide variety of enzymic and nonenzymic reactions [5].

In 1972 Misra and Fridovich showed [1] that SOD, in very low concentrations, inhibits adrenalin autoxidation and they suggested a method of determining the activity of the enzyme in biological preparations based on the degree of inhibition of adrenalin autoxidation in an alkaline medium.

The presence of bioantioxidants – nonprotein substances capable of inhibiting free-radical oxidation reactions – in the tissues makes it essential to examine their effect on the rate of adrenochrome formation during adrenalin autoxidation.

EXPERIMENTAL METHOD

Adrenalin autoxidation was studied at pH 10.2 and recorded by the formation of adrenochrome at 480 nm. The adrenochrome concentration was calculated from the molar extinction coefficients, namely 4020 M^{-1} cm^{-1} [6]. Standard determinations were carried out in 3 ml 0.05 M Na-carbonate buffer, pH 10.2, containing 10^{-4} M EDTA, in a 1-cm cell with the temperature kept constant at 25°C. The reaction mixture contained $3 \cdot 10^{-4}$ M L-adrenalin and 10^{-5} M adrenochrome. The addition of adrenochrome led to disappearance of the lag period of adrenalin autoxidation, so that the effect of the test substances could be studied on the linear portion of the graph of the rate of adrenalin autoxidation.

Adrenochrome is unstable at pH 10.2 and its oxidation is accompanied by a decrease in optical density at 480 nm. However, this oxidation proceeds much more slowly than the oxidation of adrenalin under the same

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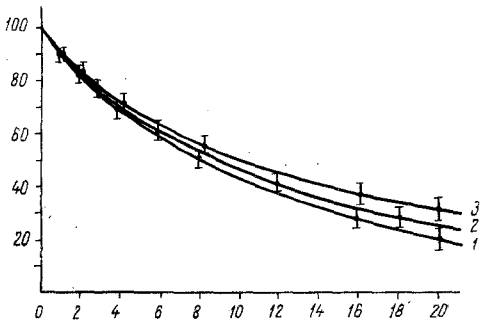

Fig. 1. Inhibition of adrenalin autoxidation by reduced glutathione (1), cysteine (2), and ascorbate (3). Abscissa, concentrations of antioxidants (in μeq/ml); ordinate, reaction velocity (in % of control).

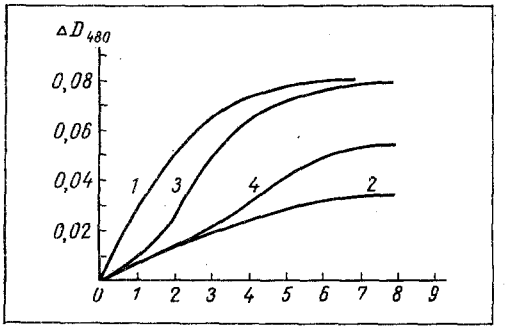

Fig. 2

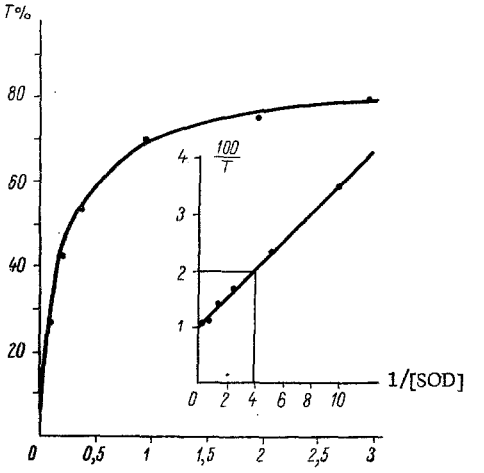

Fig. 3

Fig. 2. Kinetics of adrenalin autoxidation in the presence of reduced glutathione (2), cysteine (4), and ascorbate (3): 1) uninhibitable oxidation. Concentration of substances 20 μeq/ml. Abscissa, time of autoxidation (in min); ordinate, optical density at 480 nm.

Fig. 3. Inhibition of adrenalin autoxidation by superoxide dismutase. Abscissa, final SOD concentration (in μg/ml); ordinate, % of inhibition (T) of autoxidation.

conditions [1], and for that reason it has no significant effect on the results of the measurements. The reaction was started by the addition of 0.4 ml adrenalin solution (pH 2.5). No change in the pH of the reaction mixture was found under these circumstances. The measurable initial velocity of adrenalin autoxidation is 0.025 min^{-1}. The amount of SOD required to inhibit the initial velocity of adrenalin autoxidation by 50% under the conditions described above was taken as the unit of SOD activity.

The Unicam SP-8000 spectrophotometer (England) was used for the spectrophotometric determination.

The following preparations were used: reduced glutathione (BDH Biochemical Limited), dehydroascorbate (Serva), oxidized glutathione (Reanal), cysteine, cystine, and arenochrome (Calbiochem), adrenalin (Sigma), and EDTA (VEB Berlin Chemie Adlershof). SOD, isolated from bovine erythrocytes [4], was kindly provided by Dr. S. D. Aust (University of Michigan).

EXPERIMENTAL RESULTS AND DISCUSSION

The effect of glutathione, cysteine, and ascorbate on the initial velocity of adrenalin autoxidation at pH 10.2 is shown in Fig. 1. Appreciable inhibition of adrenochrome formation by these substances was observed if their concentration was not less than 0.5 μeq/ml. Addition of the oxidized forms of these substances, and also of α-tocopherol, caused no significant change in the velocity of the reaction studied. Oxidized

glutathione, in a concentration of 16 μM, inhibited (by 12.5%) the autoxidation reaction, but by a much lesser degree than its reduced form.

It is very important to note that reduced glutathione, ascorbate, and cysteine can react directly with free radicals [7] and remove them from the sphere of the reaction. The most likely mechanism of inhibition of adrenalin autoxidation by the products mentioned above is therefore removal of the superoxide anions and other freeradical compounds from the sphere of this reaction.

Equal concentrations of reduced glutathione, ascorbate, and cysteine give equal inhibitory effects in the initial period of the adrenalin autoxidation reaction. Later the course of the kinetic curves differed significantly (Fig. 2), probably because of the accumulation of different amounts of adrenochrome and of secondary oxidation products.

The effect of SOD on the adrenalin autoxidation reaction is illustrated in Fig. 3. For the SOD preparation used, 50% inhibition was reached with the enzyme in a concentration of 250 ng/ml. Maximal inhibition of the reaction, calculated from the graph in reciprocal coordinates, was 100%. This suggests that adrenochrome formation in this reaction takes place practically entirely by mechanisms dependent on superoxide anions.

Meanwhile hydroxyl radicals, the formation of which can take place during interaction of superoxide anions with hydrogen peroxide [8], did not participate in this reaction.

Proof of this statement was given by the ineffectiveness of ethanol, mannitol formate, and benzoate, known [9] to be "traps" of the hydroxyl radical, as inhibitors of adrenalin autoxidation at pH 10.2. These substances, in concentrations of 10^{-3} M in the reaction mixture, had no appreciable effect on the velocity of the reaction tested.

Inhibition of adrenalin autoxidation by SOD is the basis of a method of determining the activity of this enzyme [1]. The problem of the possible effect of inhibitors of free radical reactions present in the tissue on the results of determination of SOD activity has not yet been settled. Considering the content of antioxidants in the tissued [10] and the level of SOD activity [11], the ratio between them (μeq antioxidants:units of SOD activity) varies in different tissues from 1:100 to 1:300. This means that, if biological material containing 1 unit of SOD activity is added to the reaction mixture, the concentration of antioxidants in it will be at least one order of magnitude lower than the minimally active concentration.

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