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Activation of nitrate reductase by oxidation

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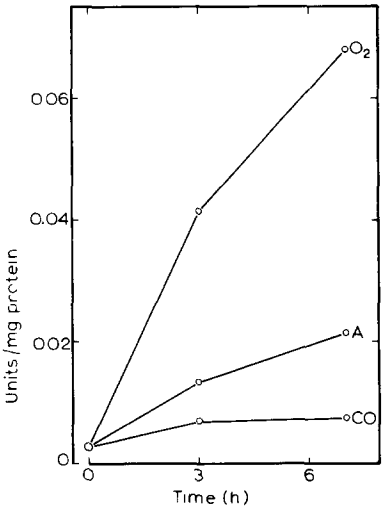
SUMMARY

The activation of the inactive form of the nitrate reductase (NADH: nitrate oxidoreductase, EC 1.6.6.1) present in cell-free extracts of *Chlorella vulgaris* Beijerinck requires an oxidizing agent. Ferricyanide causes conversion of the proenzyme to active enzyme within a few minutes, even at 0°C. Molecular oxygen causes a slow activation which requires many hours at room temperature, and never reaches the high activity level attained with ferricyanide. In unfractionated extracts, CO inhibits the activation by molecular O_2. The sensitivity of this activation to CO may account for the *in vivo* sensitivity of nitrate reduction to CO in these algae.

We have previously reported that CO inhibits the reduction of nitrate by illuminated suspensions of the green alga, *Chlorella vulgaris* Beijerinck (formerly called *Chlorella pyrenoidosa*)[1]. This inhibition was not accompanied by extensive nitrite accumulation. That is, it appeared that the reduction of nitrate to nitrite was inhibited by CO *in vivo*. The enzyme which catalyzes this reaction (NADH: nitrate oxidoreductase, EC 1.6.6.1) has been isolated and extensively purified, and found to be unaffected by CO[2]. This enzyme is obtained in extracts prepared from sonicated cells largely in an inactive form, which can be extensively activated[3,4]. The experiment shown in Fig. 1 presents evidence that the activation of the enzyme in crude extracts requires oxygen, and that it is inhibited by CO. For this experiment the cell extract was added to a medium containing phosphate buffer of pH 6.7 and nitrate, and incubated in Warburg vessels for the time indicated. Six vessels were prepared with identical reaction mixtures. Two of the vessels were gassed with CO, two with argon, and two with O_2. For each of the experimental points, a vessel was taken down, and nitrate reductase activity was measured on an aliquot of the reaction mixture[3]. The results show that the activation occurs more slowly under argon than under O_2, and still more slowly under CO than

under argon. No oxygen absorbent was used in these experiments. It would be expected that if CO were simply displacing O_2, the activation rate in CO and in argon should be the same, reflecting the activity of traces of oxygen remaining in the vessels. The quantities of oxygen taken up during the activation were too small to be measured accurately under the conditions employed.

The experiment of Fig. 1 has been repeated many times with many variations. The high phosphate concentration favors the activation. The inhibition by CO can be relieved by O_2 or by white light. These light effects are complicated by other light effects apparently associated with photooxidations. That is, one can stimulate the activation of nitrate reductase in crude extracts by light at low oxygen tensions in the absence of CO, but the effect of light in the presence of CO is substantially higher. Further work is required to establish the relationship of these *in vitro* effects of O_2 and CO to the phenomena previously observed *in vivo*[1].



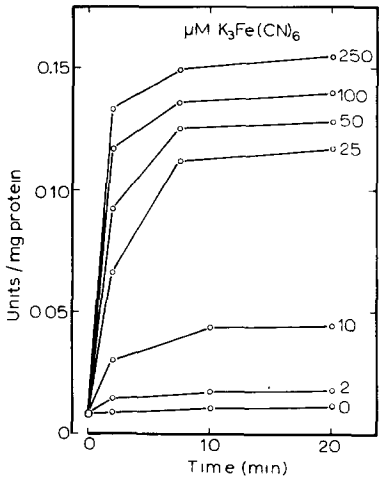


Fig.1. Change with time of nitrate reductase activity in the presence of oxygen, argon and carbon monoxide. *Chlorella* cells were cultured and harvested, and a cell-free extract was prepared by sonication and stored frozen, all as previously described[2,3]. After thawing, the extract was centrifuged at 40 000 × *g* for 30 min, and the supernatant was employed directly. Each reaction vessel contained 2.0 ml of 0.25 M sodium, potassium phosphate buffer (pH 6.7), 0.01 M KNO_3, and extract (0.4 ml) containing 5.2 mg protein. Gassing was for 10 min. Samples were incubated with shaking, in the dark, at 20°C. The ordinate shows nitrate reductase activity in units/mg protein. One unit catalyses the oxidation of one μmole of NADH by NO_3^- per min under the conditions used for assay[3].

Fig.2. Time course of the activation of nitrate reductase by various concentrations of ferricyanide. Reaction mixtures contained 0.25 M sodium, potassium phosphate buffer (pH 6.7) and the indicated concentrations of ferricyanide. Reactions were started by the addition of cell-free extract to give a final concentration of 1.45 mg of protein per ml. Cell-free extract was prepared as for the experiment of Fig.1 except that low molecular weight substances were removed by passage through a column of Sephadex G-50 equilibrated with 1 mM Tris–HCl buffer (pH 8.0). Incubations were in air at 20°C. Samples were taken for enzyme assay at the times indicated. Activation in 500 μM $K_3Fe(CN)_6$ was slightly less than in 250 μM (curve not shown).

The recognition of the fact that the activation of nitrate reductase proenzyme is stimulated by oxygen prompted the testing of artificial oxidants, and led to the development of a procedure for rapid measurement of enzyme *plus* proenzyme in unfractionated extracts, by the use of ferricyanide. The activation is conveniently carried out in 0.25 M phosphate buffer of pH 6.7. The sample to be activated is added to an aliquot of buffer containing an appropriate amount of ferricyanide. After about 10 min at room temperature the nitrate reductase activity is assayed as previously described[2,3]. The optimal amount of ferricyanide must be determined by a procedure resembling a titration. Successive activations are run, with the same amount of extract, and increasing amounts of ferricyanide. The latter should be present in slight excess, that is, should not be reduced, at the end of activation. A large excess should be avoided, because excess ferricyanide inhibits the active enzyme and also interferes with our assay procedure. Since ferricyanide is a substrate for the diaphorase component of the enzyme[2], it oxidizes NADH in the presence of enzyme more rapidly than does the nitrate in the assay system. The assay for nitrate reductase depends on the measurement of the rate of NADH oxidation. To ensure that it is nitrate reduction which is being measured, and not ferricyanide reduction, the assay can be carried out with and without added nitrate. The distinction between nitrate reduction and ferricyanide reduction is usually easy, because the latter is more rapid than the former, and the rate of oxidation of NADH changes abruptly when excess ferricyanide has been reduced.

Fig. 2 shows the time course of the activation of the enzyme by ferricyanide. For this experiment, the extract employed was passed through a column of Sephadex G-50, and the activation was carried out at 20°C. The Sephadex treatment leaves the initial enzyme activity essentially unchanged, though preparations so treated are generally more rapidly activated in air. Low molecular weight substances which inhibit the activation or cause a reversible inactivation are removed by Sephadex. Because of the presence of low molecular weight reducing substances in crude extracts, about ten times more ferricyanide is required for maximum activation prior to Sephadex treatment than after.

The sum of nitrate reductase enzyme and proenzyme was determined by the ferricyanide procedure for the experiment shown in Fig. 1, for all incubation mixtures. There was 0.18 unit of nitrate reductase enzyme plus proenzyme per mg of protein present initially, and this remained constant in all gasses for the duration of the incubation.

There appears to be a similarity between the activation of alloxanthine-inhibited xanthine oxidase (EC 1.2.3.2) by ferricyanide as described by Massey *et al.*[5] and the activation of the nitrate reductase precursor found in fresh extracts of *Chlorella vulgaris* Beijerinck.

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