Reduction of Hydroxylamine in the Root Nodules of Leguminous Plants

ARTTURI I. VIRTANEN, AUNE KEMPPI and EEVA-LIISA SALMENOJA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The formation of hydroxylamine as an intermediate has been discussed in connection with N_2-fixation. The reduction of hydroxylamine to ammonia is hereby very probable. When nitrate is reduced to ammonia hydroxylamine has been shown to be an intermediate, and Egami [1] has even proved the existence of a special hydroxylamine-reductase in nitrate reducing bacteria. From *Neurospora crassa* Zucker and Nason [2] recently isolated, and in a noticeable degree purified, the enzyme reducing hydroxylamine. This enzyme proved to be a metallo-flavoprotein.

On the other hand Colter and Quastel [3] have shown that blood hemoglobin acts as an enzyme in the dismutation of hydroxylamine to N_2 and NH_3. In the presence of cysteine or ascorbic acid hemoglobin catalyzes the reduction of hydroxylamine to ammonia. Schwyzer [4] showed in this laboratory that hemoglobin had no influence on oximes or hydroxamic acids (oxime of pyruvic acid and benzhydroxamic acid).

We have investigated the reduction of hydroxylamine and the oxime of pyruvic acid in the root nodules of leguminous plants in order to find out to what extent reduction takes place, and the role of leghemoglobin in the reduction.

Experiments were made both with water extract of root nodules and with crushed nodules. On the basis of results obtained in this laboratory N_2-fixing nodules always contain leghemoglobin, while ineffective nodules do not contain it [5]. When investigating the catalytic power of leghemoglobin + ascorbic acid in the reduction of hydroxylamine, effective root nodules of pea, clover, or soya were crushed in water, and the solution which had been clarified by centrifugation was used as agent in reduction experiments. Hydroxylamine was determined by the method developed by Csáky [6], based on Blom's principle, and ammonia by the method of Pucher. In an

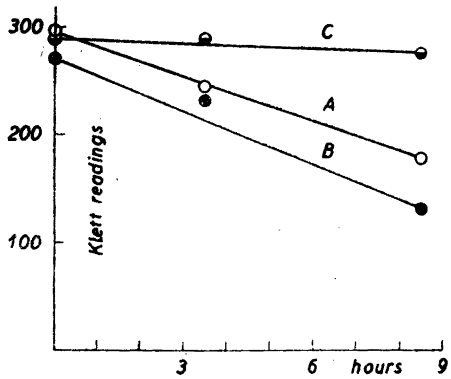

Fig. 1. Reduction of hydroxylamine. A: leghemoglobin extract from effective nodules of red clover + ascorbic acid + hydroxylamine (solution A). B: hemoglobin extract from red blood cells + ascorbic acid + hydroxylamine (solution B). C: ascorbic acid + hydroxylamine (solution C)

experiment with 3 ml of extract from 3 g of red clover nodules + 3 ml of ascorbic acid solution (= 30 mg of ascorbic acid in phosphate buffer pH 7.4) + 0.6 ml of hydroxylamine solution (10 mg $NH_2OH \cdot HCl$/ml) the loss of hydroxylamine was in 3 h at 38° C 30 %. In the control boiled nodule extract was used as agent.

In comparing the effect of leghemoglobin and blood hemoglobin on the breakdown of hydroxylamine, the content of leghemoglobin of the nodule extract was determined spectrophotometrically as pyridinehemochromogen, and a solution was prepared from red blood cells by dilution until the content of hemoglobin was the same as that of leghemoglobin in the nodule extract.

Example: From 2 g of effective root nodules of clover 5.32 ml of reddish water extract was obtained. The following solutions were prepared: 4 ml of nodule extract + 4 ml of ascorbic acid solution (= 40 mg of ascorbic acid in phosphate buffer pH 7.4) + 0.8 ml of hydroxylamine solution (10 mg $NH_2OH \cdot HCl$/ml). (Solution A)

4 ml of hemoglobin-solution of blood (1 ml red blood cells: 4 ml water, diluted to 192 ml) + 4 ml of ascorbic acid solution + 0.8 ml of hydroxylamine solution. (Solution B)

4 ml of water + 4 ml of ascorbic acid solution + 0.8 ml of hydroxylamine solution. (Solution C)

Reaction temperature t = 38° C.

The decrease of NH_2OH appears from the curves in Fig. 1. The catalytical effect of blood hemoglobin and leghemoglobin is thus practically the same.

Nodules whose red colour had changed into green (the porphin ring of leghemoglobin has opened [7]) and which thus had lost their N_2-fixing capacity [5] reduced hydroxylamine very poorly, or hardly at all. The reduction of hydroxylamine in root nodules is thus chiefly caused by leghemoglobin. This is confirmed also by the results of the experiments in which we compared the breakdown of hydroxylamine by the extract of effective nodules formed by pea bacteria, strain H 43, and ineffective nodules formed by pea bacteria, strain H VIII. In the latter nodules no leghemoglobin is to be found. Ascorbic acid was not added to the extracts. After a reaction time of 3 h the photometer readings were: in the experiment with effective nodules 83, in the experiment with ineffective nodules 152.

Formation of ammonia by breakdown of hydroxylamine was followed in some experiments. With an extract of the root nodules of soya, to which ascorbic acid had not been added, 0.28 μg of NH_2OH nitrogen disappeared during 3 h, while 0.12 μg of NH_3 nitrogen was formed, which corresponds approximately to the equation: $3\ NH_2OH = N_2 + NH_3 + 3\ H_2O$.

Experiments in which an eventual reduction of pyruvic acid oxime in root nodules was examined gave negative results. Increase of alanine as a reduction product of the oxime could not be observed in these experiments by using the paper chromatographic method. The method of determining the oxime is, however, uncertain as hydroxylamine to a great extent decomposes during hydrolysis when the solution contains organic substances like ascorbic acid.

Our findings show that an intensive breakdown of hydroxylamine takes place in N_2-fixing root nodules which contain leghemoglobin. As ascorbic acid is to be found in the nodules in relatively high concentration (2–3 times higher than in roots) [8] the reduction to ammonia is apparently practically complete in those hydroxylamine concentrations which may occur in the nodules if hydroxylamine is formed as an intermediate. In parallel experiments where the effect of red blood cells and red root nodules on the reduction of hydroxylamine in the presence of ascorbic acid was compared, the reaction velocity was found to be approximately the same. As the disappearance of hydroxylamine is very slight in ineffective and leghemoglobin-free root nodules, as well as in older ones where the leghemoglobin, because of the splitting of the porphin ring, has changed into green pigment, it is obvious that leghemoglobin is the main, or only, factor which causes the reduction of hydroxylamine in root nodules. In the case that hydroxylamine is formed as an intermediate in N_2-fixation in root nodules, leghemoglobin is thus indispensable in the reduction of hydroxylamine.

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Fixation of Molecular Nitrogen by Excised Nodules of the Alder

ARTTURI I. VIRTANEN, TAUNO MOISIO, RUSSELL M. ALLISON and R. H. BURRIS*

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland, and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

The alder tree fixes molecular nitrogen at a rate sufficient to support vigorous growth on a nitrogen-poor soil. As it now is possible to demonstrate nitrogen fixation consistently with excised nodules from leguminous plants [1,2], it seemed advisable to test whether alder nodules also can fix nitrogen apart from the host plant.

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