

Organic Hydroxylamine Compounds Formed from Nitrite in *Torulopsis utilis*

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For some twenty years the formation of hydroxylamine both in the reduction of nitrate and in the fixation of molecular nitrogen has often been dealt with¹. Bound hydroxylamine has been found to be formed in connection with both processes ("bound hydroxylamine" = a substance released on acid hydrolysis, which on oxidation with iodine gives nitrite²). On the whole, the nature of the "bound hydroxylamine" has remained unknown. Only Virtanen and Laine³ were once able to isolate an oxime compound from the "sterile" sand-substrate of pea inoculated with effective *Rhizobium*, which compound on reduction gave aspartic acid and accordingly was, or contained, the oxime of oxalacetic acid. Except oxime nitrogen "bound hydroxylamine" could also be hydroxamic acid, especially as the formation of hydroxamic acid from hydroxylamine and organic acids or their amides has been brought about with preparations made from plant- and animal organisms.

Chromatographic methods have rendered it possible to study the "bound hydroxylamine" fraction more closely. Work on this line is going on in our laboratory. The first results of these investigations are presented in this communication.

Virtanen and Csáky⁴ observed that both low- and normal-nitrogen *Torulopsis utilis*, when suspended in a nitrate containing solution, which was vigorously aerated, forms "bound hydroxylamine" very rapidly. The amount of it decreased, however, as soon as after 15–20 min., probably indicating that the enzyme needed for its reduction had time to become effective enough through adaptation. We therefore used *Torulopsis*-yeast in our experiments. As the strain used now formed only a very small amount of "bound hydroxylamine" from nitrate as well as from nitrite in aerated solutions we performed our experiments in a low oxygen solution containing nitrite, the amount of "bound hydroxyl-

amine" increasing noticeably under these conditions. At first we tried to identify the compounds containing hydroxylamine as such by paper chromatography, but this method did not turn out to be suitable, mostly because the extracts obtained contained large amounts of unknown organic substances. As the identification even of small amounts of amino acids by paper chromatography is easy we started to use the method of reducing hydroxylamine compounds to the corresponding amino acids and to identify these. The method presupposes that amino acids are beforehand removed as carefully as possible. The method we used was briefly the following:

115 g of fresh low-nitrogen *Torulopsis utilis* (N-content 6.0 % of dry materia) was suspended in 1 150 ml of a mineral nutrient solution without combined nitrogen in a Klyver flask placed in a waterbath at 30° C. Air was flushed through the suspension for 10 min., followed by nitrogen gas for 10 min. more before the addition of sodium nitrite in a concentration of 75 µg N/ml. This leads to the accumulation of about 3 µg NH₂OH-N/g fresh yeast. After 35 min. the suspension was centrifuged, the cells were washed with icecold distilled water, re-centrifuged and suspended in 96 % alcohol (3 ml/g yeast) freed from aldehydes. The whole operation after the addition of nitrite took 1 h. The suspension was kept in an ice-box overnight and then centrifuged.

The clear supernatant was evaporated *in vacuo*, lipid material extracted by *isoamyl*-alcohol, and amino acids removed by cation exchange resin (Dowex 50) in the cold (0–3°C). The solution then contained 240 µg bound hydroxylamine-N, but no nitrite or free NH₂OH. Only traces of some amino acids could be found in the solution (*cf.* below). Half of the solution was subjected to reduction by 1.5 % sodium amalgam in the cold, the other half regarded as a control. Reduction time was 6 h whereby about 70 % of bound hydroxylamine was reduced.

Amino acids were absorbed on Dowex 50, displaced by ammonia and chromatographed on Whatman No. 4 paper using the solvent system butanol-acetic acid and phenol-NH₃. As a further control 73 g of *Torulopsis*-yeast were treated in the same way, but without addition of nitrite (Figs. 2A and 2B).

Figs. 1 and 2 show a two-dimensional paper chromatogram from the experiment in which nitrite had been added to the suspension of *Torulopsis*-yeast. Fig. 1 A shows the amounts of amino acids *before*

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A Diffusion-equilibration Method as a Stage in the Determination of the Deuterium Oxide Content in 1—2 Microlitres of Fluid

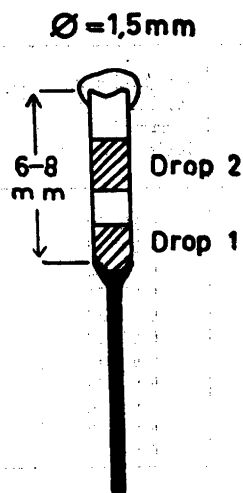
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Determination of the deuterium oxide content in fluids is generally performed utilizing the difference in density between pure water and deuterium oxide. Convenient methods have been described which allow an estimation of the deuterium oxide content in very small volumes with sufficient accuracy for most biological purposes. Thus the gradient tube method developed by Linderström-Lang¹ (see also Linderström-Lang, Jacobsen and Johansen²) requires only about 0.5 microlitre for an accurate determination. However, all methods based upon density determinations require that the fluid, the deuterium oxide content of which will be determined, contains only H₂O, HDO and D₂O, respectively. A distillation procedure is thus a necessary step before the actual measurements can be made. If the volume of the fluid to be analyzed is limited to a few microlitres, common distillation procedures become inconvenient.

The present work is a description of a simple method for "purification" of very small samples (1—2 μ l) prior to the density determination in the gradient tube.

Theory. The principle of the method is shown in Fig. 1. Sample 1, a drop of volume V_1 containing an unknown concentration C_1^0 of deuterium oxide, is allowed to attain diffusion-equilibrium in a



small closed space with sample 2, a drop of volume V_2 containing distilled water. (It is assumed, for the sake of simplicity, that the distilled water used contains no deuterium oxide. This assumption introduces a negligible error.) The air space between the drops acts as a semipermeable membrane allowing only volatile substances to pass through. After equilibrium has been attained, any volatile substance has the same activity in both drops. It may be assumed that the activity coefficients of HDO (and D₂O) in water do not significantly differ from the activity coefficients of HDO (and D₂O) in water solutions of the molar and ionic strength present in biological fluids. Therefore

$$C_1^{eq} = C_2^{eq} \quad (1)$$

where the superscripts denote that the concentrations are those at equilibrium.

Furthermore

$$C_1^0 V_1 = (V_1 + V_2) C_2^{eq} \quad (2)$$

and

$$C_2^{eq} = C_1^0 \frac{V_1}{V_1 + V_2} \quad (3)$$

If the only volatile substance present is deuterium oxide, a density determination of sample 2 at equilibrium will give the original concentration of deuterium oxide in sample 1. Equation (2) contains of course the assumption that the amount of deuterium oxide present in the air space