Organic Hydroxylamine Compounds Formed from Nitrite in *Torulopsis utilis*. II. Acetylhydroxamic Acid

ARTTURI I. VIRTANEN and NILS-ERIK SARIS

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

In an earlier paper [1] we reported results which showed that "bound hydroxylamine", which is formed in small amounts in the cells of *Torulopsis utilis* suspended in a nitrite-containing nutrient solution, contains oximes of α-keto acids. In all experiments the amount of the oxime of pyruvic acid was highest, next came the oxime of α-ketoglutaric acid, the smallest amounts being those of the oximes of oxypyruvic, oxalacetic, and glyoxalic acids. The amounts and mutual relations of different oximes varied appreciably in different experiments, as can be expected since the amount of the corresponding keto acids varies in the cells and is dependent on many different factors.

When continuing our investigations special attention was given to hydroxamic acids which could possibly be formed in addition to oximes. In the experiment described in our first paper about 70 % of "bound hydroxylamine" was reduced by sodium amalgam to amino acids. The rest of the "bound hydroxylamine" could thus consist of hydroxamic acids. In most experiments practically all the "bound hydroxylamine" passed through the Dowex 50 column. On the basis of this *Torulopsis* cells do not contain noticeable amounts of the hydroxamic acids of amino acids. The hydroxamic acids of such carboxylic acids which do not contain basic groups were instead to be expected in the solution emerging from the Dowex column. In our very first experiments we found this to be the case, as in fact a reddish spot was obtained with ferric chloride on the paper chromatogram of the solution. The chemical nature of the compound remained obscure, however, and was not therefore dealt with in our earlier paper.

A closer investigation has now been made on the nature of the hydroxamic acid formed when *Torulopsis* reduces

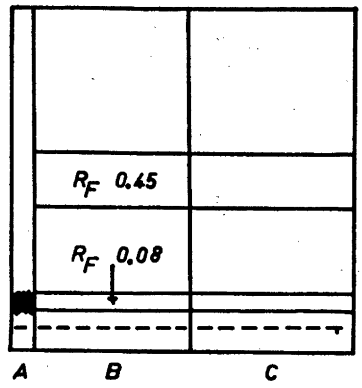

Fig. 1. Paper chromatogram of »bound hydroxylamine» in Torulopsis *cells. Solvent: amylacetate/formic acid/water (4:1:1). Dotted line = starting line.*

A. Paper strip sprayed with ferric chloride solution. Reddish spot with R_F *0.08.*

B. After hydrolysis hydroxylamine could be found mostly in the zones with R_F *0.08 and 0.45.*

C. The corresponding zones were analyzed. The zone with R_F *0.45 gave on reduction with sodium amalgam alanine and accordingly contained the oxime of pyruvic acid. The zone with* R_F *0.08 contained acetylhydroxamic acid (*cf. *Fig. 2).*

nitrite. The extract obtained from the cell mass was passed through a cellulose powder column with butanol-acetic acid as solvent (Dowex treatment was omitted). "Bound hydroxylamine" passed through comparatively quickly. The combined fractions containing "bound hydroxylamine" were evaporated to a small volume. The solution obtained was spread along a starting line at the bottom of a Whatman No. 4 paper, and using amylacetate/formic acid/water (4:1:1) as solvent the chromatogram was developed. A narrow strip cut off from the margin of the paper was sprayed with a solution of ferric chloride upon which the reddish spot appeared, R_F 0.08 (Fig. 1 A). The sheet was then cut in two lengthwise and one of the halves again was cut crosswise into 16 strips which were extracted with water (Fig. 1 B). The solutions obtained were hydrolyzed with sulphuric acid and hydroxylamine was determined by the method of Csàky. The values showed that "bound

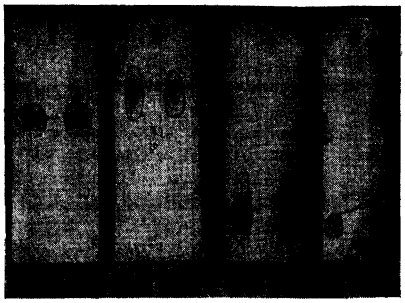

Fig. 2. Comparison between synthetic acetylhydroxamic acid and the hydroxamic acid found in Torulopsis *cells. A. Synthetic acetylhydroxamic acid. X. Hydroxamic acid found in* Torulopsis *cells. Solvents: 1 butanol/acetic acid/water (63:27:10), 2) amylacetate/formic acid (80 %)/water (4:1:1), 3) ethylacetate/butanol/water (8:2:1), 4) ethylacetate/propanol/acetic acid/water (5:2:2:1).*

hydroxylamine" had mostly divided into two zones: R_F 0.08 and R_F 0.45. From the other half of the paper (Fig. 1 C) the zones corresponding to hydroxylamine maxima were cut off and eluted with water. From the zone corresponding to R_F 0.45 alanine was obtained by reduction with sodium amalgam (identified by paper chromatography). This zone accordingly contained the oxime of pyruvic acid. The hydroxylamine compound obtained from the strip with R_F 0.08, which corresponded to the red spot, was used for several paper chromatograms with different solvents: butanol/acetic acid/water (63:27:10), amylacetate/formic acid (80 %)/water (4:1:1), ethylacetate/butanol/water (8:2:1), and ethylacetate / propanol / acetic acid / water (5:2:2:1) (Fig. 2). Corresponding paper chromatograms were made for comparison with synthetic acetylhydroxamic acid.

The synthetic acid was prepared from ethylacetate and hydroxylamine-HCl in an alkaline methanol solution at 30° C. The solution was neutralized with HCl upon which KCl precipitated. The precipitate was filtered off and the solution evaporated to dryness *in vacuo*. The residue was dissolved in ethanol, ether was added, and the precipitate was filtered off. The clear solution was evaporated to dryness and the residue crystallized from acetone. M.p. 88—89 °C. According to the determination of hydroxylamine in the preparation it is pure acetylhydroxamic acid. It gives an intensely red colour with ferric chloride.

The hydroxamic acid found in *Torulopsis* cells is according to chromatographic analyses, presented in Fig. 2, acetylhydroxamic acid.

Acetylhydroxamic acid is not reduced by sodium amalgam (1.5 %), whereas oximes are reduced quantitatively to the corresponding amino acids. On the basis of this the following experiment was performed: from the alcoholextract of the cell mass of *Torulopsis* alcohol was evaporated, and the oximes in the solution were reduced with sodium amalgam. The amount of hydroxylamine which disappeared on reduction indicated the amount of oximes. The difference between the total "bound hydroxylamine"-N and the reduced oxime-N indicates the amount of acetylhydroxamic acid. In two different experiments 30 and 50 %, respectively, of the total "bound hydroxylamine" was acetylhydroxamic acid, whereas 50—70 % of "bound hydroxylamine" thus corresponded to the oximes of keto acids, mainly to that of pyruvic acid.

In the earlier paper we reported that appreciable amounts of "bound hydroxylamine" were formed in nitrite solution only at low oxygen concentration. We have now found that *Torulopsis* cells with low N-content form "bound hydroxylamine" both from nitrate and nitrite in aerated suspensions. At best about 10—20 μg NH_2OH-N/g fresh yeast was accumulated in the cells during the first ten to twenty minutes.

Our results indicate that "bound hydroxylamine" is accumulated in *Torulopsis utilis* cells suspended in a nitrate or nitrite containing nutrient solution. In this nitrogen fraction the oximes of α-keto acids as well as acetylhydroxamic acid are found. The oximes are obviously formed through a chemical reaction between hydroxylamine and α-keto acids [2]. Acetylhydroxamic acid is probably formed either from hydroxylamine and acetyl-CoA [3] or from hydroxylamine and acetylphosphate [4].

Since the amount of oximes rapidly decreases in *Torulopsis* cells there must be some mechanism through which "bound hydroxylamine" is either reduced or hydrolyzed in the cells. Virtanen has earlier advanced the hypothesis that the oximes of α-keto acids should be reduced to the corresponding amino acids. For the present we have not been able to demonstrate this reduction. The observations made by

Yamafuji [5] on this point cannot be regarded as conclusive evidence. The question is thus still open. The other possibility that the oximes formed are hydrolyzed, and that the free hydroxylamine is then reduced to ammonia also lacks experimental evidence.

At the moment it seems very likely that the physiological function of the formation of "bound hydroxylamine" is the "trapping" of hydroxylamine in case it is not reduced to ammonia quickly enough. On the other hand, it is still unsettled if the formation of oximes at the same time constitutes a special pathway for amino acid synthesis. The significance of acetylhydroxamic acid is also obscure.

1. Virtanen, A. I. and Saris, N.-E. *Acta Chem. Scand.* **9** (1955) 337.
2. Virtanen, A. I. and Alfthan, M., *unpublished*, Ref. Virtanen, A. I. *Ann. Acad. Sci. Fennicae, Ser. A. II, Chem.* **1952** No. 43.
3. Chou, T. C. and Lipmann, F. *J. Biol. Chem.* **196** (1952) 89.
4. Lipmann, F. and Tuttle, C. *J. Biol. Chem.* **159** (1945) 21.
5. Yamafuji, K. *Nature* **167** (1951) 770; Yamafuji, K. and Omura, H. *Enzymologia* **15** (1952) 296.

Received February 15, 1956.

Activation Energies in the Alkaline Hydrolysis of Saturated Aliphatic Esters

EERO A. HALONEN

Institute of Chemistry, University of Helsinki, Finland

The volatility and low solubility of saturated aliphatic esters in water may lead to appreciable errors in the determination of the rates of hydrolysis.

In the present study, the air volume above the solution was kept small by using two ampoules, one of about 6 ml capacity and the other of about 10.5 ml, which were connected by a short length of rubber tubing. 5 ml of an 0.02 M aqueous solution of the ester was measured into the larger ampoule and 5 ml of 0.02 M aqueous sodium hydroxide into the other. After the rubber tubing was closed with a screw fastener, the ampoules were placed alongside each other in the thermostat; in this way evaporation of the ester into the alkali solution was avoided. After both ampoules had attained the thermostat temperature, the tubing was opened, the alkali was transferred into the ester solution with vigorous shaking, and the tubing was closed again. The ampoules were taken from the thermostat after suitable intervals, the tubing was removed, and the reaction mixture poured rapidly into an ice-cold hydrochloric acid solution. The excess of hydrogen chloride was titrated with baryta solution using phenolphtalein as indicator. The rate constants were calculated from the second-order rate equation. The values of the ate constants obtained are given in Table 1 (the values for ethyl formate from a publication of Tommila and Maltamo [1] have been included for comparison).

The rate constants for all the esters studied are in good agreement with the values reported by Salmi and Leimu [2], and those for ethyl acetate with the values published by Tommila and coworkers [3,4]. From the calculated activation energy and frequency factor values given in the table it is seen that for the last three esters the decrease in the rate is mainly due to a decrease in the frequency factor, and that the activation energy increases from ethyl formate to ethyl acetate and then decreases slowly in the series ethyl acetate, ethyl propionate and ethyl *iso*butyrate. On the other hand, the activation energies for ethyl propionate and ethyl *n*-butyrate do not differ from each other. The rate falls greatly from ethyl formate to ethyl acetate and the difference between the rates of ethyl propionate and ethyl butyrate is abnormally large (*cf.* Ref.[4]). The rate decreases only slightly from ethyl acetate to ethyl propionate in the temperature range studied (as calculated from the Arrhenius plot, ethyl propionate hydrolyses more rapidly than ethyl acetate at temperatures below $-3°$ C).

The differences in the rates of hydrolysis can be explained by assuming that the activation energy differences are mainly determined by hyperconjugation and that the differences in the inductive and steric effects appear mainly in the frequency factors. In a previous study [5], it was concluded that the conjugation between the