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On the Estimation of Bound Hydroxylamine in Biological Materials

TIHAMÉR Z. CSÁKY*

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

The occurence of hydroxylamine in some biological processes has been convincingly shown *e. g.* in the fixation of atmospheric nitrogen by *Azotobacter* and leguminous bacteria [1—4] and in the reduction of nitrates by *B. coli* [5] and *Torula* yeast [6]. Regarding the quantitative study of hydroxylamine the method mostly used for estimation is that of Blom [7]. This very sensitive method is based on the oxidation of hydroxylamine to nitrite in acetic acid medium with iodine and on the estimation of nitrite by means of the colour reaction with sulphanilic acid and α-naphthylamine. The chemically bound hydroxylamine (oxime) is first split off by boiling in 3 *N* sulphuric acid for 6 hours [2, 8].

In this laboratory Blom's quantitative method was recently used in the investigations on the nitrate assimilation of *Torula* yeast [6]. In the course of this work some attention was devoted to the influence of other oxidized nitrogen compounds possibly occuring in the living cells on the estimation of oxime nitrogen. The substances investigated in this respect are: nitrous, hyponitrous acid, and nitrohydroxamic acid. In the following, the results of these experiments are briefly given.

EXPERIMENTAL

Preparations. The following preparations have been used in the experiments:
Hydroxylamine hydrochloride, Merck's preparation *(pro analysi)*,
Sodium nitrite, Baker's analyzed.

* Visiting scientist from the Hungarian Biological Research Institute, Tihany, Hungary.

Sodium hyponitrite and sodium salt of nitrohydroxamic acid were prepared, the former by reducing sodium nitrate with sodium amalgam, the latter by mixing a warm saturated solution of hydroxylamine hydrochloride in alcohol with a concentrated solution of sodium ethylate in absolute alcohol, filtering off the sodium chloride and treating the filtrate with ethyl nitrate [9].

Estimation of free hydroxylamine. In the original Blom's method iodine was used for the oxidation of hydroxylamine to nitrite and sodium thiosulphate for destroying the excess of iodine. Working in acetic acid medium, thiosulphate gives mostly a turbidity which causes great difficulties in the colorimetric measurement. The turbidity can be avoided by using sodium arsenite instead of sodium thiosulphate. In this case the solution is kept excellently clear and the colour can be easily measured in a convenient colorimeter.

The modified reaction is made as follows: 1—5 ml of the solution containing 0.2—0.5 μg hydroxylamine N is placed in a 10 ml calibrated tube and 1 ml of sulphanilic acid (10 g sulphanilic acid dissolved in 1000 ml of 30 % acetic acid by heating on the water-bath) and 0.5 ml iodine solution (1.3 g iodine in 100 ml glacial acetic acid) measured to it. After 3 to 5 minutes the excess of iodine is destroyed with 1 ml of sodium arsenite solution (2 g sodium arsenite in 100 ml distilled water) and after adding 1 ml α-naphthylamine solution (3 g α-naphthylamine dissolved in 1000 ml of 30 % acetic acid) the mixture is made up with distilled water to 10 ml. After 20—30 minutes the colour is developed and measured in Klett-Summerson photoelectric colorimeter using a green filter (500 mμ) against a control containing all reagents but the solution to be tested. The amount of hydroxylamine-N can be read from a calibration curve obtained with pure sodium nitrite or hydroxylamine hydrochloride solution.

Estimation of bound (oxime-) hydroxylamine. A sample of 1 ml of the solution to be tested and 1 ml 6 *N* sulphuric acid is placed in a Pyrex glass test tube with ground-in stopper carrying a capillary tubing **. The solution is boiled in a water-bath for 6 hours, treated with charcoal if it has become coloured and transferred quantitatively into a 10 ml tube. The excess of sulphuric acid is buffered with 3 ml of 35 % sodium acetate and the free hydroxylamine estimated as above.

Effect of boiling in sulphuric acid on nitrous, hyponitrous and nitrohydroxamic acid and hydroxylamine in dilute solution. All these substances give the colour reaction without any treatment with iodine; hyponitrous acid of course

** If the solution contains nitrous, hyponitrous acid or nitrohydroxamic acid, sulphanilic acid has to be added to it before hydrolysis. In this case 1 ml of the solution to be tested is measured into the test tube together with 0.5 ml sulphanilic acid and 0.5 ml 12 *N* H_2SO_4 (*cf.* Table 3).

only in higher concentrations. In order to investigate how these substances influence the determination of bound hydroxylamine they were at first boiled separately in 3 *N* sulphuric acid. 1 ml of the solution was placed in a Pyrex glass tube carrying a capillary tubing together with 1 ml 6 *N* H_2SO_4 and boiled for several hours. After boiling the solution was transferred into a 10 ml calibrated tube, the excess of acid neutralized with 3 ml of 35 % sodium acetate and the colour reaction made as above.

Table 1 shows that nitrous, hyponitrous acid, and nitrohydroxamic acid were totally destroyed after boiling in 3 *N* sulphuric acid for 6 hours giving no traces of colour reaction whereas hydroxylamine was stable against such treatment.

Table 1. Effect of boiling in 3 N sulphuric acid on solutions of sodium nitrite (0.01 mg) sodium hyponitrite (0.05 mg) sodium salt of nitrohydroxamic acid (0.05 mg) and hydroxylamine (0.001 mg).

Time of boiling hours	Colour reaction after boiling (Extinction)			
	Nitrite	Hyponitrite	Nitrohydroxamic acid	Hydroxylamine
0	0.260	0.104	> 1.000	0.061
2		0.010	0.041	
4		0.000	0.000	
6	0.000	0.000	0.000	0.056

Effect of nitrous, hyponitrous and nitrohydroxamic acids on the estimation of hydroxylamine. A sample of 0.5 ml hydroxylamine solution and 0.5 ml of the solution of one of the three oxidized nitrogen compounds were boiled together with 1 ml of 6 *N* sulphuric acid. The concentration of nitrite, hyponitrous or nitrohydroxamic acid was always higher than that of hydroxylamine. Table 2 shows that all the substances investigated destroy hydroxylamine completely when boiled in sulphuric acid for 6 hours.

Table 2. Effect of nitrous, hyponitrous, and nitrohydroxamic acid on very small amounts of hydroxylamine when boiled in 3 N sulphuric acid.

A: 0.45 μg hydroxylamine N + 250 μg sodium salt of hyponitrous acid
B: 0.45 μg hydroxylamine N + 250 μg sodium salt of nitrohydroxamic acid
C: 2.00 μg hydroxylamine N + 100 μg sodium nitrite
boiled in 3 *N* H_2SO_4

Time of boiling hours	Colour reaction after boiling (Extinction)		
	A	B	C
0	0.061	0.061	0.730
6	0.000	0.000	0.009

Table 3 shows the results of the experiments where the concentration of hydroxylamine and of the other nitrogen compounds was the same. In this case the greatest part of hydroxylamine has been destroyed, too. If sulphanilic acid was added before boiling the destruction was smaller.

Table 3. Effect of nitrous, hyponitrous, and nitrohydroxamic acid on hydroxylamine when boiled in equal concentrations in 3 N sulphuric acid.

A : 1.0 μg hydroxylamine N + 1.0 μg nitrohydroxamic acid N
A^1 : 1.0 μg hydroxylamine N + 1.0 μg nitrohydroxamic acid N + sulphanilic acid

B : 2.0 μg hydroxylamine N + 2.0 μg nitrite N
B^1 : 2.0 μg hydroxylamine N + 2.0 μg nitrite N + sulphanilic acid boiled in 3 *N* H_2SO_4

Time of boiling hours	Found N (μg) after boiling			
	A	A^1	B	B^1
0 *	0.60	0.92		1.94
½	0.34	0.81		
1	0.35	0.74	0.32	1.66
2	0.35	0.74		
4	0.35	0.80	0.37	1.66
6	0.34	0.78	0.45	1.55

DISCUSSION

Free hydroxylamine occurs under biological circumstances possibly only in a very small amount as it reacts very easily with keto acids forming oximes. So the estimation of hydroxylamine is connected with that of oximes. As the experiments described above show, the estimation of oximes by splitting off with sulphuric acid is exact only when hydroxylamine or oximes are in the medium but no other oxidized nitrogen compounds. If other oxidized nitrogen compounds are also present they destroy hydroxylamine partially or totally depending on the concentration. The addition of sulphanilic acid before the boiling prevents a part of hydroxylamine from being destroyed. Unfortunately, we have no sensitive methods for detecting nitrite, hyponitrous or nitrohydroxamic ions separately, all three substances giving the colour reaction with sulphanilic acid and α-naphthylamine without oxidation, hyponitrous acid only in higher concentrations.

On the other hand, it is important to know whether in the case of oxime estimation hydroxylamine alone gives the colour reaction after hydrolysis

* Solution kept with sulphuric acid at room temperature for 30—60 minutes.

with sulphuric acid or whether other substances as well give the same reaction. The colour reaction with sulphanilic acid and α-naphthylamine is given only by nitrous, hyponitrous and nitrohydroxamic acid and by hydroxylamine after oxidation with iodine. The experiments show, that all the first mentioned substances are destroyed totally by boiling in 3 *N* sulphuric acid for 6 hours, whereas hydroxylamine is stable against boiling with sulphuric acid. Therefore only hydroxylamine gives the positive Blom's test after hydrolysis.

SUMMARY

The influence of nitrous, hyponitrous and nitrohydroxamic acid on the estimation of oxime nitrogen by Blom's method has been investigated. Nitrous, hyponitrous and nitrohydroxamic acid are totally destroyed by boiling in 3 *N* sulphuric acid for 6 hours giving no traces of colour reaction. Hydroxylamine is stable against boiling with sulphuric acid if it is alone in the solution, whereas it is partly destroyed in the presence of one of the three oxidized nitrogen compounds.

The values obtained in oxime estimation by Blom's method may therefore be too low, but only hydroxylamine gives the positive reaction.

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