ENZYMIC REDUCTION OF TRIMETHYLAMINE OXIDE

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By

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(*Received May 25, 1952*)

In 1939 Tarr[1] first showed that the reduction of the trimethylamine oxide is due to a bacterial enzyme, triamineoxidase, which activates the trimethylamine oxide rendering it susceptible to reduction by various dehydrogenases of the cell. At about the same time Watson[2], later Tarr[3], Neiland[4] and Castell and Snow[5] studied on the enzyme systems or the factors affecting triamineoxidase.

Recently Tsuchiya and co-workers[6] showed that the production of ammonia and trimethylamine in shark are derived from urea and trimethylamine oxide by the action of bacterial urease and triamineoxidase and that latter enzyme plays an important roll in shark spoilage.

This paper reports on a study of some of enzymic reduciton of trimethylamine oxide which has been made to obtain a fundamental knowledge for the method of preservation and utilization of shark.

Experimental

Cultures

As in the previous report[6] 3 of the 14 cultures isolated from sharkflesh were found to be able to reduce trimethylamine oxide to trimethylamine, these 3 cultures, 1 (micrococcus), 4 and 6 (proteus), were thoroughly used in this experiments.

Preparation of the Enzyme Extract

First, the preparation of the enzyme extract which reduces trimethylamine oxide was examined.

Forty ml. of a heavy suspension of cells of each culture was permitted to autolyze under aseptic conditions with 40 ml. of 0.2 M phosphate buffer at 25°C. for 10 days. The suspension was centrifuged, the supernatant liquid (a) was decanted, and the debris was then suspended in 80 ml. of water (b). Part of

solution (a) was sterilized by passing it through a Seitz filter (c). The means of these preparations to reduce trimethylamine oxide were studied as follows. Four ml. of solution (a, b or c) ; 0.5 ml. of 0.1 M trimethylamine oxide ; 1 ml. of 0.1 M trimethylamine oxide ; I ml. of 0.1 M phosphate buffer pH 7 and 1 ml. of 0.1 M sodium lactate were added to Thunberg tubes. These were evacuated and incubated for 16 hours at 25°C. The trimethylamine produced was determined by the method of Tarr.[1] The results obtained are given in Table 1.

Table 1. Trimethylamine production by various fractions of autolyzed cells.

Fraction number		γ of trimethylamine nitrogen in 4 ml. of solution		
		Culture 1	Culture 4	Culture 6
a	Control	1.2	1.7	1.3
	Trimethylamine oxide	4.1	2.4	3.6
b	Control	3.1	2.8	1.2
	Trimethylamine oxide	84.0	40.8	45.6
c	Control	2.4	1.0	1.0
	Trimethylamine oxide	3.6	1.2	1.3

It shows that the enzyme is closely associsated with the bacterial cell, and does not readily diffuse into the surrounding medium during autolysis. It exactly fits the results obtained by Tarr.

As an attempt to prepare a cell-free enzyme extract by autolysis was unsuccessful, a washed cell suspension was used as the source of enzyme in the following experiments.

Velocity of Reduction

The velocity of the reduction of trimethylamine oxide was studied hourly by culture 1 at 25°C. The reaction mixture in Thunberg tubes consisted of the following :

(1) 2 ml. of cell suspension
(2) 1 ml. of shark muscle extract (1 : 3)
(3) 1 ml. of 0.1 M trimethylamine oxide
(4) 1 ml. of 0.2 M phosphate buffer pH 7.0.

The results obtained are given in Figure 1 and 2. They show that the reaction of the reduction of trimethylamine oxide is not linear as was shown by Tarr but is sigmoid. That is, the production of trimethylamine satisfies the equation : $\log \frac{x}{a-x} = k(t-t_1)$, derived from the law of mono-molecular auto-catalytic reaction. In this equation, a stands for the total amount of trimethylamine nitrogen produced by complete reduction ; x the amount of

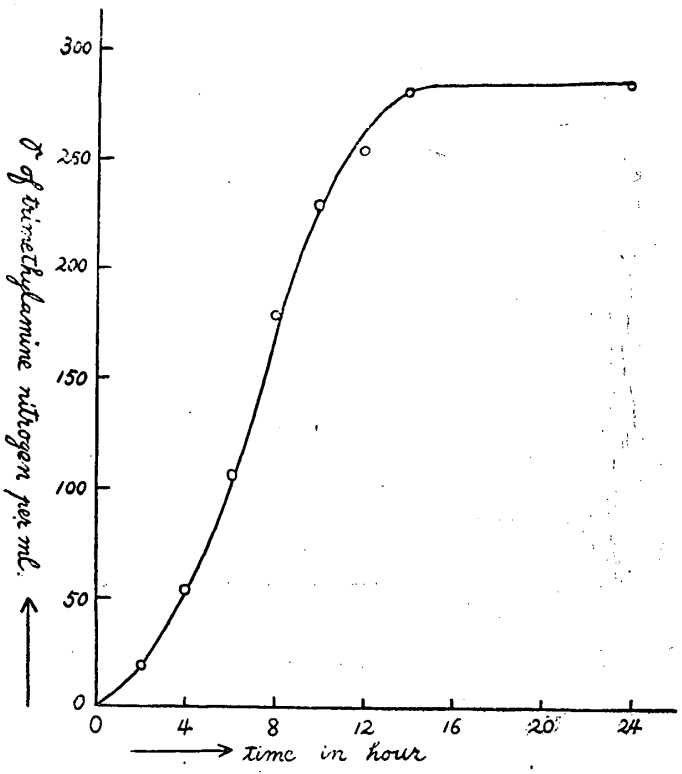

Fig. 1. Reaction velocity of reduction of trimethylamine oxide to trimethylamine.

trimethylamine nitrogen produced in a given time t; t_1 the time at $x = \frac{a}{2}$; and k represents a velocity constant, which is found as 0.216 from Figure 2.

Effect of pH

The effects of hydrogen ion concentration upon the enzymic reduction of trimethylamine oxide were examined by the following procedure.

The cell suspensions of culture 1 and 4 were incubated anaerobically in Thunberg tubes in the presence of trimethylamine oxide and sodium lactate for 2 hours at 25°C. But a variety of

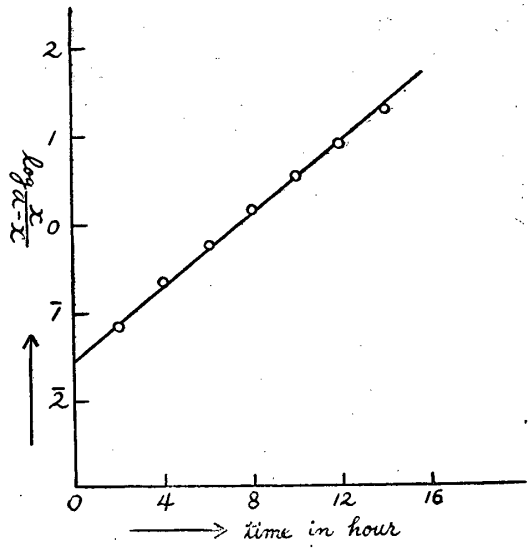

Fig. 2. Relation of $\log \frac{x}{a-x}$ and time.

pH of the medium were adjusted with McIlvaine's buffer solution of 0.2 M sodium phosphate and 0.1 M citric acid. The results obtained are shown in Figure 3.

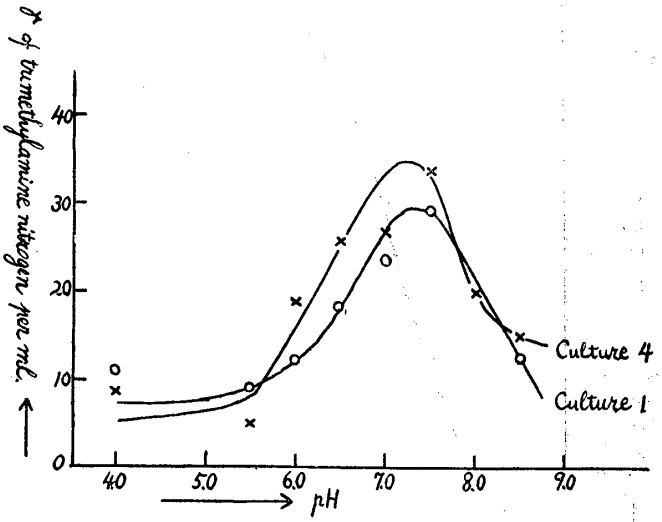

Fig. 3. The influence of pH on the rate of trimethylamine oxide reduction.

This figure shows that the effects of pH on the enzymic reduction of trimethylamine oxide were sensitive to both cultures. The oxide reduction is most active at or slightly above pH 7.3 and is greatly retarded below pH 5.5.

Effect of Temperature

The thermal effect upon the enzymic reduction of trimethylamine oxide, particularly on the inactivating effect of heat, was also examined.

Thunberg tubes were prepared with the following solutions: 2 ml. of cell suspensions of trimethylamine-non-forming culture 8: 1 ml. of 0.1 M sodium lactate: 0.5 ml. of 0.2 M phosphate buffer pH 7.0: 0.2 ml. of 0.5 M trimethylamine oxide and 1 ml. of 0.001 M Nile blue. The hollow stoppers of duplicate tubes

Table 2. Heat inactivation of the enzymic reduction of trimethylamine oxide.

Treatment	Reaction of the reduced dye
Culture 1 unheated	The reduced dye was completely re-oxidized
〃 heated	No change, the dye remained completely reduced
Culture 4 unheated	The reduced dye was completely re-oxidized
〃 heated	No change, the dye remained completely reduced
Culture 6 unheated	The reduced dye was completely re-oxidized
〃 heated	No change, the dye remained completely reduced

contained 1 ml. of either heated (80°C for 5 minutes) or unheated bacterial suspensions. The tubes were carefully evacuated and incubated at 25°C. After 2 hours the dye was reduced about 60 per cent and then the solutions were mixed. The results obtained are given in Table 2.

It indicates that the enzymic reduction is inactivated by heating for 5 minutes at 80°C.

Additional proof for this was made in the following experiment. A simple colony of each culture was aseptically transferred on agar slope in test tubes and warmed for 3 to 5 minutes at various temperatures in water bath. After incubating for 24 to 48 hours at 25°C., the bacterial growth was observed. The results obtained are shown in Table 3.

Table 3. Inhibition of bacterial growth by heating.

Temperature	Culture number		
	1	4	6
60°C for 5 minutes	+++ ++	++ ++	+++ ++
70°C 〃	−	++ ++	−
80°C for 3 minutes	−	++ ++	−
〃 5 〃	−	−	−
90°C 〃 〃	−	−	−

\+ indicates positive growth.
− 〃 negative 〃

It is almost the same as above experiment. Namely, the growth in culture 1 and 6 was inhibited by heating for 5 minutes at 70°C. and that of culture 4 was by heating for 5 minutes at 80°C. However, it should be pointed out here that the temperature at which the enzymic action is inactivated does not necessarily mean the temperature at which the bacterial growth is suspended.

Compounds Accelerating Reduction

A variety of compounds accelerating the reduction of trimethylamine oxide were examined in the presence of culture 1 for 16 hours at 25°C. The results obtained are given in Table 4.

It shows that glucose, fructose, alanine and glycine replace sodium lactate as a source of hydrogen.

Compounds Inhibiting Reduction

Table 4. A variety of compounds accelerating the reduction of trimethylamine oxide to trimethylamine.

Oxidizable substrate	γ of trimethylamine nitrogen per 1 ml. of solution
Control	264.2
0.1 M Sodium formate	306.0
〃 acetate	282.3
〃 lactate	310.6
〃 succinate	276.5
〃 Glucose	492.0
〃 Fructose	366.8
〃 Alanine	376.3
〃 Mono sodium glutaminate	281.0

A variety of compounds inhibiting the reduction of trimethylamine oxide were next examined in the presence of culture 1 for 6 hours at 25°C. The results obtained are given in Table 5.

Table 5. Compounds inhibiting the reduction of trimethylamine oxide to trimethylamine.

Inhibitor	γ of trimethylamine nitrogen in 1 ml. of solution
Control	160.8
0.01 M Hydrogen peroxide	97.2
0.001 M Sodium nitrite	135.8
〃 fluoride	109.1
〃 Mercuric chloride	46.0
〃 Cupric acetate	70.2
〃 Monoiodoacetic acid	133.6
〃 Sodium salicyrate	130.0
〃 benzoate	112.5
〃 α-dinitrophenol	120.1

It shows that the heavy metal compounds such as mercuric chloride and cupric acetate have a marked inhibitory effects on the oxide reduction. As might be expected, sodium nitrite inhibited the reduction as well as the other inhibitors. Although it is not cited in the table, potassium cyanide and toluene were also found to have inhibitory power.

Moreover the other inhibitors are now under examination with the purpose of finding suitable preservatives for fish. The details shall be discussed in papers following.

Discussion and Summary

The reduction of trimethylamine oxide by the bacterial enzyme, with special reference to the factors affecting its activity, was studied.

Under the conditions employed in this experiment the following results were established : (1) The reaction of the reduction of trimethylamine oxide was sigmoidal. The production of trimethylamine satisfied the equation, $\log \frac{x}{a-x} = k(t-t_1)$; (2) The reduction of trimethylamine oxide was weakened as the acidity of the medium increases with the optimum pH at or slightly above 7.3 ; (3) Each culture contained a heat labile enzyme which was inactivated by heating for 5 minutes at 80°C ; (4) Glucose, fructose, alanine and glycine could replace sodium lactate as a source of hydrogen : (5) The heavy metal compounds such as mercuric chloride and cupric acetate strongly inhibited the reduction of the oxide.

It should be pointed out that, in these experiments, the enzyme extract has not been prepared separately from bacterial cell. Consequently, it remaines unsolved to conclude whether it is the action of the enzyme itself or the growth of the cells that produce the enzyme, which is effected by change of pH and temperature and by inhibitory substances.

This study was made possible in part by a grant from the Research Fund of the Ministry of Education.

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