ON THE TRIMETHYLAMINE-OXIDE REDUCTASE
(THE FIRST REPORT)

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Trimethylamine and its oxide are found in certain kinds of fishes in comparatively large amount and attention to this fact has been payed for a long time. Wood have found that the trimethylamine-oxide reduction test being useful for the classification of some microorganisms, and this has been the first time for using those substances on the taxonomical point of view of the bacteriology. Fukumi and his associates have also payed attention to this subject and published two articles about this problem already. The author was interested in the nature of the active substance for this reaction and its enzymological significance, and have carried out a study using Escherichia coli and Proteus vulgaris. In this paper the results of the experiments done with so called resting bacteria of Escherichia coli will merely be reported. As for the experiments with Proteus vulgaris, the results observed were much the same.

EXPERIMENTAL METHODS

1. Measurement of trimethylamine produced: There are two methods for measuring trimethylamine under its coexistence with ammonia, namely, the formol-method and the cis-aconitic acid method. The former is not good for this experiment on the viewpoint of accuracy, and is moreover somewhat complicated and the latter method is very troublesome in procedure and considered inadequate for this kind of study. The measurement of trimethylamine has therefore been studied and established as follows: Some 0.5 cc of formaldehyde solution (about 40%), 0.5 cc of 30% KOH aqueous solution and one drop of octylalcohol (to prevent foaming) are added to the fluid to be tested (which is to be approximately 4cc), and the fluid is aerated in a water-bath of 37°C for 15 minutes to take out trimethylamine in 5 cc of 2% boric acid with indicator. This is finally titrated with N/100 HCl and the trimethylamine taken out from the fluid to be tested by aeration is calculated. With this procedure tri-
methylamine is measured in a range of 0-100 $\mu$M in the accuracy of ±3 $\mu$M.

2. Trimethylamine-oxide reduction reaction:

The experiments have usually been performed as follow, as well with some modifications according to varying purposes at times:

Suspension of resting bacteria 1cc
(made from 18 hours' growth on nutrient agar slant, approximately 0.1 mg of nitrogen)
M/10 Trimethylamine-oxide 1cc
M/10 hydrogen donor 1cc
M/5 phosphate buffer (pH 7.5) 1cc
Yeast-extract
(Supernatant of one part of dry yeast in 10 parts of distilled water, after heating at about 90°C for 20 minutes and centrifugalized)

are added and incubated at 37°C. After a certain period of time, trimethylamine produced is determined.

EXPERIMENTAL RESULTS

1. Time-after-time measurements of trimethylamine produced from its oxide by adapted and non-adapted bacteria, and the effect of yeast extract upon them.

Fig. 1
Fig. 1 shows how the addition of yeast extract influences upon the activity of trimethylamine oxide reduction. In this experiment the microorganisms were used after suspending them in trimethylamine oxide solution for 150 minutes to have them adapted to trimethylamine-oxide and washing thereafter by centrifugalizing for two times. Glucose was used as hydrogen donator and 0.1 cc yeast extract was added when its effect was to be tested. By this experiment the following three facts were made clear: (1) this reaction is concerning with a kind of adaptive enzyme which shows activity quickly after standing in the solution for about an hour, (2) that once the enzyme (molecules) is produced by adaptation, they show activity without any lag phase, and (3) that the enzyme activity is influenced considerably by yeast extract.

Fig. 2 shows the results of the experiments in which glucose, pyruvic acid and lactic acid were used as hydrogen donator. In these experiments the effect of yeast extract upon this reaction is shown very distinctly. The same result is obtained with experiments carried out anaerobically.
Fig. 3 shows the indispensability of yeast extract in this reaction. In this experiment non-adapted micro-organisms were used after dialysed in a refrigerator overnight, and glucose was used as the hydrogen donator. The effective substance in yeast extract is not attributed to diphosphopyridine nucleotide as mentioned below.
The result of the experiment, in which the effect of yeast extract was investigated with adapted microorganisms, is shown in Fig. 4. From this, it is very clear that yeast extract is working like a coenzyme, and this effect is exerted not upon the adaptation process but upon the enzyme activity itself. In this experiment, glucose was employed as substrate.
2. Optimal pH.

In Fig. 5 non-adapted microorganisms were placed in trimethylamine oxide solution under several different pHs, using glucose as hydrogen donator. The optimal pH for trimethylamine oxide reduction lies in the pH range 7.0-7.5. The factors affecting the rapidity of adaptation seem complicated, and do not always correspond with the activity of the dehydrogenase, and moreover, the order of rapidity in adaptation varies according to experimental conditions. The general information about it could be obtained rather from Fig. 2.
Fig. 6 shows the experimental results with microorganisms adapted to trimethylamine-oxide, from which it is known that the optimal pH lies around 7.5 and pH has much more remarkable influence on the acid side than on the alkaline side. In this case, as the dehydrogenase, cooperating with the reductase, was used in much larger amount than with the latter enzyme, the optimal pH of the dehydrogenase is not supposed to have any influence upon measuring the optimal pH of the reductase.

Almost compatible results were obtained with succinic acid, acetic acid, malic acid, α-alanine and some others as substrates, and in these cases also, yeast extract was found necessary as a cofactor.
3. Experiments with some dyestuffs.

Experiments were carried out to know whether neutral red, nile blue, methylene blue, resazurin, or thionin may work as a hydrogen donator or like yeast extract, but neither of oxidized nor reduced (obtained with hydrosulfite as a reductant or with catalytic reduction using Pd) forms of these dyestuffs were found effective.

4. Inhibition experiments.

Fig. 7

Several substances known generally as an inhibitive to enzymes were investigated of their inhibitory actions to the trimethylamine-oxide reductase. Those substances are known not so inhibitory to the dehydrogeneseses in general except for monooiod acetic acid at a high concentration. Fig. 7 shows the result of the experiment using glucose as a hydrogen donator. In this, the reductase is supposed of heavy-metalic nature.
The experiment shown in Fig. 8 was carried out to confirm the supposition mentioned above. As those inhibitive substance used in this experiment have little influence upon dehydrogenses, the reductase is very much likely an enzyme with a heavy metal atoms, especially iron. However, carbon mono-oxide did not inhibite the action of this enzyme under an atmospheric pressure.

5. Effective substances in yeast extract.

It is considered very important to make clear the nature of the substance or substances acting as cofactor in yeast extract not only in respect of clarifying the essential feature of the reductase itself but also in simplifying the system of the reaction. Taking dialysability of the cofactor into consideration, the following substances were tested:

1) Inorganic ions: K+, Na+, NH₄+, Ca²⁺, Mg²⁺, Mn²⁺, Fe³⁺, Cl⁻, SO₄²⁻, PO₄³⁻. All of them were found ineffective. The residual substance of yeast extract after charring was also tested in vain.
2) Vitamins: Vitamin B$_1$, B$_2$, nicotinic acid and its amide, diphosphopyridine nucleotide, pyridoxine, folic acid, pantothentic acid, anthranilic acid, paraminobenzoic acid, biotin, $\beta$-alanin, adenosin triphosphate, co-carboxylase, flavine mononucleotide, flavine adenine dinucleotide and thioglycolic acid were found all ineffective.

3) Hydrolysates of gelatine; casein and yeast nucleic acid; horse serum; chick red blood cells; heart; liver; kidney; pancreas; spleen; muscles of mouse and rabbit; haemoglobin. All of them were found hardly effective. Heated extract of silk worm pupa were found highly effective as cofactor.

6. The nature of the cofactor in yeast extract (preliminary experiment).

1) The cofactor is dialysable.

2) It is not precipitated by barium hydroxide, lead acetate, mercury sulfate or silver nitrate.

3) It is not precipitated with 3 volumes of alcohol from aqueous solution.

4) Not soluble in ether or chloroform.

5) It is 30% destroyed in 1/N HCl or NaOH, or 80% destroyed in 2/N by heating at 100°C for an hour or 100% destroyed at room temperature in 10 minutes.

6) It is not destroyed by catalytic reduction using Pd, or diazonium salt of paraminobenzoic acid.

7) It is not destroyed by an hour’s treatment at 37°C with homogenates in phosphate buffer of heart, liver, kidney, pancreas, spleen or muscle of mouse in the presence of 10$^{-4}$ M MgSO$_4$.

7. Isolation of the reductase from cells.

The reductase is quite sensitive to procedures for taking it out of cells. It is made ineffective by autolysis at 37°C for 20 hours, by lyophilization, or by ultrasonic vibration method of usual way. It is, however, made free from cells by ultrasonic vibration of 550 KC for 20 minutes at 0°-10°C, and yet in comparatively good yield.

**SUMMARY**

The following results were obtained concerning the trimethylamine oxide reductase of Escherichia coli and Proteus vulgaris:

1) The enzyme is of adaptive nature.

2) Yeast extract is needed for the manifestation of the enzymatic activity.
3) The optimal pH of the enzyme is 7.5.
4) The enzyme is considered to contain a certain heavy metal molecule, perhaps iron.
5) Studies in the nature of cofactor in yeast extract is now under way.
6) “Cell free” enzyme was obtained by ultrasonic vibration at low temperature.

REFERENCES

2) Fukumi, H., Tomizawa, J. and Sunakawa, S. Read at the scientific meeting in the National Institute of Health, March 25, 1948.
3) Fukumi, H., and Nakaya, R., in press.