**Biological Formation of Formaldehyde and Dimethyl-
amine in Fish and Shellfish—VIII.
Requirement of Cofactor in the Enzyme System***

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It has shown in the previous paper¹⁾ that the formation of FA and DMA from TMAO by the tissue homogenates of cod varieties was enhanced by an addition of a small quantity of methylene blue. In this report authors will add that a similar formation in the presence of methylene blue proceeds also in the reaction mixture containing crude enzyme prepared by means of salting-out, and a need of cofactor bearing heat-tolerant nature in this enzyme action.

Experimentals

Materials. Crude enzyme solutions examined in this study were prepared principally from pyloric caeca of Alaska pollock, *Theragra chalcogramma* and in addition, liver of cod, *Gadus macrocephalus* and midgut gland of cuttlefish, *Sepia esculenta* were employed. These samples were stored in a laboratory freezer at temperature of either -20°C or -80°C until to be used, and those kept at -20°C were treated with time priority to avoid the loss in the enzyme activity during storage. No virtual loss of the activity in the frozen tissues after one year's storage at -80°C was confirmed in the separate experiment.

Crude enzyme. To a 100 ml portion of the tissue homogenate, which had been prepared by the procedure described in the previous report¹⁾, was added 10 g of ammonium sulfate and the mixture was centrifuged at 17,000 G for twenty minutes. The first precipitate containing insoluble debris of the tissue was discarded and to a 50 ml portion of the supernatant was added 7.5 g of ammonium sulfate and 30 g of filter powder (Hyflo Super-cel) and stood for about one hour until precipitation was settled. The precipitate of crude enzyme was collected after filtered through Buchner funnel and was washed three times with a small volume of ammonium sulfate solution (25 g/100 ml). The preparation was always carried out below 3°C so as to avoid enzyme inactivation at a higher temperature.

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Abbreviation used: FA, formaldehyde; DMA, dimethylamine; TMAO, trimethylamine oxide.

Assay method of enzyme activity. The washed precipitate was dissolved in a small quantity of water and transferred to volumetric flask and filled up with water to 50 ml. A 0.5 ml portion of this crude enzyme solution was placed in a hollow stopper of Thunberg tube, while in a main tube were placed 0.5 ml of the supernatants obtained from the tissue homogenate after salting-out processes by varied strength of ammonium sulfate, 0.5 ml of 0.1 M TMAO, and 0.25 ml of 0.001 M methylene blue. And final volume of the reaction mixture was made to 5 ml by adding phosphate buffer of pH 6.1, including crude enzyme solution in the hollow stopper.

After the Thunberg tube was evacuated, the enzyme solution was tipped into main tube and was then incubated at 26°C for two hours. At an interval of 20 or 30 minutes, the enzyme reaction was stopped by adding 5 ml of 20% trichloroacetic acid and the trichloroacetic-acid extract obtained was subjected to FA and DMA determinations.

Determinations of protein, FA and DMA. Protein concentration in the crude enzyme solution was determined by Folin phenol method²⁾ using bovine albumin (Daiichi Chemicals Co.) as a standard. And, FA and DMA were determined by the methods of NASH³⁾ and DYER⁴⁾, respectively.

Results and Discussion

Salting-out effect of the enzyme. Concentration of ammonium sulfate required to precipitate the enzyme protein from the homogenate of pyloric caeca of Alaska pollock is shown in Fig. 1. Most of the enzyme can be precipitated when the quantity

Fig. 1. Salting-out effect of the enzyme participating in the formation of FA and DMA from TMAO.

System, anaerobic condition using Thunberg tubes, incubation at 26°C for two hours, 0.5 ml of supernatant in hollow stoppers, and 0.5 ml of 0.1 M TMAO, 0.25 ml of 0.001 M methylene blue, 3.75 ml of phosphate buffer (pH 6.1) in main tubes. Protein determination was done in supernatant fractions.

of ammonium sulfate reaches 2.5 g in 10 ml of the homogenate, which is roughly equivalent to 1/3 saturation of the salt. However, in the tissues of cod or cuttlefish, a little larger amount of ammonium sulfate was needed to salt out the enzyme fraction; that was 5.0 g of the salt to 10 ml of the homogenates (about 2/3 saturation). But, a high activity of the enzyme was detected in the fraction precipitated at the concentration of ammonium sulfate able to precipitate the pollock enzyme.

The presence of cofactor in pyloric caeca of Alaska pollock.

Whether the specific enzyme under study requires cofactor or not was examined in the following procedure. The crude enzyme solution from Alaska pollock was taken into hollow stopper of Thunberg tube and the supernatant part obtained after removal of enzyme fraction was placed in main tube together with TMAO solution, methylene blue, and phosphate buffer as described above. The results of this experiment are given in Fig. 2. It is quite obvious that no production of either FA or DMA can be seen when the supernatant is omitted from the reaction mixtures and this indicates that cofactor is indispensable in this enzyme system.

Fig. 2. Participation of supernatant fraction in the formation of FA and DMA from TMAO.

System, 0.5 ml of crude enzyme solution, 0.5 ml of supernatant (excluded in control sample), 0.5 ml of 0.1 M TMAO, 0.25 ml of 0.001 M methylene blue, phosphate buffer (pH 6.1) to 5 ml. Protein concentration of crude enzyme solution, 0.6 mg/ml. Continuous line, the mixture containing supernatant; interrupted line, control sample.

Fig. 3. Distribution of the cofactor in shark liver.

System, 0.5 ml of crude enzyme solution (excluded in control sample), 0.5 ml of shark-liver homogenate, 0.5 ml of 0.1 M TMAO, 0.25 ml of 0.001 M methylene blue, phosphate buffer (pH 6.1) to 5 ml. Protein concentration of crude enzyme solution, 0.6 mg/ml. Continuous line, the mixture containing shark-liver homogenate; interrupted line, control sample.

The existence of the cofactor in shark liver. As the need of cofactor was confirmed in the foregoing experiment, another examination was undertaken whether the cofactor distributes in a fish tissue which does not possess this enzyme. Liver tissue was sampled from a shark, *Deania eglantina*, which was excellent in freshness, and in a similar series of experiment the homogenate of the liver was used instead of the enzyme-free supernatant from Alaska pollock. Fig. 3 shows that the shark liver also contains cofactor, though the tissue does not exhibit the enzyme activity. The shark liver employed in this experiment was stored at -80°C for two months before it was tested.

Heat stability of the cofactor. The supernatant prepared from Alaska pollock was heated at the temperatures of 30°C , 50°C , 75°C and 100°C for 30 minutes before they were added to the crude enzyme solution from the same fish and the formation of FA and DMA was examined. Table 1 indicates that heat treatment of the protein-

Table 1. Heat-stability of the cofactor participating in the formation of FA and DMA from TMAO.

Treatment	Formation	
	FA($\mu\text{g/ml}$)	DMA-N($\mu\text{g/ml}$)
Not heated	37	20
Heated at 30°C	40	23
50°C	39	18
75°C	40	18
100°C	40	20

System, anaerobic condition using Thunberg tubes, incubation at 26°C for two hours, 0.5 ml of crude enzyme solution, 0.5 ml of supernatant, 0.5 ml of 0.1 M TMAO, 0.25 ml of 0.001 M methylene blue, 3.25 ml of phosphate buffer (pH 6.1). Protein concentration of crude enzyme solution, 0.8 mg/ml.

free supernatant has destroyed none of activity. The supernatant of this experiment was prepared by adding ammonium sulfate to the filtered homogenate from which crude enzyme had been removed at the concentration of 3 g in 10 ml.

In previous paper¹⁾, authors demonstrated in the pyloric-caeca homogenate of Alaska pollock that the formation of FA and DMA discontinued by dialysis. TOKUNAGA⁵⁾ reported also that no formation was found in the muscle extractive of the same species after dialysis and subsequent frozen storage. Accordingly, the heat-tolerant cofactor may be dialysable. For the fact of no formation of FA and DMA in the mixture of both dialysate and diffusate¹⁾, the inactivation of enzyme itself would be postulated as a cause, due to the prolonged storage of these isolates.

Summary

The participation of a cofactor was confirmed in the biological formation of formaldehyde and dimethylamine from trimethylamine oxide. The cofactor is of heat-tolerant nature and occurs not only in the tissue of cod-like fish, but also in the shark liver not having the enzyme concerned.

Literature

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