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Title	Cysteamine as a metabolite in marine mollusc, Mytilus edulis
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Citation	北海道大學水産學部研究彙報, 18(2), 88-94
Issue Date	1967-08
Doc URL	https://hdl.handle.net/2115/23307
Type	departmental bulletin paper
File Information	18(2)_P88-94.pdf

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Cysteamine as a metabolite in marine mollusc, *Mytilus edulis*

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Introduction

The metabolism of sulfur amino acids and sulfur amines has been studied in mammals. Generally, taurine formed as the end product from methionine or cysteine via cysteine sulfinic acid and hypotaurine. But hypotaurine was also formed enzymatically from cysteamine by D. Cavallini[1] in 1966. There are hardly any reports in marine invertebrates, although a large amount of taurine[2] or a considerable amount of hypotaurine[3] are found in some molluscs. J. Awapara[4] reported that methionine was converted to taurine *in vivo* by using an isotope in *Mytilus edulis*, but taurine was observed only in a small amount. But in molluscs no report has been published on cysteamine as the metabolite of the system.

To study from what substances taurine can be formed in molluscs, cysteine and cysteamine were compared as substrate in these systems with an enzyme prepared from muscle ligaments of *Mytilus edulis*. The amounts of the thiol group of cysteine and cysteamine during the incubation were determined sepctrophotometrically with N-ethylmaleinimide (NEM), using the method of E. Roberts and G. Rouser[5]. The results of these experiments show that cysteamine is more useful as a substrate than cysteine under the same conditions. The products of the experiment which reacted positively to ninhydrine and iodoplatinate, have not been strictly identified. This is only a preliminary experiment on thiol metabolism in molluscs.

Experimental

Animals and Materials

The marine molluscs, *Mytilus edulis*, which were collected at Nanaehama, Kamiisocho, Hokkaido in the winter of 1966–1967, were used in this experiment. N-Methylmaleinimide (NEM) was purchased from the Aldirch Chemical Co. Inc. Milwauckee, Wis., USA. Cysteamine hydrochloride was a product of the Tokyo Chemical Industry Co., Ltd. and cysteine was obtained from the Takeda Pure Chemicals, Ltd. All other compounds were commercial products.

The spectrophotometric determination of thiols by NEM was reproduced under the same conditions described by Roberts and Rouser.

Methods

The ligaments muscles of the animals were homogenized with 10 volumes of 0.05 M Tris buffer (pH 7.4) and centrifuged at 3000 r.p.m. for 10 minutes in a cold

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chamber at 4°C. The supernatant, which was kept at 0°C or stored at −20°C until the experiment, was used as the enzyme. Incubations were carried out in test tubes at 37°C. The standard reaction mixture contained 0.25 ml of 2.0×10^{-2} M cysteine or cysteamine hydrochloride and 0.10–0.20 ml of enzyme solution, and the final volume was 2 ml with water. In the case of cysteamine, methylene blue and sulfide were used following to the method reported by D. Cavallini[6]. The test tubes were shaken violently about once in fifteen minutes during incubation. The reaction was stopped by addition of equivolume of 3.0×10^{-3} M NEM solution of which medium was 0.5M phosphate buffer at pH 6.0. The reaction ceased because the residual thiol compounds were immediately trapped by NEM. Standing for one hour, the degenerated protein with excess NEM had separated as sediment and the absorbancy of the solution at 300mμ was measured by Shimadzu's Beckman spectrophotometer QV 50.

As for an assay of the products, the reaction mxiture contained 1.0 ml of 2.0×10^{-2} M cysteamine and 0.8 ml of enzyme solution and 0.2 ml of water. It was incubated again in the same way as described above. After incubation the reaction was stopped by the addition of 0.5 ml of 50% trichloroacetic acid, and the precipitate was centrifuged off. The supernatant was shaken three times with an equal volume of ether to remove the excess trichloroacetic acid. After being alkalinized with 0.1 ml of 2N ammonia the solution was shaken with about 0.3 g of dry Amberlite IRC–50 hydrogen form then a part of the cysteamine was removed. In this we followed the methods of D. Cavallini and his co-workers[7]. Aliquots (40μl) of the liquid were spotted on a strip of Toyo No. 51 filter paper and electrophoresis was then carried out at pH 3.1 in pyridine-acetic acid-water for 40 min. at 1.0 mA/cm. After becoming dry the strips were dipped in ninhydrine solution (0.2% in acetone) and then dried again. Next, these strips were dipped in melted paraffin (mp 46–48°C) and a semi-quantitative assay was carried out by a densitometer, the Toyo Direct Reading Densitometer. More over, to detect the sulfur reducible compounds the dried strips were sprayed with iodoplatinate reagent.

Results

Spectrophotometric determination of thiol

The use of NEM for quantitative spectrophotometric detection of thiols gave good results. The starting solution contained 6.0 μmoles NEM in 4.0 ml of 0.25 M phosphate buffer (pH 6.0). The specific absorption of NEM at pH 6.0 and changes of the absorbancy by adding the thiol compound as determined by the Shimadzu Autorecording Spectrophotometer SV 50 are shown in Fig. 1. The relationship between the concentration of thiol compound and the absorbancy at 300mμ is shown in Fig. 2.

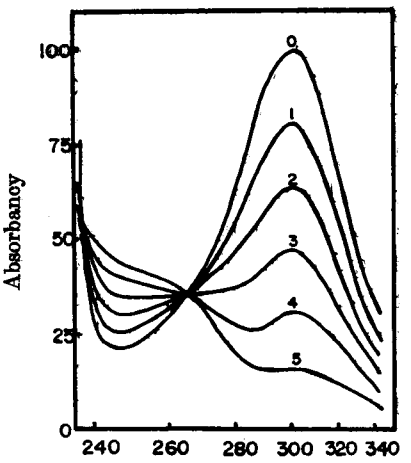

Fig. 1. Specific absorption and decrease of absorbancy at 300 mμ. with the addition of cysteine.
0, 6.0 μmoles of NEM only.
1, 6.0 μmoles of ENM plus 1.0 μmoles of cysteine.
2, 6.0 μmoles of NEM plus 2.0 μmoles of cysteine.
3, 6.0 μmoles of NEM plus 3.0 μmoles of cysteine.
4, 6.0 μmoles of NEM plus 4.0 μmoles of cysteine.
5, 6.0 μmoles of NEM plus 5.0 μmoles of cysteine.

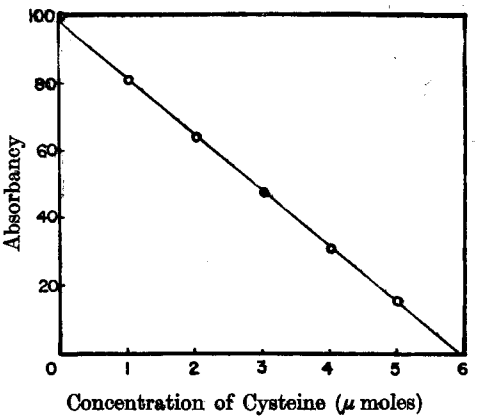

Fig. 2. Standard graph of cysteine. Decrease in absorbancy of NEM as a function of the amount of cysteine. Absorbancy: -log $T \times 10^2$ at 300mμ. Medium: 0.25 M phosphate buffer pH 6.0.

Changes in cysteine during incubation

In order to estimate the influence of the substrate and the enzyme solution on the absorbancy at 300mμ of NEM, two blank tests were carried out as shown in (A) and (B) of Table 1 and the difference of the values was negligible. Another blank test (C) was carried out to check the value of the absorbancy of the reaction

Table 1. Composition of blank test Cysteine solution, 2.0×10^{-2}M cysteine; Buffer, 0.5M phosphate buffer pH 6.0; NEM solution, 3.0×10^{-3} M NEM (dissolved in the buffer). Enzyme solution, the supernatant of 10% homogenate of fresh muscles.

	Incubation mxiture (ml)			Added solution
	Water	Cysteine solution	Enzyme solution	
(A)	1.65	0.25	0.10	2.00 ml of Buffer
(B)	1.90	0	0.10	2.00 ml of Buffer
(C)	1.90	0	0.10	2.00 ml of NEM solution

Table 2. Changes in cysteine determined by NEM. The composition is same as Table 1 (A) and the conditions of incubation are detailed in text.

	Time (min)	Absorbancy at 300 mμ	Residual cysteine (μmoles)
(A)		0.000	5.00
(B)		0.008	4.94
	0	0.018	4.87
	2	0.015	0.88
	8	0.027	4.81
	15	0.042	4.71
	30	0.043	4.70
	60	0.046	4.68
	90	0.048	4.66
	120	0.045	4.69
	180	0.060	4.57
	240	0.098	4.30
(C)		0.689	0.00

mixture when the substrate was absent.

The decrease of cysteine was observed and the result is shown in Table 2 and the change in the amount of cysteine is shown in Fig. 3. In the first 30 min. the cysteine decreased about 4%, in the next 90 min. the value did not change and in the next 120 min. the substrate decreased 8% more; consequently the cysteine decreased only 12% in all.

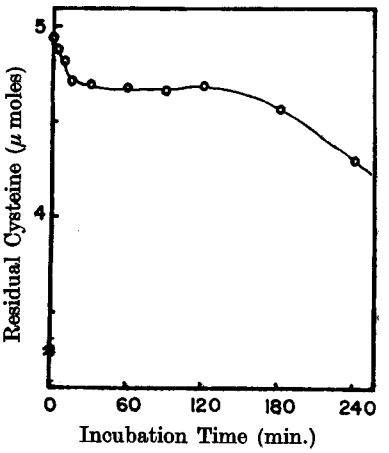

Fig. 3. Changes in cysteine during incubation. The standard assay system contained 0.1 ml of enzyme solution of fresh ligament muscle.

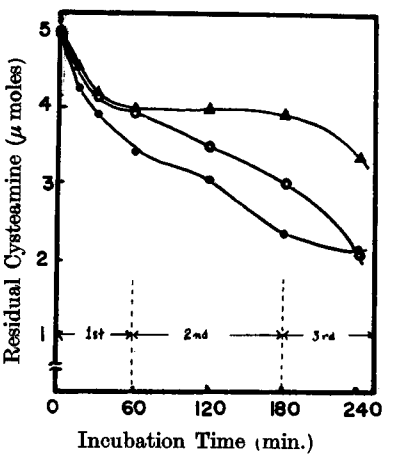

Fig. 4. Changes in cysteamine during incubation. The standard assay system contained 0.2ml of enzyme solution of fresh ligament muscle (-o-o-); 0.05 μmoles of methylene blue contained (-•-•-); 0.60 μmoles of sodium sulfide contained (-▲-▲-).

Changes in cysteamine during incubation

In this experiment methylene blue and sodium sulfide were employed as cofactor like substances as shown in the hypotaurine forming system described by D. Cavallini[6]. The results fall into three stages as shown in Fig. 4 and some different reactions seemed to take place in each stage. Methylene blue activated the decrease of cysteamine at the first and second stages. Sodium sulfide disturbed the reaction at the second stage but not at the first or third stage. However, the reaction continued without cofactor like substances, and the cysteamine had decreased about 60% after 240 min. of incubation.

With preserved enzyme solution, the cysteamine decreased a very small amount in the first 60 min. but in the next 180 min. the decrease was appreciable (Fig. 5), during the 240 min. incubation the cysteamine decreased about 90%.

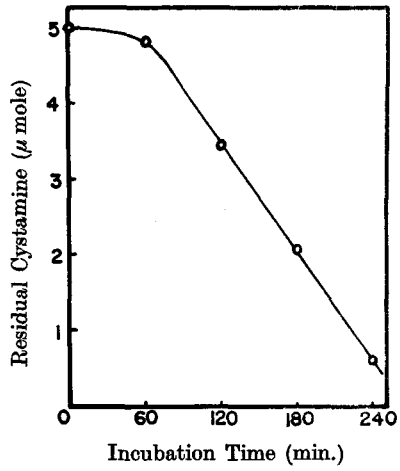

Fig. 5. Changes in cysteamine during incubation. The standard assay system contained 0.2 ml of enzyme solution which was preserved at -20°C for one week. No cofactor like substances were used.

Identification of the products of the reaction

In order to examine the products of the reaction, semi-quantitative comparison was carried out paper electrophoretically as followed the description in the method. The results are shown in Fig. 6 and Fig. 7.

In this experiment reducible substances, not belong to thiol, clearly increased during the incubation (Fig. 7). One of the unidentified compounds was located at the same position as taurine (about 4 cm from origin) and became purple when ninhydrine reagent was applied, and the other was about 6 cm from origin and became yellow in this experiment (Fig. 6).

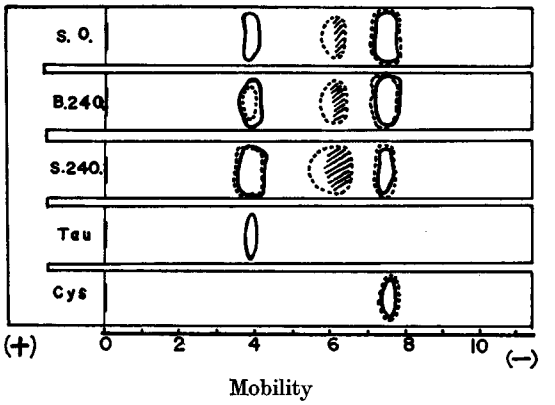

Fig. 6. Electrophoretic comparison of reaction products. Electrophoresis at 1 mA/cm for 40 min. under 0–4°C. Standard assay system contained 20 μmoles of cysteamine and 0.8 ml of enzyme solution of fresh ligament muscle. S.O., standard without incubation; B. 240., boiled enzyme was used and 240 min. incubation; S. 240., standard 240 min. incubation; Tau, taurine; Cys, cysteamine. Full line, ninhydrine positive (shadow, yellowcolor). Dotted line, iodoplatinate positive.

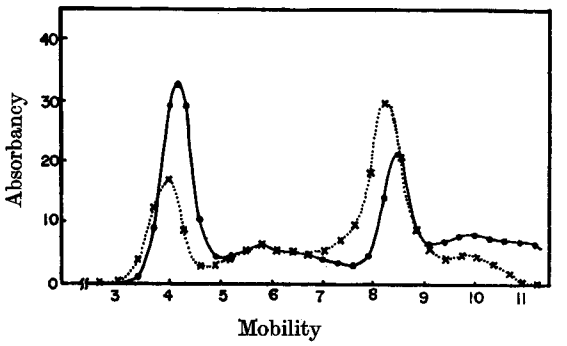

Fig. 7. Semi-quantitative comparison of the reaction products by densitometer. Full line, S. 240. and dotted line, S.O. in Fig. 6. Absorbancy, -log $T \times 10^2$ at 570 mμ. correspondence to the concentration of products.

Discussion

For determining thiol compounds, high reproducibility was obtained from some experiments by using NEM, which is a very useful method for the purpose of this investigation. With this method the rates of decrease of cysteamine and cysteine during incubation were compared and as a result it was found that the cysteamine had decreased by a much larger amount. Cysteamine is more changeable during incubation than cysteine. It is impossible to explain from this experiment that the difference in the decrease patterns of cysteamine in fresh enzyme solution and in preserved (Fig. 4 and Fig. 5), but perhaps freezing or keeping it at –20°C for one

week effected the decrease pattern. Methylene blue and sulfide, as cofactor like substances for hypotaurine forming system, were not indispensable, although some effects on the pattern of decrease were observed. From the assay of the products of the reaction, the amount of unidentifide compounds increased during incubation but not without incubation. A slight increase in the reducible compounds also occured when the enzyme solution was boiled.

Consequently, it is concluded that cysteamine is a metabolite, at least cysteamine has some specific physiological activity in the ligament muscle of *Mytilus edulis*. The substances detected after 240 min. of incubation must be derivertives of cysteamine and may be sulfinic compounds or thiosulfonic compounds derived from cysteamine. In other to identify those products, further experiments are needed.

Summary

1. NEM was used to detect the decrease of cysteamine and cysteine in *Mytilus edulis* preparation.

2. Cysteamine was more changeable than cysteine during incubation.

3. Two reducible compounds were detected with ninhydrine and iodoplatinate after 240 min. of incubation. One of them shows purple and the other shows yellow when ninhydrine reagent is applied.

4. Methylene blue and sulfide were not indispensable in this experiment, but they seemed to have some effect on the process and to change the patterns of the decreasing graph.

5. From the results of the above experiments it seems that cysteamine is a metabolite in ligament muscle of *Mytilus edulis*.

References

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