ISOLATION OF A VITAMIN B_{12}-REPLACING FACTOR FOR *OCHROMONAS MALHAMENSIS* AND ITS IDENTIFICATION AS 5'-METHYLTHIOADENOSINE

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1. Introduction

It has been known that *Ochromonas malhamensis* exhibits a similar specificity in vitamin B_{12} requirement to animals [1]. Methionine has been demonstrated to spare the vitamin B_{12} requirement partially, but not to replace the vitamin completely [2]. During the course of our studies on the metabolic roles of vitamin B_{12} in *Ochromonas malhamensis*, we have found that an unidentified vitamin B_{12}-replacing factor was contained in commercial yeast extract powder. This communication reports the isolation of the vitamin B_{12}-replacing factor. The results of qualitative tests, ultraviolet- and mass spectrometry, microbiological activity for the protozoan and other tests confirmed the identity of the factor with 5'-methylthioadenosine.

2. Materials and methods

The yeast extract powder used in this study was purchased from Daigo Nutritive Chemicals, Osaka, Japan. Cyanocobalamin was obtained from Glaxo Lab., Greenford, UK. 5'-Methylthioadenosine was prepared by degradation of S-adenosylmethionine according to the procedure of Schlenk and DePalma [3]. 5-Methylthioribose was prepared by acid hydrolysis of 5'-methylthioadenosine according to the method of Smith and Schlenk [4]. Other materials were reagent-grade commercial products and used without further purification.

Ochromonas malhamensis (Pringsheim strain) was supplied by Prof. T. Kamikubo, Hiroshima University, Japan. The original strain was provided from Prof. K. Bernhauer, Stuttgart University. The protozoan was maintained under illumination at 20–30°C in a set of flasks containing 2 ng of cyanocobalamin and the components of Ford medium [1] in a total vol of 10 ml. The culture was transferred every 4 days. The vitamin B_{12}-replacing effect was estimated by a similar method to that of the microbiological assay of vitamin B_{12} by *Ochromonas malhamensis* [1].

Acid- or alkali-treatment of the factor was carried out as follows: Three mg of the factor dissolved in 1 ml of 0.1 N HCl was heated in a sealed tube for 2 hr in a boiling water bath or the same amount of the factor dissolved in 0.1 N NaOH was incubated for 30 min at 30°C. After neutralization, these hydrolyzates were used for the subsequent experiments.

3. Results and discussions

Commercial yeast extract powder (20 g) was dissolved in 40 ml of water. The solution was applied to a Sephadex G-10 column equilibrated previously with water, and then the column was washed with a large volume of water. Elution of the vitamin B_{12}-replacing factor (referred to as Factor X) was achieved with 0.1 N NH_4OH. The eluate was evaporated to dryness in vacuo. Further purification was performed with P-cellulose (H^+) and TEAE-cellulose (OH^-). Since Factor X was easily adsorbed on P-cellulose (H^+) and not on

TEAE-cellulose (OH^-), separation of the factor from contaminants was achieved by the use of these ion-exchangers. The basic fractions containing Factor X were collected, evaporated to dryness in vacuo and the resulting residue was dissolved in a small amount of hot ethanol. The ethanol solution was applied to a Silicic acid column equilibrated with hexane. Factor X adsorbed on the column was eluted with ethanol. The eluate was concentrated to a small volume. Final purification of the factor was carried out by thin-layer chromatography on 0.75 mm-layer of Silica GF_{254} (E. Merck AG, Darmstadt, F. R. Germany). The zone of Factor X corresponded to the highest UV-absorbing zone. Its R_f value was 50–70 when Solvent A (ethanol–chloroform (1:3, v/v)) was used as developing agent. After extracted with water, the factor was crystallized from the resulting aqueous solution, yielding white needles. About 18 mg of crystals was obtained from 20 g of the yeast extract powder used as starting material.

The purity of the crystals was examined by thin-layer chromatography. When Factor X was developed on a Silica GF_{254} plate with the following solvent systems, a single UV-absorptive spot was observed on each chromatogram. The R_f values were as follows: Solvent A, 53; Solvent B (1-butanol–acetic acid–water (60:15:25, v/v)), 64; Solvent C (ethyl acetate–2-propanol (65:35, v/v)), 50; Solvent D (ethanol–acetic acid–water (75:5:25, v/v)), 70. On exposure of the plates to iodine vapor, a single spot was detected at the same position as that of Factor X on each chromatogram.

Chemical properties of the factor is summarized in table 1. The factor contained a sugar moiety, but not a deoxyribose. It also contained sulfur. But neither phosphorus, halogen, amino group nor carbonyl group was involved. Factor X melted at 204–205°C, leaving a brown coloured liquid. The melting point resembled those of nucleosides. These results indicated that Factor X would be a sulfur-containing nucleoside derivative. In order to elucidate the structure of Factor X, some chemical and physical analyses were performed. The result of elemental analysis was as follows:

	$C_{11}H_{15}O_3N_5S$
Calculated:	C 44.43, H 5.08, N 23.55, S 10.78
Found:	C 44.21, H 5.04, N 23.50, S 10.80

Its molecular weight was 297 as judged from the mass spectrum analysis. The chemical formula was similar to the empirical one. The results of NMR and IR studies showed that Factor X contained a terminal thiomethyl group. After the treatment with acid or alkali, the resulting hydrolyzates were chromatographed on a Silica GF_{254} plate with Solvent A and B, respectively (table 2). The factor was stable in basic conditions, but not in acidic conditions. When treated with acid, it was perfectly split into new compounds. The R_f values of these products were 32 and 66 in Solvent A, respectively. The spot of R_f 32 absorbed light in the ultraviolet region and showed negative responses toward sugar- and sulfur-tests. The other spot of R_f 66 did not absorb light in the ultraviolet region and was positive to sugar- and sulfur-tests. These spots of R_f 32 and 66 coincided with authentic adenine and 5-methylthioribose, respectively. The-

Table 1
Some chemical properties of Factor X

Reagent	Test for	Occurrence of reaction
Orcinol	Sugar	+
Anisaldehyde	Sugar	+
Diphenylamine	Deoxyribose	–
Platinic iodide	Sulfur	+
Molybdate	Phosphorus	–
Beilstein reagent	Halogen	–
Ninhydrin	Amino group	–
Dinitrophenylhydrazine	Carbonyl group	–

Table 2
Properties of the hydrolyzation products of Factor X

Compound	R_f value[a]	UV-absorption	Sugar test[b]	Sulfur test[c]
Factor X	53	+	+	+
Hydrolyzates	32	+	–	–
with acid	66	–	+	+
Incubation product with alkali	53	+	+	+

[a] R_f values on the thin layer chromatogram on Silica GF_{254} developed with Solvent A (ethanol–chloroform, 1:3 v/v).
[b] See Table 1.
[c] See Table 1.

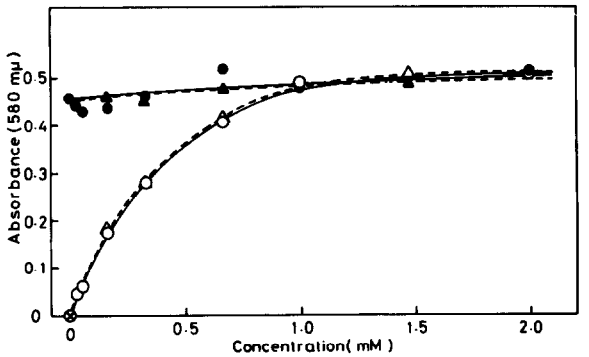

Fig. 1. Effects of graded amounts of Factor X and of 5′-methylthioadenosine on the growth of *Ochromonas malhamensis* in the presence and absence of vitamin B_{12}. Assay media contained the indicated amounts of 5′-methylthioadenosine or Factor X with or without addition of vitamin B_{12} (0.2 ng cyanocobalamin per ml of media). Some details were described in the text. (○——○) 5′-methylthioadenosine; (△--△) Factor X; (●——●) 5′-methylthioadenosine plus vitamin B_{12}; (▲--▲) Factor X plus vitamin B_{12}.

se results indicated that Factor X would be 5′-methylthioadenosine.

The R_f values of authentic 5′-methylthioadenosine on Silica GF_{254} plates developed with Solvents A and B were in good agreement with those of Factor X. Fig. 1 shows the effects of graded amounts of Factor X and of authentic 5′-methylthioadenosine on the growth of *Ochromonas malhamensis* in the presence or absence of saturated level of vitamin B_{12}. Namely, the vitamin B_{12}-replacing effect of Factor X was nearly equal to that of 5′-methylthioadenosine. Thus, the vitamin B_{12}-replacing factor isolated from commercial yeast extract powder was identified as 5′-methylthioadenosine. In the presence of 1 mM 5′-methylthioadenosine, vitamin B_{12} did not exhibit any effect on the growth of *Ochromonas malhamensis*. In other words, 1 mM 5′-methylthioadenosine replaced the vitamin B_{12} requirement of the protozoan completely.

The results presented here would provide interesting information on metabolic role(s) of 5′-methylthioadenosine in *Ochromonas malhamensis*, especially on the interrelationship to the function of vitamin B_{12} in the protozoan. Studies on the physiological activity of 5′-methylthioadenosine have been reported with mammals [5] and a protozoan, *Endamoeba histolytica* [6], but biochemical elucidation on the metabolic implication of the compound has not been performed as yet. Only Schlenk et al. [7,8] have described that 5′-methylthioadenosine served as a methyl donor by yielding S-adenosylmethionine in growing cells of *Candida utilis*. However, we have found that the growth supporting activity of S-adenosylmethionine for *Ochromonas malhamensis* was lower than that of 5′-methylthioadenosine. Moreover, we have found that 5′-methylthioadenosine was not utilized by *E. coli* 215, a vitamin B_{12} or methionine auxotroph. Taking into consideration that the protozoan has a similar specificity in vitamin B_{12} requirement like mammals, the uptake and metabolic fate of 5′-methylthioadenosine in *Ochromonas malhamenis* is now under investigation.

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