hydrolysis as used to treat the purified protein, a hydrolysis product appears which co-migrates with the unidentified methylated amino acid (figures 1 and 2). There may be an isotopically labeled product of S-adenosyl methionine which is specifically incorporated into myosin and the L-10 ribosomal protein[4], or S-adenosyl methionine may be a nonspecific contaminant which is then acid-hydrolyzed with the purified protein. The latter seems unlikely since in the case of the L-10 ribosomal protein[4] it is recovered in repeatable stoichiometric amounts[4]. Nevertheless, we believe we have partially identified this methylated amino acid which co-migrates with the neutral amino acids.

We also show here that the methylated amino acid, trimethyllysine, present in myosin, is isotopically labeled following 10 days of tissue culture of fetal canine cardiac cells when myosin has been shown to be present[2].

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Active transport of [^{32}P]thiamine diphosphate in *Escherichia coli*[1]

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Summary. Active uptake of [^{32}P]thiamine diphosphate by *E. coli* was analyzed using an improved method of gel filtration chromatography. The radioactive coenzyme was accumulated without dephosphorylation. From this result it was concluded that thiamine kinase is not involved in the membrane transport of thiamine in *E. coli*.

In the course of our study on the mechanism of thiamine (T) uptake by bacterial cells[4,5], we have noticed that in *Leuconostoc mesenteroides* less than 30% of the actively-accumulated T is phosphorylated[6]. In the active transport of T by *E. coli*, on the other hand, intracellular T is phosphorylated almost quantitatively, and it has been claimed that T kinase is involved in the membrane transport[7,8]. It has also been shown in *E. coli* that thiamine diphosphate (TDP) shares the same route of membrane transport with T. This is based on the following observations; 1. that growth on TDP of a mutant of *E. coli*, which requires TDP for its growth (70-23-107), is competitively inhibited by T[9]; 2. uptake of [^{14}C]T by washed cells of *E. coli* in a specified reaction mixture is inhibited by TDP competitively[10]; and 3., binding of [^{14}C]T by T-binding protein (which is supposed to be an essential component of T uptake system) is shown to be inhibited by about 50% in the presence of 1:1 amount of TDP to [^{14}C]T[4]. If T kinase was involved in T uptake by *E. coli*, TDP should be dephosphorylated before the membrane transport[11]. In this paper we describe evidence that T kinase is not involved in T uptake, using the technique of a gel filtration chromatography[12] for the determination of T phosphates. The determination of T phosphates has been improved, and [^{32}P]TD can be separated from T triphosphate (TTP) and more highly phosphorylated T.

Materials and methods. Synthesis and purification of [^{32}P]TDP. The method described below has been developed and shown to be applicable for the synthesis of [^{32}P]TDP and [^{32}P]TMP. 40 µl of 85% H_3PO_4 was added to 10 mCi of carrier free [^{32}P]H_3PO_4 and heated on a small flame for circa 10 min. 70 mg of P_2O_5 was then added and the mixture kept in a 150 °C oil bath for 10 min. This was then cooled down to 120 °C and 25 mg of T HCl was added to it[13]. The reaction mixture was kept at 120 °C for 15 min and then cooled down to room temperature. 10 ml of 99.5% C_2H_5OH (4 °C) was added and the reaction mixture kept at 4 °C for 2 h. After decantation, the residue was washed again with 10 ml of ethanol and then twice with 10 ml of ethyl ether. The residue was now dissolved in 0.1 ml of 20 mM β-mercaptoethylamine (MEA) at 4 °C and precipitated twice with 10 ml of ethanol. This ethanol precipitation was repeated 2 more times from 0.2 ml of 0.2 N HCl containing MEA. The precipitate obtained was dissolved in 2 ml of 0.3 M potassium phosphate buffer at pH 5.2 containing 5 mM MEA and 1 mM NaCl, and applied to a column of Sephadex G-25 (1.35 × 195 cm) pre-equilibrated with 0.3 M potassium phosphate buffer, pH 7.5, containing 5 mM MEA and 1 mM NaCl. The column was developed using the same buffer at about 15 °C in a dark room, at the rate of 8 g/h. One peak fraction of TDP was selected on the basis of the radioactivity and absorption ($A_{233\ nm}$) maxima of the column eluate. 0.17 ml of 99.5% ethanol was added to each 4-g fraction and the sample stored at −80 °C. A 0.8-ml aliquot of the selected fraction was applied to filter paper (30 cm wide × 60 cm long, Toyo No. 50) which had been soaked in 0.3 M potassium phosphate buffer, pH 5.2, containing 5 mM MEA and 1 mM NaCl (this was also used as the electrode buffer). Electrophoresis was carried out for

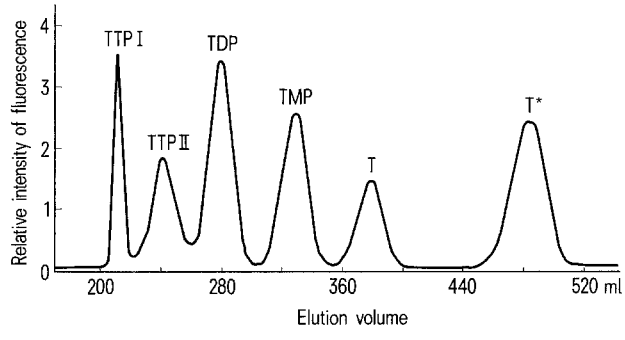

Fig. 1. Separative determination of T and T phosphates on Sephadex G-25. A 1.5-ml sample containing 10 µg of TTP, TDP, TMP, and T was oxidized by BrCN and Nagoh, neutralized and developed as described under 'methods'. The fraction with the maximum fluorescence in TTP I was re-chromatographed in the same position as TTP II. The column was developed at a flow rate of 8 g/h and fractionated into 4-g fractions. Standard thiochrome solutions for the determination of recoveries were prepared at the same time as the sample was prepared, and stored in the dark during the development of the column. * In $K_3Fe(CN)_6$-NaOH oxidation method, this peak is absent.

3 h at 4 °C at the voltage of 1.5 kV (120 mA at the start). After the run, the paper was monitored for UV absorbing zones. [^{32}P]TDP, which had moved 6–9 cm toward the anode, was cut out and eluted with 6–10 ml of 5 mM MEA at 4 °C to get ca. 80% elution (circa 2 h). Ethanol was added to 4% (v/v) and the sample was stored at −80 °C. 18–21 μCi of [^{32}P]TDP (1.8 μmoles) was obtained from the 4-g fraction of Sephadex eluate. The radiochemical purity was analyzed by paper chromatography, thin layer chromatography, and high voltage paper electrophoresis and always found to be 93% or above.

Bacterial strains. A mutant of *E. coli* W 70-23, 70-23-107, was isolated and characterized by H. Nakayama and R. Hayashi[14] and donated to us by H. Nakayama. *E. coli* strain 70-23 lacks phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7) and strain 70-23-107 lacks T monophosphate kinase (EC 2.7.4.16) as well as having the defect of 70-23.

Gel filtration analysis of T and its phosphates. T and its phosphates were first oxidized to give their fluorescent derivatives or thiochrome and thiochrome phosphates by BrCN and alkali. 1.5 ml of BrCN (Br_2-saturated H_2O was titrated with 10% KCN to the point of decolorization) and 1.0 ml of 20% NaOH were added successively to about 3 ml of a sample which contained 3 nmoles or more of each of TTP, TDP, TMP, and T, or one of them, in 0.1 M sodium acetate buffer, pH 4.5. TTP was a generous gift of Dr T. Yusa of Sankyo Co., Tokyo. The reaction mixture was neutralized to pH 7 with 6 N HCl and applied to a column containing Sephadex G-25 (1.35 × 195 cm), which had been equilibrated with 0.3 M potassium phosphate buffer pH 7.5 (standard buffer), and the column was developed with the standard buffer. The column and fraction collector were shielded from light by covering them with black paper.

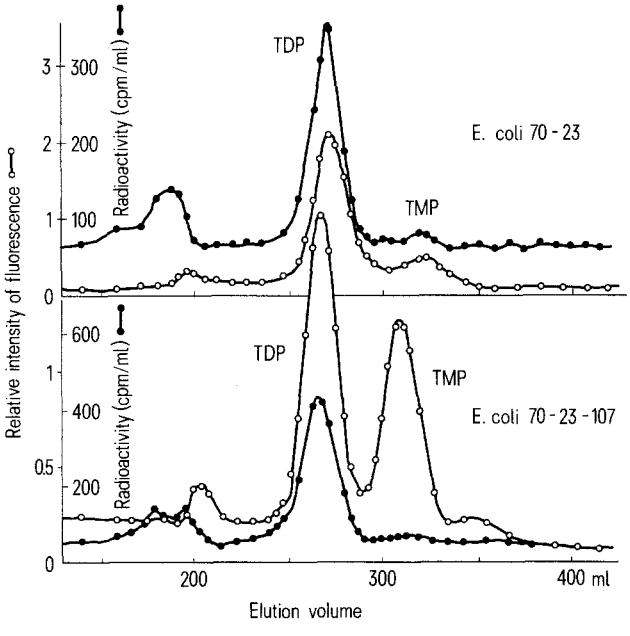

Fig. 2. Gel filtration analysis of [^{32}P]TDP accumulated in *E. coli*. The uptake-reaction mixture containing 2.9 mg dry weight of cells/ml, 0.32% glucose, 0.24 M Tris-HCl buffer of pH 7.4 (total 5.0 ml/sample) was preincubated at 37 °C for 5 min. [^{32}P]TDP was then added to the concentration of 3×10^{-6} M. After 15 min of shaking at 37 °C, 15 ml of 0.25 M Tris-HCl of pH 7.4 was added and poured immediately onto 0.45 μm millipore filter. The cells collected on the filter were extracted with 7.0 ml of 20 mM sodium acetate buffer of pH 4.5 at 100 °C for 3 min and then at 70 °C for 10 min. After centrifugation (6000 × g for 5 min at 4 °C), the clear cell extract was concentrated under vacuum below 50 °C to 0.5 ml. This concentrate was oxidized and applied to the gel column. Specific activity for the TDP fractions combined was calculated as 11.7 and 12.4 μCi/μmole in *E. coli* 70-23 and 70-23-107, respectively. The specific activity of [^{32}P]TDP used was 10.8 μCi/μmole. These 3 values are supposed to be within the technical error. The amounts of TDP, TMP, and T by fluorometry were 0.208, 0.041, 0 and 0.150, 0.173, and 0 (nmole/mg) for *E. coli* 70-23 and 70-23-107, respectively.

Results. Effect of gel and pH on the separation of T and its phosphates in gel filtration analysis. Before we decided on the final procedure described in materials and methods' section, we tested the following gel materials: Sephadex G-15 (fine), G-25 (fine, superfine), G-50 (fine), and Bio-Gel P-6. The best result was obtained with Sephadex G-25 (fine). Sephadex G-15 gave incomplete separation of TDP and TTP, G-50 gave nearly similar elution volumes for the types of T samples, and Bio-Gel P-6 gave partial separation of 3 peaks of TTP, TDP, and TMP in a very small elution volume (between 135 ml and 165 ml in a 1.35 × 145 cm column). G-25 (superfine) gave a slower flow rate with no improvement of separation between peaks. The effect of pH of the developing buffer was tested in Sephadex G-25 (fine) columns. TDP was eluted in 2 peaks when it was developed with 0.2 M potassium phosphate buffer of pH 6.6. The 2 peaks were shifted and fused in the former or in the latter when eluted with more acidic or more alkaline buffer, respectively.

Anomaly in the elution pattern of TTP in gel filtration analysis. TTP was eluted in 2 peaks when certain amounts of other thiochromes were present in the same sample (figure 1). The main factor influencing the separation of TTP was the amount of coexisting thiochromes. The concentration of buffer had only a minor effect. The amount of the faster eluting peak increased in proportion to the amount of co-chromatographed thiochromes up to 3 times that of thiochrome triphosphate. A competition of thiochrome triphosphate with other thiochromes for a limited number of binding sites on the gel matrix could be a reason for the separation. Both peaks of TTP were re-chromatographed in the same position as that of the slower moving peak (240 ml). The above mentioned separation of TTP peaks could be minimized when the column was eluted with a high ionic strength buffer (e.g. the standard buffer).

Analysis of model samples by the gel filtration method. Sample solutions (1.5 ml) which contained the 4 thiochromes (10 μg of each) were analyzed and a typical result is shown in figure 1. Average recoveries with the standard deviation and the range for TTP, TDP, TMP, and T were 101.2 ± 5.36 (93.2–108), 101.5 ± 5.01 (96.0–110), 97.1 ± 4.07 (94.0–104), and 106.9 ± 7.10 (99.3–117), respectively, in 6 experiments. Samples (1.5 ml) containing 1 μg of the 4 compounds were also analyzed and nearly the same results were obtained. However, even the slightest turbidity in the developing buffer (microorganisms, gel matrix, etc.) disturbed the analysis. Hence the fluorometer readings for the fraction which contained no thiochrome were routinely measured and subtracted from the readings of each fraction. The 2 peaks of free T were concluded to be 2 different compounds[6]. When the sample was oxidized by alkaline $K_3Fe(CN)_6$, the free T was eluted in 1 peak only at circa 380 ml.

Active accumulation of [^{32}P]TDP without dephosphorylation. [^{32}P]TDP was incorporated into *E. coli* cells, which required T (70-23) or TDP (70-23-107) for its growth, against the concentration gradient. The accumulated TDP was analyzed by the gel filtration method. The result (figure 2) suggests that, in *E. coli* strain 70-23, TDP was incorporated without dephosphorylation, but in *E. coli* strain 70-23-107 part of the [^{32}P]TDP was dephosphorylated first and then monophosphorylated with cold phosphate

(the non-radioactive TMP peak) because this mutant cannot phosphorylate TMP to TDP. This process is, however, not essential for the uptake of TDP by this strain, hence the specific radioactivity of [^{32}P]TDP was not reduced during the membrane transport ([^{32}P]TDP peak of the figure, strain 70-23-107).

Discussion. Kawasaki and Yamada[15] attempted to demonstrate the uptake of free T by *E. coli* using a T kinase defective mutant. In their mutant, however, the active uptake of T occurred only during 2–3 min after the start of reaction. On the basis of their data they considered the negative charge on TDP to be important for the accumulation of intracellular T. Hence the participation of T kinase in T uptake by *E. coli* has not completely been excluded. But the data presented in this report, showing that TDP is transported into *E. coli* strain 70-23 without the participation of T kinase, exclude this uptake system from the scope of so called 'group translocation'. The accumulation of cold TMP in strain 70-23-107 could possibly be explained by the preliminary data that the activity of alkaline phosphatase is enhanced to some extent in this strain. As alkaline phosphatase is known to be localized in the periplasmic space TDP is possibly dephosphorylated by this enzyme outside the cell membrane. In addition to studies of uptake, a method for the synthesis of [^{32}P]TDP and [^{32}P]TMP with high specific radioactivity is described in this paper. The activities obtained were 10 and 5 mCi/mmole for [^{32}P]TDP and [^{32}P]TMP, respectively.

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Isolation of the two paragonial substances deposited into the spermatophores of *Acanthoscelides obtectus* (Coleoptera, Bruchidae)

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Summary. Aqueous extracts from spermatophores of *Acanthoscelides obtectus* (Coleoptera, Bruchidae) have been chromatographed on Sephadex G 25 and G 15 columns, the active fractions being further purified by preparative SiO_2 TLC. The isolation and properties of 2 biologically active compounds are reported. The paragonial substance A has a favourable action upon oogenesis, is a low molecular weight molecule (500–1000) and gives 1 single amino acid on hydrolysis, corresponding to glycin on TLC. The paragonial substance B is toxic, or an inhibitor of oogenesis at lower concentration, thus showing an antagonistic effect.

Previous research[2-5] has shown that male secretions produced by one of the accessory glands (tubular glands) of the Coleopterae (Bruchidae) *Acanthoscelides obtectus* influence female reproductive activity. These products, the so-called paragonial substances[3], are deposited into the spermatophores during mating and migrate into the hemolymph some h later. The isolation of the crude active compounds[3] was achieved from aqueous extracts of spermatophores, collected at the end of copulation. It has been possible to isolate 2 paragonial substances, substance A which stimulates oogenesis, and substance B which on the contrary inhibits oogenesis and is toxic at higher concentrations. In the present work, which involved different fractionations of the extracts from some 8000 spermatophores obtained through dissection, we report the final isolation of the 2 chromatographically homogeneous products, which should later on permit the determination of their chemical nature.

The experiments were carried out on spermatophores collected by dissection of the female abdomen, just after copulation. They were immediately extracted with water at 20–40 °C, followed by vacuum concentration. Sephadex G 25 coarse, G 25 superfine and Sephadex G 15 were successively used for chromatographic fractionation. Aliquots from each fraction were taken and applied to SiO_2 TLC for control. After developing with butanol-acetic acid-water 4:1:1, staining with ninhydrin at 100 °C (1% in acetone in spray) the active compounds were looked for in connection with previous results[3] before the activity was tested. Column chromatography was performed with help of an automatic Elugraph apparatus with double recording of the absorbance at 220 and 280 nm. For preparative TLC, SiO_2 plates of 0.25 mm thickness were self-made from Merck HR silica gel, following the usual method. For analytical TLC, commercial plates (Merck) were used directly. Final purification steps were accomplished through repetition on previously washed plates, using acetone and water for washing before development. Finger printing of purified samples was carried out by 2-dimensional TLC developing with butanol-acetic acid water 4:1:1 and then 70% propanol in water.

For the biological activity determination, the purified products were dried under vacuum and dissolved in physiological serum before injection in the abdomen of 4-day-old virgin females, each receiving 1 µl of solution. After injection, the females were reared in presence of bean seeds during 10 days at 27 ± 2 °C and 70% relative humidity. The