5b (22.4%) ^{1}H-NMR δ: 0.9 (d, 3H), 0.95 (d, 3H), 2.2 (m, 9H), 2.6 (t, 2H), 3.7 (m, 7H), 4.6 (bs, 1H); I.R. (liquid film): 1065, 1125, 1135, 1670, 2220, 3460 cm^{-1}.
6b (99.7%): ^{1}H-NMR δ: 0.9 (d, 3H), 1.9 (bs, 13H), 2.4 (m, 3H), 3.5 (m, 6H), 4.5 (bs, 1H); I.R. (liquid film) 1030, 1080, 1120, 1710, 3400 cm^{-1}.
2b (54.5%): b.p. 99 °C 24 mm Hg: ^{1}H-NMR δ: 1.1 (d, 3H), 1.15 (d, 3H), 1.9 (m, 9H), 3.75 (m, 4H): I.R. (liquid film): 800, 845, 1005, 1040, 1120, 1380, 1440 $cmxl^{-1}$.
The compounds synthesized so far were first submitted to a GLC analysis utilizing the same capillary column as described above, but a more accurate temperature program. The results are summarized in table 3; the peaks corresponding to compound **1** and to one of the diastereoisomers of compound **2a**, and that of the natural product, displayed the same retention time.
Looking at the main fragmentations (table 4) of the same substances, again no appreciable differences were detectable, therefore the structure of the natural spiroketal could not hitherto be assigned.
A further GLC analysis, employing a less polar stationary phase (WCOT glass capillary column, OV 101, 25 m, 0.2 mm ID) achieved a satisfactory resolution of all the synthesized substances; compound **1** showed the same RT of the natural product and their identity was confirmed by an injection of the 2 combined samples.
While our work was in progress, a paper appeared by Baker et al.[16] in which the identification of the same spiroketal **1** in female rectal glands was described. Such a compound is claimed by the authors to be the major sex pheromone component emitted by the fly.
An analogous GLC-MS analysis of the rectal glands of *Dacus oleae* males did not show any of the substances found in the females. Only some peaks corresponding to C_{10}-C_{18} aliphatic hydrocarbons, which were not further investigated, were detected.

1 Part of this work has been presented at ESOC II, Stresa, June 1981, and at the II Meeting on *Dacus oleae,* Perugia, March 1982.

2 Acknowledgments. We thank Dr G. Vita, Laboratori CNEN, Casaccia (Roma) for insects supplying and helping with dissection techniques. We are grateful to Prof. G. Jommi, Laboratorio di Chimica Organica, Università di Milano, for helpful discussion. CNR, special 'ad hoc' program 'Fitofarmaci e fitoregolatori', subproject 2, is acknowledged for financial support.

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0014-4754/83/050502-04$1.50 + 0.20/0

Inability of thiamine phosphates transport in isolated rat hepatocyte[1]

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Summary. The hapatic transport of thiamine phosphates was studied in isolated rat hepatocytes. No significant radioactivity of ^{14}C-thiamine monophosphate and ^{14}C-thiamine pyrophosphate was incorporated into liver cells freshly isolated using the collagenase-perfusion technique. The result indicates that neither thiamine phosphates nor their thiamine moiety are available for liver cells.

Evidence has been accumulated which shows that the uptake of thiamine by various living cells occurs by active transport. Two reports recently have appeared on thiamine transport in isolated rat hepatocytes, in which thiamine was shown to be transported into the cells in an unaltered form by an active, sodium dependent process[3,4]. Thiamine is known to be present in living cells in 4 forms: free, mono-, pyro- and tri-phosphate[5,6]. Nakayama and Hayashi provided evidence that both thiamine monophosphate and thiamine pyrophosphate can be utilized intact by *Escherichia coli* mutants auxotrophic for thiamine phosphates[7]. Consequently Nishimune and Hayashi clearly demonstrated using ^{32}P-labeled thiamine pyrophosphate that the coenzyme was accumulated by *E. coli* without dephosphorylation[8]. On the other hand, no conclusive evidence has been obtained on the availability of thiamine phosphates for animal cells. With respect to this problem, Schaller and Höller suggested that splitting of thiamine pyrophosphate at the surface on the intestinal mucosa or in mucosal fluid and active transport of thiamine are interrelated with each

other[9], and an identity of intestinal thiamine pyrophosphatase with alkaline phosphatase was further proposed by Iwata et al.[10].

In this paper, we describe that a carrier function specific for thiamine transport in isolated hepatocytes is hardly inhibited by thiamine phosphates such as thiamine monophosphate and thiamine pyrophosphate and no significant radioactivity of ^{14}C-thiamine phosphates is transported into liver cells.

Materials and methods. [^{14}C]Thiamine([thiazole-2-^{14}C]thiamine hydrochloride, 24.3 Ci/mole) was purchased from the Radiochemical Center, Amersham (England), bovine serum albumin (fraction V, Powder), alkaline phosphatase (type I), pyrithiamine hydrobromide and thiamine monophosphate chloride from Sigma Chemical Co., collagenase (CLS IV) from Worthington and thiamine hydrochloride and thiamine pyrophosphate chloride (Cocarboxylase) from Nakarai Chemicals Ltd, Kyoto. Acid phosphatase (Takadiastase B) from Sankyo Co. Ltd, Tokyo. Dimethialium (3-2'-methyl-4'-aminopyrimidyl-(5')-methyl-4,5-dimethylthiazolium chloride hydrochloride) was a gift from Takeda Chemical Industries Ltd, Osaka. All other chemicals were reagent grade.

Hepatocytes were isolated from fed male Wister rats (200 ~ 300 g) by the procedure of Seglen[11]. Cell viability was checked routinely by trypan blue dye exclusion and was over 95%. All experiments were carried out by a modification procedure described by Lumeng et. al.[4] as follows: Freshly isolated hepatocytes ($1.3 \sim 3.8 \times 10^6$ cells per ml) were preincubated for 15 min in Krebs-Henseleit medium containing 25 mg/ml of dialyzed bovine serum albumin, streptomycin (100 µg/ml) and penicillin G (100 units/ml). After preincubation thiamine transport was initiated by the addition of ^{14}C-thiamine of indicated concentration. The preincubation and incubation were carried out at 37 °C, and the incubation media were equilibrated with 95% O_2 and 5% CO_2 at all times. At appropriate time intervals, 0.5-ml aliquots of the cell suspensions were removed and diluted with 1 ml of ice cold incubation medium containing nonradioactive thiamine of same concentration as that in incubation medium and centrifuged at 15,600 × g at room temperature for a few seconds (Eppendorf centrifuge 5412, Hamburg, FRG). After centrifugation the cell pellets were washed with 1 ml of the same medium and immediately recentrifuged. The total pellets were extracted by addition of 1 ml of 6.7% trichloroacetic acid and the radioactivity was measured in 15 ml of Tritosol liquid scintillant by means of a liquid scintillation spectrometer. Experiments were repeated at 3 times and each experimental condition was performed in duplicate. Results have been corrected to account for the viability of the cell suspensions. Comparison of sample means were made using Student's t-test. p-Values less than 0.05 were considered to be significant.

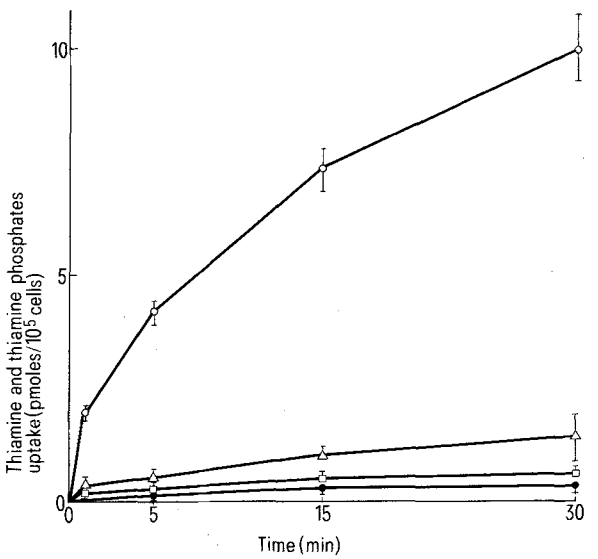
^{14}C-Thiamine monophosphate and ^{14}C-thiamine pyrophosphate were synthesized from ^{14}C-thiamine (24.3 Ci/mole) according to the method previously described[12]. The radiochemical purity of ^{14}C-thiamine monophosphate and ^{14}C-thiamine pyrophosphate estimated by paper chromatography in isopropanol: 0.5 M sodium acetate buffer (pH 4.5): water (65:15:20) were 96.7% and 96.1%, respectively.

Results and discussion. It has been demonstrated that thiamine transport in isolated rat hepatocytes is Na^+- and energy-dependent[3,4]. The table shows the effects of several thiamine analogs and derivatives on ^{14}C-thiamine uptake by isolated liver cells in the presence of Na^+. Pyrithiamine at 10-fold concentration of ^{14}C-thiamine markedly inhibited the ^{14}C-thiamine uptake by liver cells as already reported[3,4]. Furthermore, dimethialium, a derivative of thiamine which has a methyl group in place of hydroxyethyl group at the 5'-position of the thiazole moiety, showed stronger inhibitory effect on the uptake of ^{14}C-thiamine by rat hepatocytes. These inhibitory analogs of thiamine transport have been reported to be transported and accumulated in eucaryotic cells [13,14]. These results also support previous findings suggesting that a carrier specific for thiamine is involved in thiamine transport in rat liver cells[3,4]. In contrast with these compounds, ^{14}C-thiamine uptake by rat hepatocytes was hardly inhibited by thiamine monophosphate or thiamine pyrophosphate. This fact strongly suggested that thiamine phosphates may not be taken up by liver cells. Therefore, the uptake of thiamine phosphates of physiological concentration by rat hepatocytes was investigated using radiolabeled thiamine monophosphate and thiamine pyrophosphate synthesized from [thiazole-2-^{14}C]-thiamine.

Effect of thiamine analogs and derivatives on thiamine transport in freshly isolated hepatocytes

Addition	^{14}C-Thiamine uptake	
	pmole/10^5 cells/5 min	%
None	21.03 ± 0.51	100
Thiamine	8.24 ± 0.27[a]	39
Pyrithiamine	8.52 ± 0.70[a]	41
Dimethialium	4.74 ± 0.40[a]	23
Thiamine monophosphate	20.14 ± 0.93[b]	96
Thiamine pyrophosphate	19.99 ± 1.13[b]	95

The uptake of ^{14}C-thiamine was assayed as described in the text. Nonlabeled thiamine, thiamine analogs and derivatives (200 µM) were added to the liver cell suspensions simultaneously with 20 µM ^{14}C-thiamine and the solution mixture was incubated for 5 min. Each value represents the mean ± SD of 6 determinations. [a]Significantly different ($p < 0.001$) from none. [b]Not significantly different from none.



Uptake of thiamine, thiamine monophosphate and thiamine pyrophosphate in isolated rat hepatocytes. A suspension of liver cells in Krebs-Henseleit medium was preincubated for 15 min at 37 °C. The uptake was initiated by adding 1 µM ^{14}C-thiamine (– ○ –), ^{14}C-thiamine monophosphate (– △ –) and ^{14}C-thiamine pyrophosphate (– □ –) and was continued at 37 °C for the indicated times. Subsequent procedures are described in the text. (– ● –), 0 °C in the presence of 1 µM ^{14}C-thiamine. Each point represents the mean ± SD of 6 determinations. The difference between ^{14}C-thiamine and ^{14}C-thiamine monophosphate or ^{14}C-thiamine pyrophosphate at each time is significant ($p < 0.001$).

As shown in the figure, no significant incorporation of ^{14}C-thiamine phosphates into the liver cells were observed, whereas ^{14}C-thiamine was appreciably taken up by the cells. After the addition of the medium treated ^{14}C-thiamine pyrophosphate with acid phosphatase (Takadiastase) or the addition of alkaline phosphatase to the incubation medium, the radioactivity corresponding to 5.12 and 1.36 pmoles/10^5cells of ^{14}C-thiamine, respectively, was detected in the liver cells. These results indicate that neither thiamine phosphates themselves nor their thiamine moiety are essentialy available for the liver cells. Recently, we have reported that both thiamine monophosphate and thiamine pyrophosphate could not directly translocate yeast cell membrane[15]. From these facts it is conceivable that eucaryotic cells such as yeast and hepatocytes are incapable of taking up phosphate ester of thiamine in contrast with *Escherichia coli.* Therefore, it is concluded that rat liver cells utilize only free thiamine in the plasm to synthesize thiamine coenzyme, although thiamine is present both in free and monophosphate form in rat plasm[16].

1 This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.
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0014-4754/83/050505-03$1.50+0.20/0

Detergent solubilization of cardiac 5′-nucleotidase

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Summary. Nonionic, anionic and zwitterionic detergents were employed for solubilization of 5′-nucleotidase from acetone powder preparations of rat heart. Zwittergents effectively solubilized the enzyme and may allow now its further identification and purification by electrophoretic techniques.

5′-Nucleotidase (EC 3.1.3.5) is a predominantly membrane-bound ecto-enzyme. Although its function in different cells and tissues is not understood, interest in this enzyme arises from the multitude of pharmacological and possible physiological effects of adenosine, the product of hydrolysis of 5′-AMP.

Several attempts to purify the enzyme from cardiac muscle have involved the use of detergents for solubilization including anionic deoxycholate and nonionic Triton X-100[1,2]. Problems encountered or expected with these detergents include modification of electrical charges of protein, and thus interference with electrophoretic techniques, and difficulty of detergent removal during and after purification[3]. Some authors have combined different types of detergents which perhaps minimizes some of these problems[4]. Recently, zwitterionic surfactants, i.e. sulphobetaine derivatives, have become available and were reported to possess effective membrane solubilization properties without accompanying charge effects thus allowing electrophoresis or isoelectric focussing of the solubilized proteins in their presence[5,6]. Zwittergent 3-14 has already been used successfully to solubilize 5′-nucleotidase from cultured fibroblasts and the subsequent identification of the enzyme by electrophoretic techniques[7].

In the following we report data concerning detergent effects on cardiac 5′-nucleotidase, comparing effects of zwitterionic with those of several ionic and nonionic detergents.

Methods and materials. Rat heart acetone powder was prepared and suspended in 50 mM TRIS-HCl (pH 7.5) containing 0.15 M KCl as described previously[8]. To 2 ml portions of suspended extract (13 mg/ml protein) detergents were added in concentrations of 1% (g/100 ml) each unless otherwise stated, and suspensions were then stirred for 4 h at 4 °C. Enzyme activity was determined in the detergent-treated suspensions and, following centrifugation at 100,000 × g for 1 h, in the supernatants and the resuspended pellets. Assays were performed with 5′-IMP as substrate and measurement of phosphate formation[8]. Assays contained, in a total of 0.25 ml, 50 mM TRIS-HCl, pH 8.5, 8 mM $MgCl_2$, 20 mM IMP and about 0.06 mg protein. Incubations were for 60 min at 37 °C. In the case of detergent-treated preparations, the detergents were diluted to 0.25% in enzyme assays and were identical in assays of suspended pellets and supernatants. Triplicate assays were done throughout and resulted in variations of less than 5%. Protein was measured by the method of Lowry et al.[9] and in the case of detergent-containing preparations no interference with protein measurements was observed at the resulting dilutions (0.02% or less) in these assays. The following detergents were used: Nonionic-Tween (type 20, 40, 60 and 85), octylglucose, Lubrol WX and Lubrol PX; anionic-deoxycholate; zwitterionic-Zwittergents 3-10, 3-12, 3-14 and 3-16. Detergents were purchased from Sigma Chemical Co., St. Louis, Mo., USA, except for Zwittergents