ACTIVE TRANSPORT OF THIAMINE BY FRESHLY ISOLATED RAT HEPATOCYTES\textsuperscript{1,2}

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(Received November 28, 1977)

Summary Rat hepatocytes were freshly prepared from adult animals using the collagenase-perfusion technique. The hepatic transport of thiamine was studied in isolated liver cells. The process was found to be saturable with an apparent $K_t$ of 0.31 mM and a $V_{\text{max}}$ of 0.7 $\mu$moles/ml intracellular fluid/5 minutes. However, at higher substrate concentrations, the process proceeded in a linear fashion. Both pyrithiamine and oxythiamine were inhibitory on the hepatic uptake of thiamine, the latter showed much weaker activity than the former. The system required the presence of sodium ions and was sensitive to ouabain. Anaerobic condition and metabolic inhibitors, e.g., 2,4-dinitrophenol, cyanide, and iodoacetate suppressed the uptake rate of thiamine. Addition of ethanol in the incubation medium also caused significant reduction of thiamine uptake. Efflux studies indicated that a portion of intracellular thiamine is readily available for exodus. Chromatographic analyses showed that thiamine was only slightly metabolically altered during the transport process. It is suggested that thiamine is transported into isolated hepatic cells by an active, sodium-dependent process.

Although a large volume of information is available in regard to specific, carrier-mediated transport processes of thiamine in the small intestine (1–14), central nervous system (15–18), red blood cells (19), and microorganisms (20–23), the mechanism of thiamine transport in the liver, especially isolated hepatocytes has not been investigated. The use of collagenase prepared hepatocytes for various biochemical and pharmacological studies has gained wide acceptance since its introduction nearly a decade ago (24). Isolated liver cells are able to transport a variety of solutes through a carrier-mediated active process (25–28). This report

\textsuperscript{1} Supported by The University of Connecticut Research Foundation Grant # 35-252.
\textsuperscript{2} Portions of this paper were reported at the 1977 Fall Meeting of American Society for Pharmacology and Experimental Therapeutics. August 21–25, 1977, Columbus, Ohio (Pharmacologist, 19, 205, 1977).
presents evidence demonstrating that thiamine is also actively transported into freshly isolated adult rat hepatocytes.

MATERIALS AND METHODS

A. Material. [Thiazole-2-14C]thiamine (14 mCi/mmole) was purchased from Amersham/Searle. The radiochemical purity was ascertained to be over 95% by thin-layer chromatography. Non-labeled thiamine was obtained from Mallinckrodt. Ouabain, 2,4-dinitrophenol, N-ethylmaleimide, oxythiamine and pyrithiamine were obtained from Sigma Chemical. Iodoacetic acid and potassium cyanide were purchased from Nutritional Biochemical Co. All chemicals were reagent grade and used without further purification.

B. Methods. Male Charles River rats weighing 200 to 300 g were housed in groups in wire-bottomed cages in an air-conditioned room (23°C). They received regular laboratory chow and water ad libitum.

The method of liver perfusion and cell isolation was previously described (25, 26). The cell pellet, following the final wash of the cell isolation procedure, was resuspended in 10 volumes of Krebs-Ringer's bicarbonate buffer (pH 7.4) containing 1% gelatin. Uptake studies were performed by placing 5 ml of the final cell suspension (50–80 mg wet wt/ml suspension) into 25 ml plastic Erlenmeyer flasks. Under control conditions, a trace amount of [thiazole-2-14C]thiamine (0.01 µCi/0.7 nmoles/ml suspension) and 0.1 µmole/ml of non-labeled thiamine were added to the suspension. The flasks were shaken in a metabolic shaker at 37°C under an atmosphere of 95% O2–5% CO2. In experiments with anaerobic conditions, the incubation medium was gassed with humidified 95% N2–5% CO2 prior to the incubation and the vessels were capped during the incubation period. In experiments with Na+ free medium, Tris-buffer was used and the NaCl was replaced by Tris chloride (25, 26). At the end of incubation period, 1 ml of the cell suspension was removed and placed into a pre-weighed microcentrifuge tube (Brinkmann Co.) and was centrifuged for 30 seconds in a microcentrifuge (Centrifuge 3200, Brinkmann Co.). The supernatant was removed and the tube weighed. The cell pellet was resuspended in 150 µl of distilled water and transferred into an 8 ml counting vial and digested at room temperature with 0.5 ml of Unisol (Isolab., Akron, Ohio) for 16 to 24 hours or until a clear solution was obtained. Two hundred and fifty microliters of absolute methanol was added with mixing and 5 ml of Unisol-Complement (Isolab., Akron, Ohio) was added. An aliquot of the supernatant was placed into an 8-ml scintillation vial containing 6 ml of Scintisol (Isolab.). The extracellular aqueous volume was determined by incubating hepatocytes in the presence of 14C-polyethylene glycol for varying lengths of time. Results were corrected on this basis to account for extracellular trapping and non-specific entry of thiamine. The distribution ratio (I/M) is referred to as the ratio of intracellular to medium (or extracellular) concentrations.
The extent of macromolecule-thiamine binding was estimated by the following procedures. At the end of 5 minutes and 60 minutes of incubation, 10 ml of cell suspension was centrifuged at 1,000 rpm (Sorval GLC-1) for 1 minute; the supernatant was removed by pipette. The cell pellet was washed with 10 volumes of ice-cold Krebs bicarbonate buffer. The cell pellet was then resuspended in an equal volume of 0.05 M phosphate buffer (pH 7.2) and sonicated. One-half milliliter of the cell extract was placed on a 0.9 × 8 cm Bio-Gel P-2 column equilibrated with 0.05 M phosphate buffer (pH 7.2) and eluted with the same buffer. One-half milliliter fractions were collected and counted in 6 ml of Scintisol.

The possible formation of thiamine metabolites during the transport process was tested by thin-layer chromatography using a solvent system of water : pyridine : acetic acid (80 : 20 : 2). The sonicated solution was deproteinized with 5 volumes of absolute methanol. The methanol solution was concentrated by a stream of air before being placed on the thin-layer plate. Authentic thiamine was co-chromatographed with all samples tested.

In efflux studies, isolated hepatocytes were preincubated for one hour at 37°C in the presence of thiamine. At the end of the preincubation period, cells were resuspended in fresh media in the presence and in the absence of 5 × 10^-4 M ouabain. Cells were incubated for different periods of time and the radioactivity remaining in hepatocytes was determined.

RESULTS

1. The uptake of thiamine

When isolated hepatocytes were incubated under normal conditions with thiamine, the initial rate of uptake was very rapid. As shown in Fig. 1, the I/M ratio exceeded unity after 5 minutes of incubation and was greater than 5 after 60 minutes of incubation. The presence of sodium ions was also essential for the accumulation of thiamine by the hepatocytes; complete replacement of sodium ions with Tris caused a profound drop in the accumulating capacity (Fig. 1). An adequate oxygen supply was also important for the transport processes; elimination of oxygen during the incubation period resulted in a significant decrease on the uptake of thiamine (Fig. 1). These results strongly indicate that thiamine is actively taken up by isolated hepatocytes.

2. Identification of thiamine in the tissue extract

Hepatocytes which had been incubated with thiamine for 5 minutes and 60 minutes respectively, were extracted as described in METHODS. When tissue extracts were chromatographed on small columns of Bio-Gel P-2 in order to separate free and bound thiamine derivatives, about 2% and 8% of the radioactivity was associated with the high molecular weight material after 5 and 60 minutes of incubation respectively (Fig. 2). Thin-layer chromatography of the tissue extract (60
Fig. 1. Time course of thiamine uptake under various conditions. Isolated rat liver cells were incubated in Krebs-Ringer's bicarbonate solution (pH 7.4) containing $^{14}$C-thiamine (0.01 μCi/ml suspension) and 0.1 μmole/ml of non-labeled thiamine. Incubation conditions are described in the text. At different time intervals, incubations were terminated and the distribution ratio between intracellular fluid and medium (I/M) was measured (control ---; N₂ ------; Tris --- ---). Values are the mean of 4 determinations with the standard errors shown as vertical bars.

Fig. 2. Chromatographic identification of free and bound thiamine. Radioactivity found in the aqueous extract of hepatocytes after 5 minutes (□) and 60 minutes (▲) of incubation with labeled thiamine was chromatographed on 0.9 × 8 cm Bio-Gel P-2 columns equilibrated with 0.05 M phosphate buffer (pH 7.2) and eluted with the same solvent. Fractions of 0.5 ml were collected. Authentic $^{14}$C-thiamine was co-chromatographed as a control (○).
Fig. 3. Chromatographic identification of thiamine metabolites. Radioactivity found in the aqueous extract of hepatocytes after 60 minutes (∆) of incubation with labeled thiamine was chromatographed on silica gel thin-layer plate with a solvent system of water–pyridine–acetic acid (80:20:2). Authentic thiamine was co-chromatographed as a control (●).

Fig. 4. The distribution ratio (I/M) of thiamine with varying concentrations of non-labeled thiamine in the medium after 5 minutes of incubation. The incubation media contained 0.01 μCi of 14C-thiamine per ml of cell suspension. Each value represents the mean of 4 determinations ± S.E.

minutes) showed that more than 90% of the radioactivity taken up by hepatocytes co-chromatographed in a single peak with authentic thiamine (Fig. 3).

3. Kinetics of thiamine transport

The capacity of the concentrating mechanism of isolated liver cells was examined by incubating hepatocytes with increasing concentrations of non-labeled
thiamine in the medium. Measurements of I/M ratio as well as total uptake were made after 5 minutes of incubation in order to study initial rates. As shown in Fig. 4, the thiamine accumulating system in the hepatocytes became saturated about 1 mM. The observed $K_i$ for thiamine obtained from best fitted regression analysis on the double reciprocal plot was $0.31 \times 10^{-3} \text{M}$. The apparent $V_{max}$ was 0.7 μmoles/ml intracellular fluid/5 minutes (Fig. 5). At high thiamine concentration, 1 to 10 mM, the hepatic uptake of thiamine became linear fashion (Fig. 6).
4. Effect of thiamine analogues on the transport of thiamine

In order to establish the specificity of the uptake system, the effect of thiamine analogues on the hepatic thiamine transport was examined by incubating isolated hepatocytes with $10^{-4}$ M of pyrithiamine or oxythiamine. The analogues were added to the incubation medium simultaneously with $^{14}$C-thiamine and followed by up to 60 min incubation. As shown on Table 1, the uptake of thiamine was significantly inhibited in the presence of pyrithiamine and oxythiamine, although the latter showed much weaker activity than the former.

### Table 1. Effect of thiamine analogues on the uptake of $^{14}$C-thiamine by isolated liver cells.

| Incubation time (min) | Control | Oxythiamine ($10^{-4}$ M) | Pyrithiamine ($10^{-4}$ M) |
|----------------------|---------|---------------------------|---------------------------|
| 5                    | 100 ± 1.2 | 92.4 ± 1.2**               | 67.4 ± 0.6**               |
| 30                   | 100 ± 1.1 | 88.4 ± 3.0*                | 40.2 ± 0.7**               |
| 60                   | 100 ± 1.4 | 95.4 ± 1.7                | 49.6 ± 0.7**               |

* Each value represents the percent of control with mean ± S.E. ($N=4$).
* * Significantly different from control at $p<0.05$.
** ** Significantly different from control at $p<0.001$.

Table 2. Effect of inhibitors on thiamine transport by isolated hepatocytes.*

| Addition of inhibitor | Concentration | Percent of control$^b$* |
|----------------------|---------------|-------------------------|
| None                 |               | 100.0 ± 2.2             |
| Ouabain              | 20 µM         | 83.0 ± 0.9              |
|                     | 100           | 65.3 ± 1.5              |
|                     | 500           | 58.5 ± 0.8              |
| 2,4-Dinitrophenol    | 25            | 70.8 ± 1.7              |
|                     | 100           | 68.2 ± 1.4              |
|                     | 250           | 51.5 ± 1.5              |
| N-Ethylmaleimide     | 100           | 77.7 ± 1.2              |
|                     | 500           | 78.0 ± 1.6              |
| Iodoacetic acid      | 1,000         | 66.3 ± 1.2              |
|                     | 5,000         | 55.3 ± 1.6              |
| Potassium cyanide    | 2,000         | 66.7 ± 1.2              |
|                     | 5,000         | 63.1 ± 2.4              |
| Ethanol              | 0.95%         | 64.1 ± 1.7              |
|                     | 1.90          | 53.2 ± 1.9              |
|                     | 3.80          | 44.6 ± 0.4              |

* Hepatocytes were incubated at 37°C for 60 minutes in the presence of $^{14}$C-thiamine and various concentrations of inhibitors.

$^b$ Each value is expressed as mean ± SEM from 4 determinations.
* All values are significantly different from control at $p<0.005$. 
5. Effect of inhibitors on thiamine transport

To test whether the transport of thiamine is dependent on intracellular energy supplies or not, the uptake of thiamine was studied in hepatocytes that had been incubated with varying concentrations of metabolic inhibitors. As shown on Table 2, the uptake of thiamine was significantly suppressed in the presence of ouabain \((2 \times 10^{-5} \text{M} - 5 \times 10^{-4} \text{M})\) and 2,4-dinitrophenol \((2.5 \times 10^{-5} \text{M} - 2.5 \times 10^{-4} \text{M})\). Table 2 also shows that the transport of thiamine was inhibited by the addition of N-ethylmaleimide \((0.1 \text{ and } 0.5 \text{mM})\), iodoacetic acid \((1.0 \text{ and } 5.0 \text{mM})\), potassium cyanide \((2.0 \text{ and } 5.0 \text{mM})\) and ethanol \((0.9-3.8\%)\).

6. Efflux of thiamine

In order to assess the existence of a readily exchangeable pool of intracellular thiamine, efflux studies were performed. As shown in Fig. 7, there is a rapid exit of thiamine from the cellular compartment during the first 15 minutes of incubation. In the presence of \(5 \times 10^{-4} \text{M}\) of ouabain, the rate of thiamine exit was significantly accelerated (Fig. 7).

![Fig. 7. Efflux of thiamine from pre-loaded hepatocytes. Isolated hepatocytes were pre-incubated for one hour at 37°C in the presence of thiamine. At the end of the pre-incubation period, cells were resuspended in fresh media in the presence (----) and in the absence (-----) of \(5 \times 10^{-4} \text{M}\) ouabain. Values are nmoles of thiamine per g-wet wt remaining in hepatocytes, with the standard errors shown as vertical bars.](image)

DISCUSSION

The results of our present studies suggest the existence of a carrier-mediated, energy-dependent transport system for thiamine in freshly prepared hepatic parenchymal cells at lower concentrations. By contrast, the hepatic uptake of thiamine is proportional to its concentration at higher external substrate
concentrations. Similar results were also obtained in other preparations (4, 11, 18). The nature of the transport system is similar to that demonstrated in the small intestine (1–14), central nervous system (15–18) and microorganisms (20–23). The transport system for thiamine requires the presence of oxygen and is sodium dependent. As shown in Fig. 1, the distribution ratio (I/M) exceeded unity after five minutes of incubation. The I/M ratio continued to increase with time, and at 60 minutes, hepatocytes achieved a concentration gradient of more than fivefold. The accumulation of thiamine was significantly suppressed under a nitrogen atmosphere. Replacing Na⁺ with Tris chloride resulted in a complete inhibition of thiamine accumulation by hepatocytes.

A small portion of thiamine taken up by hepatocytes was bound to cellular macromolecules (Fig. 2). NOSE et al. (29) also observed a thiamine-binding protein in E. coli, although the exact role of the protein in the transport process is not known. The possible relationship between thiamine transport and phosphorylation in isolated hepatocytes was not investigated in the present study. It is known that phosphorylation of thiamine is catalyzed by thiamine pyrophosphokinase which is primarily associated with a soluble fraction of the subcellular preparations (8, 30, 31), however, several studies have indicated that this process may not play any significant role in the transport of thiamine across cellular membrane (11, 19, 21). In the present study, there seemed no significant metabolic alteration of thiamine during the experimental periods (Fig. 3), although some newly formed thiamine phosphates present in hepatic cells at the end of an experiment may be split rapidly by a highly active phosphatase during our extraction and chromatographic procedures.

The competitive inhibition of thiamine analogues on the transport of thiamine has been reported in various biological systems (6, 7, 11, 18, 21). The present observation that hepatic thiamine uptake was significantly reduced in the presence of pyrithiamine, and, to a lesser degree, in the presence of oxythiamine (Table 1) was in complete agreement with previous reports.

The uptake of thiamine was dependent on the availability of metabolic energy. As shown on Table 2, the inclusion of an uncoupling agent (2,4-dinitrophenol), cellular respiration poison (potassium cyanide) or sulfhydryl blocking agents (iodoacetate, N-ethylmaleimide) in the incubation medium caused significant but variable degrees of inhibition. Ouabain, which is known as a “sodium pump” inhibitor, was also inhibitory. The addition of ethanol in the cell suspension produced a profound decrease of thiamine transport capability. Although the inhibitory effect of ethanol on the intestinal transport of thiamine is well established (12, 32), a stimulatory effect of ethanol on active absorption of thiamine has also been reported (10). SORRELL et al. (33) reported that ethanol is capable of causing exodus of the vitamin from the hepatic cells. They suggested that liver cells exposed to ethanol release the vitamin from their stores and cannot rebind the vitamin in the presence of ethanol. Although present studies cannot exclude this
possibility, the exact mechanism of the inhibitory effect of ethanol has not yet been defined (10, 12).

The observed $K_t$ value of 0.31 mM is much greater than that observed in other cellular systems. A $K_m$ value between 0.4 and 0.6 µM was reported in the small intestine (2, 6, 11), while IWASHIMA et al. (21) reported a $K_m$ value of 0.18 µM in yeast cells. In brain slices the apparent $K_m$ of the uptake for thiamine was 0.36 µM (18). Although isolated hepatocytes were able to accumulate neutral amino acids without significant change in their affinity (25), the reason for the observed differences between the present studies and others remain to be solved. HORNE et al. (26) also reported a significant decrease in affinity of isolated hepatocytes for methotrexate.

Efflux studies (Fig. 7) indicated that a portion of intracellular thiamine is readily exchangeable with the extracellular medium. This rapid loss of thiamine from the cell is suggestive of the presence of an intracellular pool of free thiamine which is readily available for exodus, however, firm evidence is lacking on this point. In the presence of ouabain (5 x 10^{-4} M), thiamine exhibited a greater exit rate. It is possible that the thiamine reuptake mechanism of hepatocytes is quite efficient in the absence of a Na-pump inhibitor. KAWASAKI and YAMADA (34) also observed a rapid exit of thiamine from E. coli.

The results obtained with ouabain, Na^+-free medium, competition, metabolic inhibitors, anaerobiosis together with those on thiamine accumulated by hepatocytes against a concentration gradient all lead to the suggestion that thiamine is conveyed into hepatic cells by an active (energy-requiring) system which is also dependent on the sodium pump.

The author wishes to thank Drs. Philip Rosenberg and Steven D. Cohen for their critical review of the manuscript.

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