Transport and Metabolism of Thiamin in Isolated Rat Hepatocytes*

Lawrence Lumeng, James W. Edmondson, Steven Schenker, and Ting-Kai Li

From the Departments of Medicine and Biochemistry, Indiana University School of Medicine and Veterans Administration Hospital, Indianapolis, Indiana 46223, and Vanderbilt University Medical School and Veterans Administration Hospital, Nashville, Tennessee 37203

This study examines thiamin transport in isolated rat hepatocytes and its relationship to thiamin phosphorylation. In an Na+ medium, [35S]thiamin, 3 μM, was accumulated rapidly by the cells, and a near steady state intra-/extracellular distribution ratio of 3 was attained in 1 min. However, the uptake of radioactivity continued to increase with time owing principally to the accumulation of [35S]thiamin pyrophosphate (TPP). In a choline, Li+ or K+ medium, the steady state intra-/extracellular distribution ratio of [35S]thiamin was decreased to ≤1.1. Accordingly, the rate of formation of [35S]TPP also decreased. Ouabain and uncouplers of oxidative phosphorylation significantly lowered the distribution ratio of intra-/extracellular [35S]thiamin. These data indicate that thiamin transport in liver is concentrative, Na+-dependent, and dependent on biological energy. Additionally, they suggest that thiamin transport plays a significant role in governing the rate of synthesis of TPP. Neither pyri-thiamin, an inhibitor of thiamin pyrophosphokinase, nor o-benzoylthiamin disulfide, a permeable thiamin analog, affected the distribution ratio of intra-/extracellular [35S]thiamin, but preferentially inhibited the phosphorylation of [35S]thiamin. By contrast, amprolium primarily inhibited uptake. These data suggest that thiamin transport and phosphorylation can be differentiated by the action of appropriate inhibitors.

The thiamin transport carrier and thiamin pyrophosphokinase in Escherichia coli are both located in the bacterial membrane fraction (1-2). This fact, together with the lack of accumulation of thiamin in the bacterium, has led to the conclusion that thiamin transport in E. coli is tightly coupled to thiamin phosphorylation. By contrast, in mammalian cells, thiamin pyrophosphokinase is localized in the cytosol (3). It is, therefore tempting to postulate that thiamin transport and phosphorylation in mammalian cells are dissociable and independent events. However, experiments addressing this point have thus far produced inconclusive results (4).

Because the liver is an important storage site for thiamin and related compounds in the body, we have examined the relationship of thiamin transport and thiamin phosphorylation in isolated rat hepatocytes. By using a reliable method for the separation of thiamin and its phosphoesters, the experiments reported herein indicate that thiamin phosphorylation occurs extensively in isolated rat hepatocytes and that this metabolic step is separable from thiamin uptake according to time course and by appropriate inhibitors. These findings differ significantly from those of a recent report (5) which suggested that thiamin is not phosphorylated after its uptake by isolated liver cells.

MATERIALS AND METHODS

Preparation of Isolated Hepatocytes—Male Sprague Dawley rats weighing 200 to 300 g were fed a standard rat chow until 24 h before use. Isolated rat liver cells were prepared as described previously (6). The cell preparations consistently contained >95% parenchymal cells. Immediately following isolation, 93 to 98% of the cells excluded trypan blue, and 85 to 90% of the cells continued to exclude the dye following ½-h preincubation and 1-h incubation. Cells prepared in this manner exhibited high rates of gluconeogenesis, maintained intracellular K+ concentrations ranging between 130 to 150 meq/liter of cell water, and were active in amino acid transport (6).

Incubation Conditions—Freshly isolated hepatocytes, 30 to 60 mg wet weight per ml, were preincubated for 30 min in 7.5 ml of Krebs-Henseleit medium containing 10 mM lactate and dialyzed, fatty acid-free bovine serum albumin, 25 mg/ml. To study thiamin transport in the presence of Na+, the experiments were initiated after preincubation by the addition of 3 μM [35S]thiamin (120 μCi/μmol). In experiments where the effect of Na+ deletion on thiamin uptake was examined, the cells were sedimented after preincubation and then resuspended in the same volume of one of the following incubation media: 1) choline medium prepared by isosomotic substitution of NaCl and bicarbonate in the Krebs-Henseleit medium with their respective choline salts; 2) Li+-containing medium prepared by using choline bicarbonate and LiCl as equivalent replacements for the respective Na+ salts in the Krebs-Henseleit medium; and 3) K+-containing medium prepared by isosomotic substitution of NaCl and bicarbonate in the Krebs-Henseleit medium with their respective K+ salts. These incubation media also contained 3 μM [35S]thiamin. 10 mM lactate, and dialyzed, fatty acid-free bovine serum albumin, 25 mg/ml. The incubations and incubations were carried out at 37°C, and the mixtures were equilibrated with 95% O2 and 5% CO2 at all times. In some experiments, 1.0 μM [35S]thiamin was employed.

At appropriate time intervals, 1-ml aliquots of the cell suspension were removed and immediately centrifuged at 15,600 × g for 30 s at 4°C (Eppendorf centrifuge 5412, Hamburg, Germany). After separation of the medium from the cell pellet, the latter was washed with 1 ml of icecold incubation medium containing 3 μM nonradioactive thiamin and immediately recentrifuged to separate the supernatant from the washed cell pellet. The total pellet radioactivity was extracted by addition of 1 ml of 10% trichloroacetic acid. This washing step effectively removed most of the radioactivity from the extracellular space in the cell pellet. Radioactivity of 0.1-ml aliquots of the pellet extract, 0.1 μl of the incubation medium, and 0.5 μl of the wash supernatant was measured in PCS liquid scintillator (Amersham/ Searle, Arlington Heights, IL) by means of a liquid scintillation spectrometer. Internal standardization was employed for quench and efficiency corrections. Other methods of cell separation, e.g., the separatory tube (7) and the silicon layer (8) were also tried, but the procedure described above consumed less time, provided the lowest extracellular fluid radioactivity background, and had the best reproducibility between experiments.

Separation of the Thiamin Compounds by Paper Electrophoresis—An aliquot, 0.05 ml, of a solution containing 1 mg each of...
nonradioactive thiamin. TMP, TP, and TTP was added to 0.9 ml of the pellet extract. The trichloroacetic acid in this mixture was then removed by ether extraction. Thiamin and its monophosphate, pyrophosphate, and triphosphate were separated by electrophoresis on Munkel S311 paper in a pyridine-glacial acetic acid-water solution (20:8:2972, v/v), pH 3.2, at a constant current of 8 mA for 35 min. The thiamin compounds were then detected by spraying with an alkaline ferricyanide reagent (9) and visualized under long wavelength ultraviolet light. The bands were cut and immersed in liquid scintillant for radioactivity measurement. The recovery of 14C counts invariably exceeded 85%.

**Determination of Intracellular Water Space—**Measurements of intracellular and extracellular spaces and thiamin uptake were performed in parallel for each experiment as described previously (6). Briefly, in the flasks for measurement of H2O and [14C]inulin spaces, cells were incubated in the appropriate media containing nonradioactive thiamin, H2O (0.15 μCi/ml), and [14C]inulin (0.15 μCi/ml). Separation of medium from cell pellet was performed by centrifugation as described above, but the cell pellet was not washed. The intracellular water space was calculated as the difference between the H2O and [14C]inulin distribution spaces in the cell pellet. The intracellular water space was 32 ± 2% (mean ± S.E.; n = 25) of the total pellet water content.

**Expression of Results—**Cellular radioactivity of thiamin was obtained by subtracting the radioactivity in the extracellular space of the washed pellet from the total thiamin count of the washed pellet. The latter was determined by the radioactivity of the thiamin band on paper electrophoresis. The validity of this calculation was based on the fact that >90% of the radioactivity in the medium and in the extracellular space of the washed pellet was in the form of thiamin. The radioactivity of the thiamin in the extracellular space was calculated from the volume of the extracellular space of the pellet and the thiamin radioactivity per ml of the supernatant of the washed pellet. Cellular content of the thiamin compounds was calculated by dividing the cellular radioactivity by the volume of intracellular water and was expressed as nanomoles per ml of cell water, with the assumption that the thiamin compounds were homogeneously distributed within the intracellular space. The results presented are either the mean values of three experiments or representative of at least four experiments.

**Materials—**Type II collagenase and hyaluronidase were purchased from Worthington Biochemicals, Freehold, NJ. Thiamin, TMP, TP, pyrithiamin, ouabain, dinitrophenol, choline chloride, choline bicarbonate, LiCl, fatty acid-free bovine serum albumin, and N,N′-dimethylformamide were purchased from Sigma Chemical Co. TTP, amorpil, and o-benzoylthiamin disulfide were kindly provided as gifts by the Central Research Laboratories, Sankyo Co. (Tokyo, Japan), Merck Sharp & Dohme Research Laboratories (Rahway, NJ), and Tanabe Seiyaku Co. (Osaka, Japan). [35S]Thiamin and PCS liquid scintillant were purchased from Amersham/Searle, Arlington Heights, IL. The purity of [35S]thiamin was verified by paper electrophoresis. Carboxyl cyanide p-trifluoromethoxyphenyldrazone was obtained from Calbiochem (La Jolla, CA).

**RESULTS**

**Transport and Metabolism of [35S]Thiamin by Isolated Hepatocytes and the Effect of Na+**—In an Na+ medium containing an initial concentration of 3 μM [35S]thiamin, isolated hepatocytes accumulated [35S]thiamin rapidly, and the distribution ratio of intra-/extracellular [35S]thiamin concentration was greater than 3 within 1 min (Fig. 1). Owing to the cellular uptake and metabolism of [35S]thiamin, its concentration in the medium decreased about 50% in the 1 h of incubation. This decrease was accompanied by a small and gradual decline in the intracellular concentration of [35S]thiamin. Thus, the mean distribution ratio of intra-/extracellular [35S]thiamin remained constant at about 3.6 throughout the incubation. Importantly, the cellular accumulation of [35S]thiamin reached a near steady state more rapidly than the rise in intracellular [35S]TPP, but the latter exhibited the largest increment, reaching 42 nmol/ml of cell water in 1 h (Fig. 1). By contrast, the increase in intracellular [35S]TMP and TTP was considerably smaller. When 1.0 μM [35S]thiamin was used in other experiments, a similar profile of change in intracellular [35S]thiamin and its phosphoesters as a function of time was observed. However, the distribution ratio of intra-/extracellular [35S]thiamin was reduced to 2.0, and the formation of [35S]TPP was decreased to 9 nmol/ml of cell water in 1 h. Equilibrium dialysis indicated that thiamin is not bound to either bovine serum albumin or to cytosolic proteins (data not shown). It is concluded, therefore, that thiamin transport by the hepatocytes is concentrative.

In order to demonstrate the dependence of [35S]thiamin transport on medium Na+, liver cells were incubated in a medium containing choline (Fig. 2). Cellular [35S]thiamin concentration increased slowly, and the distribution ratio of intra-/extracellular [35S]thiamin did not exceed 1.1 even in 2 h of incubation. Presumably owing to the low intracellular level of [35S]thiamin, the formation of labeled TPP, TMP, and TTP was also reduced in the absence of Na+ in the medium. Similar data were obtained with isolated liver cells incubated in either an Li+ or a K+ medium.

The addition of ouabain (2.5 mM) to isolated liver cells incubated in an Na+ medium reduced the distribution ratio of intra-/extracellular [35S]thiamin from 3.3, in the control, to 1.5 (data not shown). Thus, the concentrative process of thiamin transport in liver is coupled to Na+ flux.

**Energy Dependence of [35S]Thiamin Transport—**Uncouplers of oxidative phosphorylation, carbonyl cyanide p-trifluoromethoxyphenyldrazone and dinitrophenol, decreased the intracellular levels of [35S]thiamin and [35S]TPP in a dose-dependent manner (Fig. 3). These data indicate that both the transport and the phosphorylation of thiamin in liver are dependent on biological energy.

**Effect of Thiamin Analogs on [35S]Thiamin Transport and Metabolism**—o-Benzoylthiamin disulfide is a thiamin analog that permeates plasma membranes very rapidly by simple diffusion and can be converted intracellularly to thiamin (10). On the other hand, pyrithiamin is a potent inhibitor of thiamin pyrophosphokinase (11), but its effect on thiamin...
mM concentration it decreased the phosphorylation of [35S]thiamin, and at 0.3 mM and higher concentrations, it completely abolished this reaction. These data, therefore, indicate that thiamin transport in liver is dissociable from thiamin phosphorylation. Additionally, they strongly suggest the existence of a thiamin channel (or carrier) in the liver cell membrane which can be distinguished from thiamin pyrophosphokinase by the action of pyrithiamin.

Amprolium is a thiamin analog that lacks a hydroxyethyl group and is not a substrate of thiamin pyrophosphokinase. It has been shown to inhibit thiamin absorption in ligated duodenal loops of hens (12). Amprolium differs from pyrithiamin and o-benzoylthiamin disulfide in that it decreased the distribution ratio of intra-/extracellular [35S]thiamin concentration (Fig. 5). It also decreased the rates of [35S]TPP formation. transport in mammalian tissue has not been defined. o-Benzoylthiamin disulfide, up to 0.3 mM, affected only slightly the distribution ratio of intra-/extracellular [35S]thiamin concentrations (Fig. 4). It decreased [35S]TPP formation presumably because of dilution of radiolabeled thiamin by nonradioactive thiamin. These findings are consistent with the interpretation that o-benzoylthiamin disulfide traverses the liver cell membrane by simple diffusion and, in so doing, does not interfere with thiamin transport. Pyrithiamin, up to 0.3 mM, also did not alter significantly the concentrative gradient of [35S]thiamin across the liver cell membrane (Fig. 4). However, at 0.03

![Fig. 2. Transport and metabolism of [35S]thiamin by isolated liver cells in choline medium.](image)

Liver cells, 59 mg wet weight per ml, were incubated for 2 h in choline medium. The other conditions are identical with Fig. 1.

![Fig. 3. Effects of uncouplers of oxidative phosphorylation on the concentrations of [35S]thiamin and [35S]TPP in isolated hepatocytes after 15-min incubation.](image)

Liver cells, 50 mg wet weight per ml, were incubated as described in Fig. 1. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) dissolved in N,N'-dimethylformamide, 0.01 ml, was added. This solvent per se had no effect on thiamin uptake and metabolism. Similar data were obtained after 30-min incubation. Cell viability based on trypan blue exclusion remained >80% with these concentrations of uncouplers. DNP, dinitrophenol.

![Fig. 4. Effects of pyrithiamin and o-benzoylthiamin disulfide on the cellular concentration of [35S]thiamin and [35S]TPP in isolated hepatocytes after 30-min incubation.](image)

Liver cells, 55 mg wet weight per ml, were incubated as in Fig. 1. o-Benzoylthiamin disulfide dissolved in 0.01 ml of N,N'-dimethylformamide or the solvent alone was added. Similar data were obtained after 15-min incubation.

![Fig. 5. Effect of amprolium on the cellular level of [35S]thiamin and [35S]TPP in isolated hepatocytes.](image)

Hepatocytes, 62 mg wet weight per ml, were incubated as described in Fig. 1.
presumably by lowering the intracellular level of $[^{14}S]$thiamin. Whether or not it inhibits the thiamin pyrophosphokinase reaction in liver has not yet been studied.

**Effects of Ethanol on $[^{14}S]$Thiamin Transport and Metabolism**—The effect of ethanol (87 mM) on $[^{14}S]$thiamin transport and metabolism was also examined in Na$^+$ medium. Ethanol oxidation did not affect the distribution ratio of intra-/extracellular $[^{14}S]$thiamin concentrations. It also did not exert any significant effect on $[^{14}S]$thiamin phosphorylation.

**DISCUSSION**

In agreement with a recent report (5), we find that thiamin transport in isolated liver cells is concentrative, Na$^+$-dependent, and dependent on biological energy. However, our results indicate that $[^{14}S]$thiamin is extensively phosphorylated to TPP, TMP, and TTP after its uptake into the cells. This finding differs significantly from that of Chen (5), who found that $[^{14}S]$thiamin remained largely unmetabolized for as long as 1 h of incubation. This difference may be explained by his use of sonication and methanol for terminating the incubation. It has been speculated that under these conditions the phosphoesters of thiamin were hydrolyzed to thiamin by cellular phosphohydrolases (5).

Thiamin transport in mammalian tissue has been studied most extensively in the small intestine (13-24). Because it has not been possible to reliably dissociate thiamin uptake and phosphorylation in such studies, some investigators have expressed the view that, as in E. coli, these processes in mammalian cells are tightly coupled (4). Although several earlier studies have shown that the distribution ratio of intra-/extracellular thiamin concentration exceeds unity when mammalian tissues are incubated in the presence of thiamin (18, 20-22), it was observed that TPP accumulated more rapidly than thiamin, TMP, and TTP after its uptake into the cells. This difference may be explained by the amphoteric nature of this enzyme activity in liver (25), the levels of intracellular thiamin compounds as determined herein by radioactivity measurements have probably underestimated the true concentrations owing to dilution of radiolabel. Studies are now in progress to measure both the distribution ratio and the radioactivity of all the thiamin compounds in the medium and in hepatocytes. In this manner, the relative rates of turnover of different intracellular pools of thiamin, TMP, TPP, and TTP, if they exist, can also be assessed.

The finding that amprolium significantly lowers the distribution ratio of intra-/extracellular thiamin concentrations (Fig. 5) suggests that amprolium may compete with thiamin in its uptake. A less likely possibility is that amprolium may accelerate thiamin efflux. Since amprolium lacks a hydroxyl group and is not phosphorylated, it appears that amprolium can be used as a nonmetabolizable thiamin analog in the further characterization of thiamin transport. In accord with this expectation, our preliminary data indicate that the $[^{14}C]$amprolium transport in isolated hepatocytes is, indeed, highly concentrative and is Na$^+$-dependent.

Chen (5) has also reported that ethanol, in the range of 206 to 824 mM, decreased the rate of thiamine transport 36 to 55%.

Since these enormously high ethanol concentrations never occur *in vivo*, we have examined the effect of 87 mM ethanol, already a near lethal concentration. This level of ethanol did not produce any discernible effect of thiamin transport or metabolism in isolated hepatocytes.

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