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# The inhibitory effect of choline and other quaternary ammonium compounds on thiamine transport in isolated rat hepatocytes

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**Quaternary ammonium compounds, such as choline and acetylcholine significantly inhibited thiamine uptake in isolated rat hepatocytes. Kinetic analysis using Lineweaver-Burk and Dixon plots of inhibition experiments revealed that choline and acetylcholine were purely competitive inhibitors for thiamine uptake with $K_i$ values of 0.61 mM and 0.31 mM, respectively. Among quaternary ammonium compounds, hemicholinium-3 and curare were the strongest inhibitors, and kinetic studies showed that these compounds were also purely competitive inhibitors with $K_i$ values of 12.5 $\mu$M and 4.3 $\mu$M, respectively. These results indicate that choline, acetylcholine and their structural analogs share a common binding site with thiamine in isolated rat hepatocytes. On the other hand, choline uptake by isolated rat hepatocytes occurred by a saturable mechanism with a $K_t$ of $162 \pm 3.85$ $\mu$M and $V_{max}$ of $80.1 \pm 1.30$ pmol/$10^5$ cells per min as well as by a nonsaturable mechanism. Thiamine, pyrithiamine, oxythiamine, chloroethylthiamine and dimethialium inhibited choline uptake, while thiamine phosphates such as thiamine monophosphate and thiamine pyrophosphate insignificantly inhibited uptake. Although a Lineweaver-Burk plot of choline uptake in the presence of thiamine showed that thiamine also competitively inhibited choline uptake, a Dixon plot of the inhibition experiment was hyperbolic and indicated that the inhibition of choline uptake by thiamine was 'pseudo-competitive'. On the basis of these results, it is suggested that in isolated rat hepatocytes thiamine and choline do not share common transport sites.**

## Introduction

Chen [1], Lumeng et al. [2] and previous papers from this laboratory [3–5] demonstrated that thiamine is transported via an active and $Na^+$-dependent process in isolated rat hepatocytes. The kinetic studies indicated that there is a single carrier system for thiamine in isolated rat hepatocytes with a $K_t$ of 34.1 $\mu$M and $V_{max}$ of 20.8 pmol/$10^5$ cells per 30 s [4]. Using rat perfused liver, Zeisel et al. [6] showed that choline is taken up by a saturable mechanism with a $K_t$ of $0.17 \pm 0.07$ mM and by a nonsaturable mechanism. It has also been shown that thiamine and choline mutually inhibited their transport across plasma membrane in kidney and small intestine; Rennick [7] reported that thiamine competitively inhibits renal tubular choline transport in the chicken, and Sung and Johnstone [8] obtained results with rat kidney which show the inhibition by thiamine of choline transport. Moreover, Herzberg and Lerner [9] demonstrated that the transport of choline and thiamine are mutually antagonistic in the small intestine of the chick. The results of a kinetic study, however, indicated that there is probably not a single common site for choline and thiamine

absorption in the intestine. Our previous report [4] showed that naturally occurring quaternary ammonium compounds such as choline and acetylcholine are competitive inhibitors of thiamine uptake in isolated rat hepatocytes, while betaine and carnitine which have a negatively charged group in their molecules do not inhibit thiamine uptake.

In this paper, further primarily kinetic studies of the effects of quaternary ammonium compounds on thiamine uptake, particularly of the interaction of thiamine and choline uptake in isolated rat hepatocytes are presented.

## Materials and Methods

*Chemicals*

[$^{14}$C]Thiamine ([*thiazole*-2-$^{14}$C]thiamine hydrochloride, 24.3 Ci/mol) and [$^{14}$C]choline ([*methyl*-$^{14}$C]choline chloride, 58 Ci/mol) were purchased from the Radiochemical Centre, Amersham (U.K.). The following agents were used; collagenase (CLS IV) from Worthington, and bovine serum albumin (Fraction V powder) from Miles. Dimethialium (3-[2′-methyl-4′-aminopyrimidyl-(5′)-methyl]-4,5-dimethylthiazolium chloride hydrochloride) and chloroethylthiamine (3-[2′-methyl-4′-aminopyrimidyl-(5′)-methyl]-4-methyl-5-chloroethylthiazolium chloride hydrochloride) were the gifts from Takeda Chemical Industries, Ltd. (Osaka) and Sankyo Co, Ltd. (Tokyo), respectively. All other chemicals were reagent grade.

*Preparation of isolated rat hepatocytes and assay of thiamine and choline uptake*

Hepatocytes were prepared from 200–300 g Wistar male rat fed ad libitum as previously reported [4] according to a minor modification of the procedure of Seglen [11]. Cells isolated in this manner showed a viability exceeding 90% by the Trypan blue exclusion method.

After preincubation for 15 min at 37°C, the transport of thiamine and choline were initiated in a Corning centrifugation tube (50 ml) by the addition of [$^{14}$C]thiamine or [$^{14}$C]choline in 3 ml of cell suspended ($3.5 \cdot 10^6$ cells/ml) in the Krebs-Henseleit medium containing dialysed bovine serum albumin (25 mg/ml), streptomycin (100 μg/ml) and penicillin G (100 units/ml). The experiments were terminated by the addition of 15 ml of ice-cold medium. After separation of the medium from the cell pellets by centrifugation for 5 s at $700 \times g$, the cell pellets were washed with 10 ml of ice-cold medium, and then recentrifuged for 5 s at $700 \times g$ as described previously [4]. Blank tubes were routinely determined as follows: [$^{14}$C]thiamine or [$^{14}$C]choline was added to the cell suspensions at 0°C, and then immediately diluted, centrifuged and washed by the procedure described above. Cell pellets were extracted by the addition of 1 ml of 6.3% trichloroacetic acid and the radioactivity was measured in a liquid scintillant containing Triton X-100 by means of liquid scintillation spectrometry.

*Determination of intracellular water space*

The intracellular water space was determined as the difference of $^3H_2O$ and [$^{14}$C]inulin distribution ratio in the cell pellet [3] in parallel for each experiment, and calculated to be $2.62 \pm 0.239$ μl/$10^6$ cells (n = 42, mean ± S.E.).

*Statistical estimation of data*

The data of transport obtained are presented as means ± S.E. Kinetic parameters ($K_t$ and $V_{max}$) and S.E. for these values were calculated as described by Wilkinson [13]. Significant differences were assessed by the Student's two-tailed *t*-test.

## Results and Discussion

A previous paper from this laboratory [4] showed that choline and acetylcholine competitively inhibited thiamine uptake in isolated rat hepatocytes with a $K_i$ of 0.61 mM and 0.31 mM, respectively. However, at least two cases of competitive inhibition are defined by a Lineweaver-Burk plot; one is purely competitive inhibition, while the second is 'pseudo-competitive' as defined by Robinson and Alvarado [10]. The two forms of competition can be distinguished by a Dixon plot. Namely, the reciprocal of the substrate uptake ($1/v$) is linearly related to the concentration of inhibitor ([$I$]) in the case of purely competitive, whereas this relation is hyperbolic in the case of 'pseudo-competitive' inhibition [10]. Therefore, the inhibitory effects of choline and acetylcholine on the initial rate of thiamine uptake from a medium

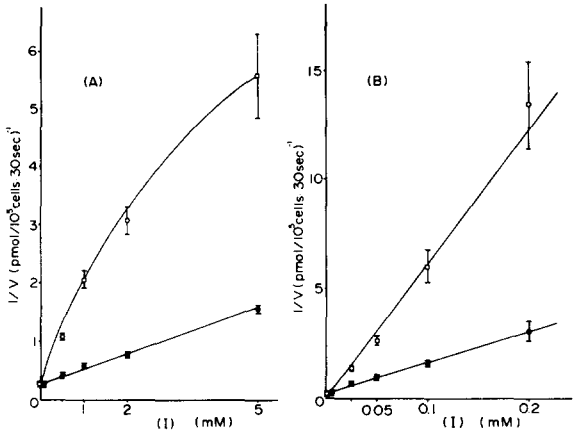



Fig. 1. (A) Inhibition of thiamine uptake (at a concentration of 10 $\mu$M) by choline (●——●) and acetylcholine (○——○). (B) Inhibition of thiamine uptake (at a concentration of 10 $\mu$M) by hemicholinium-3 (●——●) and curare (○——○). Dixon plots. The data presented in (A) and (B) were corrected for the contribution of nonsaturable uptake. Each value is the mean ± S.E. of three experiments.

containing 10 $\mu$M thiamine were analysed by Dixon plot to determine whether the inhibition produced by choline or acetylcholine was competitive or 'pseudo-competitive'. As shown in Fig. 1A, the reciprocal of the rate of thiamine uptake vs. choline concentration was linear. In the case of acetylcholine, the Dixon plot was not entirely linear; high concentrations of acetylcholine inhibited thiamine uptake almost completely. It is, therefore, likely that acetylcholine is also a purely competitive inhibitor of thiamine uptake in isolated rat hepatocytes [12]. These results suggest that choline, acetylcholine and thiamine share a common binding site in the plasma membrane of rat hepatocytes.

The effect of a number of quaternary ammonium compounds on thiamine uptake is presented in Table I. Among the compounds examined, tetraethylammonium and hemicholinium-15 were the most potent inhibitors. These compounds at the concentration 1 mM inhibited thiamine uptake 92 and 93%, respectively. Choline, acetylcholine, carbachol and tetramethylammonium inhibited thiamine uptake 68, 83, 62 and 36%, respectively. Among bis-quaternary ammonium compounds, decamethonium inhibited thiamine uptake 60%, whereas hexamethonium and

TABLE I

EFFECT OF QUATERNARY AMMONIUM COMPOUNDS ON THIAMINE UPTAKE

The uptake of [$^{14}$C]thiamine was assayed as described in the text. Quaternary ammonium compounds (1 mM) were added to the cell suspensions simultaneously with 10 $\mu$M [$^{14}$C]thiamine, and the mixtures were incubated for 30 s. The data presented were corrected for the contribution of the nonsaturable uptake. The distribution ratio is the molar ratio of intracellular thiamine to thiamine in The medium. the results presented are the means ± S.E. of three experiments.

| Addition | [$^{14}$C]Thiamine uptake (pmol/$10^5$ cells per 30 s) | Distribution | |
|---|---|---|---|
| | | Ratio | % |
| None | 2.430 ± 0.023 | 1.141 ± 0.011 | 100 |
| Choline | 1.021 ± 0.021 | 0.479 ± 0.010 | 42 |
| Acetylcholine | 0.414 ± 0.028 | 0.194 ± 0.013 | 17 |
| Carbachol | 0.928 ± 0.035 | 0.436 ± 0.016 | 38 |
| Hexamethonium | 2.401 ± 0.046 | 1.127 ± 0.022 | 99 |
| Succinylcholine | 2.113 ± 0.083 | 0.992 ± 0.039 | 87 |
| Decamethonium | 0.973 ± 0.028 | 0.457 ± 0.013 | 40 |
| Tetramethylammonium | 1.549 ± 0.071 | 0.728 ± 0.033 | 64 |
| Tetraethylammonium | 0.201 ± 0.007 | 0.094 ± 0.003 | 8 |
| Hemicholinium-15 | 0.168 ± 0.005 | 0.079 ± 0.002 | 7 |

succinylcholine were less effective inhibitors of thiamine uptake. On the basis of these studies, it is unclear why hexamethonium and succinylcholine were inefficient inhibitors of thiamine uptake. This effect may be due to the differences in carbon chain length between the quaternary nitrogen atoms and the lipophilicity of the compounds. Table II shows a comparison of the inhibitory effects of thiamine derivatives with a quaternary nitrogen atom (Fig. 2) and quaternary ammonium compounds which are effective inhibitors of thiamine uptake. Hemicholinium-3, a choline analog, and curare, an acetylcholine analog (at concentrations of 50 $\mu$M), inhibited thiamine uptake 76 and 89%, respectively. The inhibition produced by these compounds was greater than that produced by dimethialium, the strongest inhibitor among thiamine derivatives [4]. Hemicholinium-3 and curare were also purely competitive inhibitors as shown in Figs. 1B and 3. The values of $K_t$ and $V_{max}$ for thiamine in the absence and presence of 25 $\mu$M hemicholinium-3 were 58.5 ± 0.58 and 182

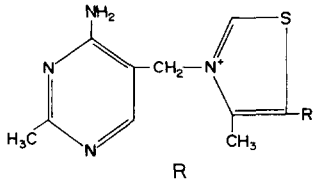
TABLE II

EFFECT OF QUATERNARY AMONIUM COMPOUNDS AND THIAMINE DERIVATIVES ON THIAMINE UPTAKE

The uptake of [$^{14}$C]thiamine was assayed as described in the text. Quaternary ammonium compounds and nonradioactive thiamine derivatives (50 $\mu$M) were added to cell suspensions simultaneously with 10 $\mu$M [$^{14}$C]thiamine, and the mixtures were incubated for 30 s. The data presented were corrected for the contribution of nonsaturable uptake. The distribution ratio is the molar ratio of intracellular thiamine to thiamine in the medium. The results presented are the means ± S.E. of three experiments.

| Addition | [$^{14}$C]Thiamine uptake (pmol/$10^5$ cells per 30 s) | Distribution Ratio | % |
|---|---|---|---|
| None | 3.608 ± 0.055 | 1.377 ± 0.021 | 100 |
| Tetraethylammonium | 1.715 ± 0.078 | 0.655 ± 0.030 | 48 |
| Hemicholinium-15 | 1.245 ± 0.086 | 0.475 ± 0.033 | 35 |
| Hemicholinium-3 | 0.876 ± 0.008 | 0.334 ± 0.003 | 24 |
| Curare | 0.401 ± 0.007 | 0.153 ± 0.003 | 11 |
| Thiamine | 1.811 ± 0.079 | 0.691 ± 0.030 | 50 |
| Dimethialium | 0.906 ± 0.019 | 0.346 ± 0.007 | 25 |

± 23.0 $\mu$M ($p < 0.001$), 29.4 ± 0.17 and 30.5 ± 2.77 pmol/$10^5$ cells per 30 s (n.s.), respectively. In the case of curare inhibition, the values of $K_t$ and $V_{max}$ in the absence and presence of 10 $\mu$M curare were 47.4 ± 1.86 and 146 ± 20.6 $\mu$M ($p < 0.001$), 29.4 ± 0.67 and 27.2 ± 2.60 pmol/$10^5$ cells per 30 s (n.s.), respectively. For these inhibitors, hemicholinium-3 and curare, the $K_i$ values were found to be 12.5 $\mu$M and 4.3 $\mu$M, respectively. These results indicate that choline and its analogs share a common binding site with thiamine in the membranes of isolated rat hepatocytes. However, the affinities of these compounds for the thiamine binding site are markedly different; $K_i$ values for




| R | |
|---|---|
| Thiamine | -CH$_2$-CH$_2$-OH |
| Chloroethylthiamine | -CH$_2$-CH$_2$-Cl |
| Dimethialium | -CH$_3$ |

Fig. 2. Structure of thiamine derivatives.

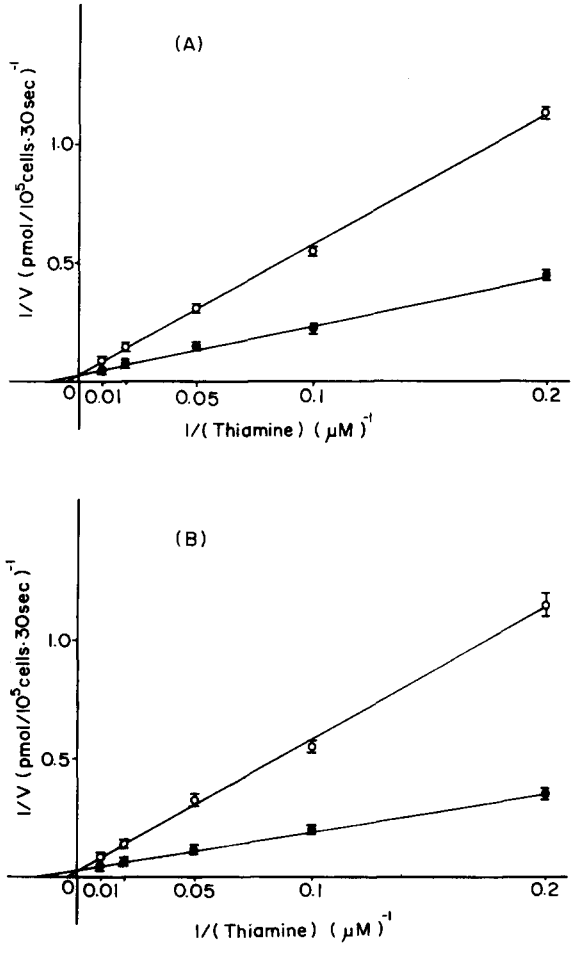



Fig. 3. (A) Lineweaver-Burk plot of thiamine uptake in the absence (●———●) and presence (○———○) of 25 $\mu$M hemicholinium-3. (B) Lineweaver-Burk plot of thiamine uptake in the absence (●———●) and presence (○———○) of 10 $\mu$M curare. The data presented in (A) and (B) were corrected for the contribution of nonsaturable uptake. Each value is the mean ± S.E. of three experiments.

hemicholinium-3 and curare were one-third and one-eighth of the $K_t$ for thiamine, respectively [4], and almost same and one-third of that of dimethialium, respectively [3]. Furthermore, hemicholinium-15, tetraethylammonium, decamethonium and tetramethylammonium were also competitive inhibitors with $K_i$ values of 16.3 $\mu$M, 24.2 $\mu$M, 0.61 mM and 0.96 mM, respectively. Although tetraethylammonium was a more potent inhibitor of thiamine transport than tetramethylammonium, the strength of the electropositivity of quaternary nitrogen atom may not play a signifi-

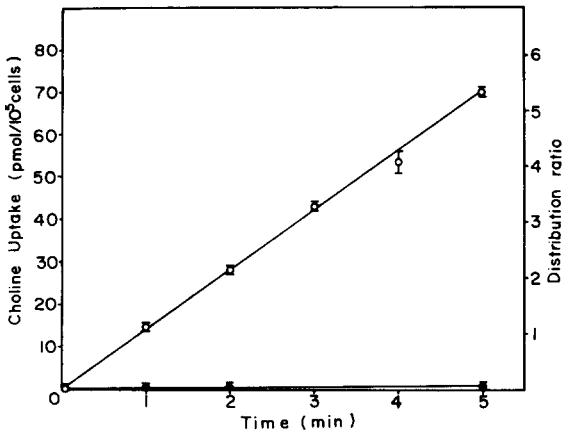



Fig. 4. Time-course of the uptake of choline by isolated rat hepatocytes. The uptake of 50 $\mu$M [$^{14}$C]choline was studied at 37°C (○——○) and 0°C (●——●). The transport assay were carried out as described in the text. The results presented are the means ± S.E. of three experiments.

cant role in their affinities for the thiamine transport system in isolated rat hepatocytes.

Fig. 4 shows the time course of [$^{14}$C]choline uptake at a concentration of 50 $\mu$M. The uptake was linear for as long as 5 min of incubation at 37°C, whereas the rate of uptake gradually slowed thereafter (data not shown). Therefore, this incubation was carried out for 1 min to measure the initial velocities of choline uptake. At 0°C, the uptake of [$^{14}$C]choline was insignificant.

Table III shows the inhibitory effect of thiamine and thiamine analogs (at concentrations of 1 mM) on the initial uptake of [$^{14}$C]choline. Pyrithiamine, oxythiamine and nonradioactive thiamine inhibited choline uptake 49, 48 and 38%, respectively, whereas among thiamine derivatives oxythiamine was a weak inhibitor of thiamine uptake in isolated rate hepatocytes [4]. Chloroethylthiamine and dimethialium, which do not have a hydroxy group in the thiazole moiety, also inhibited choline uptake 45 and 40%, respectively. On the other hand, thiamine monophosphate and thiamine pyrophosphate, which have a negatively charged group in their molecules, were much less inhibitory , an effect which is similar to their effect on thiamine uptake in isolated rat hepatocytes [4,5].

In isolated perfused rat liver, Zeisel et al. [6] demonstrated that choline was transported by two

TABLE III

EFFECT OF THIAMINE DERIVATIVES ON CHOLINE UPTAKE

The uptake of [$^{14}$C]choline was assayed as described in the text. Thiamine derivatives (1 mM) were added to the cell suspensions simultaneously with 50 $\mu$M [$^{14}$C]choline, and the mixtures were incubated for 1 min. The data presented were corrected for the contribution of nonsaturable uptake. The distribution ratio is the molar ratio of intracellular choline to choline in the medium. The results presented are the means ± S.E. of three experiments.

| Addition | [$^{14}$C]Choline uptake (pmol/10$^5$ cells per min) | Distribution | |
|---|---|---|---|
| | | Ratio | % |
| None | 18.5 ±0.715 | 1.321±0.051 | 100 |
| Thiamine | 11.5 ±0.297 | 0.821±0.021 | 62 |
| Thiamine monophosphate | 17.2 ±0.355 | 1.229±0.025 | 93 |
| Thiamine pyrophosphate | 16.2 ±0.781 | 1.157±0.056 | 88 |
| Pyrithiamine | 9.50±0.419 | 0.679±0.030 | 51 |
| Oxythiamine | 9.67±0.079 | 0.691±0.006 | 52 |
| Chloroethylthiamine | 10.3 ±0.236 | 0.736±0.017 | 55 |
| Dimethialium | 11.1 ±0.455 | 0.793±0.033 | 60 |

mechanisms; one is a saturable mechanism with a $K_t$ of 0.17 ± 0.07 mM and $V_{max}$ of 0.84 ± 0.16 $\mu$mol/min per g dry weight; the second is a nonsaturable mechanism. These investigators suggested that choline oxidase activity did not always limit choline uptake by the liver. In our studies, the initial uptake of [$^{14}$C]choline as a function of choline concentration in isolated rat hepatocytes also exhibited saturable and nonsaturable mechanisms for choline uptake (data not shown). Lineweaver-Burk analysis of the saturable component of choline uptake in the presence and absence of 1 mM thiamine is shown in Fig. 5A. The apparent $K_t$ for saturable component of choline uptake was 162 ± 3.85 $\mu$M and $V_{max}$ was 80.1 ± 1.30 pmol/10$^5$ cells per min; this $K_t$ value is comparable with that of perfused rat liver [6]. The inhibitory effect of thiamine on choline uptake was also competitive as was the case of choline on thiamine uptake. The values of $K_t$ and $V_{max}$ for choline in the presence of 1 mM thiamine were 286 ± 1.27 $\mu$M ($p < 0.001$) and 81.6 ± 1.95 pmol/10$^5$ cells per min (n.s.). These results are also similar to those described with the small intestine of the chick [9]

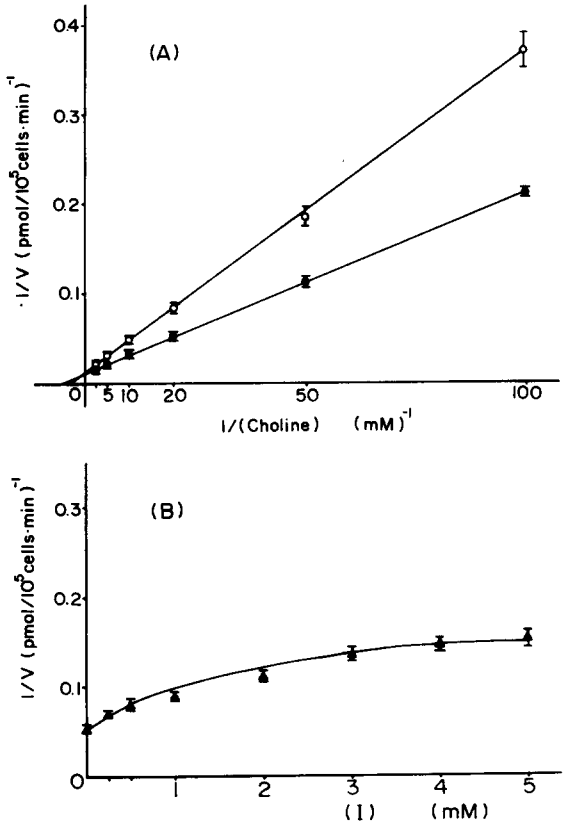



Fig. 5. (A) Lineweaver-Burk plot of choline uptake in the absence (●———●) and presence (○———○) of 1 mM thiamine. (B) Inhibition of choline uptake (at a concentration of 50 μM) by thiamine (▲———▲). Dixon plot. The data presented in (A) and (B) were corrected for the contribution of nonsaturable uptake. Each value is the mean ± S.E. of three experiments.

and the renal tubular excretion in the chicken [7]. However, in contrast to the effect of choline on hepatocyte thiamine uptake, a Dixon plot of the reciprocal of choline uptake vs. thiamine concentration exhibited a hyperbolic pattern (Fig. 5B). These data, therefore, indicated that the inhibition of thiamine on choline uptake is 'pseudo-competitive'. Thus, the uptake systems for thiamine and choline in isolated rat hepatocytes are mutually antagonistic by Lineweaver-Burk analyses; however, Dixon plots of this inhibition showed that the systems have different properties. These results strongly suggest that choline shares a common binding site in the thiamine uptake system, whereas thiamine binds at a site different from the site for choline in the choline transport system.

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