4 L. E. HOKIN, *J. Gen. Physiol.*, 54 (1969) 327.
5 R. WHITTAM AND M. E. AGER, *Biochem. J.*, 93 (1964) 337.
6 M. MAIZELS, *J. Physiol. London*, 195 (1968) 657.
7 J. S. WILLIS, *Biochim. Biophys. Acta*, 163 (1968) 516.
8 J. S. WILLIS, *J. Gen. Physiol.*, 49 (1966) 1221.
9 J. S. WILLIS, *Biochim. Biophys. Acta*, 163 (1968) 506.
10 J. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
11 J. S. WILLIS AND N. M. LI, *Am. J. Physiol.*, 217 (1969) 321.
12 A. KLEINZELLER AND A. KNOTKOVA, *J. Physiol. London*, 175 (1964) 172.

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Subcellular distribution of thiamine pyrophosphokinase activity in rat liver and erythrocytes

The transport of thiamine across the cell surface membrane is of basic importance for the study of thiamine metabolism in mammalian tissues. Until now only little is known about the mechanism of uptake of thiamine into the cell.

Since thiamine pyrophosphokinase was found exclusively in the membrane fraction (spheroplasts) of *Escherichia coli*, the participation of this enzyme in the transport of thiamine across the membrane barrier has been postulated[1].

The aim of the present study was to check whether this hypothesis was applicable to mammalian cells. Therefore the distribution of thiamine pyrophosphokinase in the subcellular fractions of rat liver and rat erythrocytes was investigated. The results provide evidence that thiamine pyrophosphokinase is localized in the soluble phase of both types of cells. A preliminary report on the fractionation of erythrocytes has been published[2].

Male albino Sprague-Dawley rats of 220–280 g weight, raised on a commercial stock diet, were decapitated after they had been starved for 12 h.

Nucleotides and phosphorylated sugars were obtained from C. F. Boehringer & Soehne, 68 Mannheim, Germany. Bovine serum albumin was a product of the Behringwerke, 355 Marburg, Germany and Dextran 150 of Pharmacia, Uppsala, Sweden. Thiamine(thiazole-2-^{14}C)hydrochloride was purchased from the Radiochemical Centre, Amersham, England.

Rat liver cell fractionation was carried out according to the method of DE DUVE *et al.*[3]. For the fractionation of erythrocytes, blood was collected in heparinized glass vessels. The white blood cells were removed almost completely with the "buffy layer". The erythrocytes were washed twice with 0.15 M NaCl and hemolysed by addition of 4 vol. of distilled water. The suspension was frozen and thawed twice. Hemolysate supernatant was obtained by centrifugation of the hemolysate at 105000 × *g* for 1 h. The erythrocyte membrane fraction (ghosts) was prepared according to the method of REGA *et al.*[4]. Leucocytes were obtained by the procedure of HOLLDORF *et al.*[5].

Thiamine pyrophosphokinase (EC 2.7.6.2) activity was determined as described previously[6,7], NAD nucleosidase (EC 3.2.2.5) activity according to the method of KAPLAN[8], phosphoglucomutase (EC 2.7.5.1) activity by the method of NOLTMANN AND BRUNS[9], acid phosphatase (EC 3.1.3.2) activity by the procedure of GIANETTO AND DE DUVE[10], and glucose-6-phosphatase (EC 3.1.3.9) activity by the method of DE DUVE *et al.*[3] but with 0.05 M histidine in the incubation medium. Cytochrome oxidase (EC 1.9.3.1) was assayed by the method of APPELMANS *et al.*[11]. Absorbance at 546 nm was followed with an "Eppendorf" recording spectrophotometer.

Inorganic phosphate was estimated by the procedure of KUTZBACH AND JAENICKE[12]. Bovine serum albumin was used as a standard for the determination of protein by the method of LOWRY *et al.*[13]. The preparation of ^{14}C-labelled thiamine pyrophosphate, the determination of thiamine and thiamine pyrophosphate and the estimation of thiamine pyrophosphate cleavage have been previously published[6,7].

Subcellular distribution of rat liver thiamine pyrophosphokinase activity: The distribution pattern of thiamine pyrophosphokinase activity is shown in the lower part of Fig. 1. More than 98 % of the activity was found in the soluble phase. As can be inferred from the distribution of cytochrome oxidase (see upper part of Fig. 1), no leakage of mitochondrial enzymes had occurred during the fractionation process. Acid phosphatase and glucose-6-phosphatase activities, which showed the expected distribution, were completely absent from the soluble fraction. This indicated that no enzymatic activities had been released from the particles into the soluble phase. Even after solubilization of the particulate fractions with 2 % deoxycholate, no

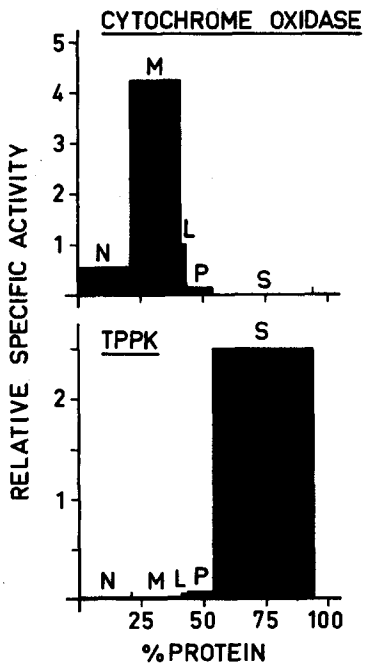

Fig. 1. Subcellular distribution of rat liver cytochrome oxidase and thiamine pyrophosphokinase (TPPK). N = nuclear fraction; M = mitochondrial fraction; L = lysosomal fraction; P = microsomal fraction; S = soluble fraction. Protein recovery = 93.6 %.

thiamine pyrophosphokinase activity was detected. ATPase, which might be present in these fractions, interferes with the determination of thiamine pyrophosphokinase activity, since it reduces the concentration of ATP in the assay mixture. Furthermore, ADP and AMP arising from the action of ATPase, strongly inhibit thiamine pyrophosphokinase[6,7]. Therefore the concentration of ATP in the assay was varied. With increasing ATP concentration, thiamine pyrophosphokinase activity in the particles remained negligible. In the soluble phase the optimal ATP concentration was the same as that reported recently for rat liver cytosol[6,7].

Subcellular distribution of rat erythrocyte thiamine pyrophosphokinase activity: Table I demonstrates that more than 99% of thiamine pyrophosphokinase activity was observed in the supernatant obtained by centrifugation of the hemolysate at 105000 × *g* for 1 h. Less than 1% of the activity was found in the sediment or in the ghost preparation. The marker enzymes of the soluble phase (phosphoglucomutase) and of the membrane fraction (NAD nucleosidase) indicated that no mutual contamination existed and that the membranes were intact. By treatment of the hemolysate sediment and the ghosts with 1% deoxycholate, no thiamine pyrophosphokinase activity was released from the membranes. ATPase, which is localized in the membranes of erythrocytes, could be removed from the hemolysate by centrifugation at 105000 × *g* for 1 h and was inactivated by treatment of the fractions with deoxycholate or Triton X-100. Even after inactivation of ATPase, no thiamine pyrophosphokinase activity was observed in the membranes. Because all fractions lacking thiamine pyrophosphokinase activity cleaved less than 10% of added thiamine pyrophosphate, it was obvious that these fractions were actually free of thiamine pyrophosphokinase.

In contrast to earlier findings[14] no thiamine pyrophosphokinase activity was observed in leucocytes, even on treatment with deoxycholate.

TABLE I

Subcellular distribution of rat erythrocyte thiamine pyrophosphokinase, NAD nucleosidase and phosphoglucomutase. (100% corresponds to the following specific activities: thiamine pyrophosphokinase, $8 \cdot 10^{-4}$ mU/mg protein, at 37°; NAD nucleosidase, $3 \cdot 10^{-2}$ mU/mg protein, at 25°; and phosphoglucomutase, $2.7 \cdot 10^{-1}$ mU/mg protein, at 25°. The last column gives the percentage of thiamine pyrophosphate which is cleaved by the fractions during the incubation period.)

Fraction	*Relative enzymatic activity*			*Thiamine pyrophosphate cleavage* (%)
	Thiamine pyrophosphokinase	*NAD nucleosidase*	*Phosphoglucomutase*	
Hemolysate	13			2
+ 0.5% deoxycholate	98			0
+ 1.0% Triton X-100	95			
Hemolysate supernate	100	0	100	0
+ 0.5% deoxycholate	97			0
Hemolysate sediment	<1			3
+ 1.0% deoxycholate	<1			0
Ghost preparation	<1	98	0	1
+ 1.0% deoxycholate	<1	100	0	7

The data presented here provide substantial evidence that thiamine pyrophosphokinase is localized exclusively in the soluble fractions of rat liver cells and erythrocytes. This is in agreement with the observation of LEUTHARDT AND NIELSEN[15], who found thiamine pyrophosphokinase activity only in the 25000 × *g* supernatant of rat liver homogenate. It seems most unlikely that the fractionation procedure released a loosely held particulate enzyme into the soluble fraction, because the same thiamine pyrophosphokinase activity was present in the supernatant when rat liver homogenate had been centrifuged directly. Since there is no appreciable thiamine pyrophosphate-cleaving activity and since there is no thiamine pyrophosphokinase activity detectable in the particulate fractions when ATPase is inhibited, thiamine pyrophosphokinase is considered to be a completely soluble enzyme.

Consequently, thiamine pyrophosphokinase cannot participate in the transport of thiamine across the membranes of rat liver and erythrocytes. The question arises whether there exists a specific "thiamine permease" in mammalian cell membranes or not.

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1 I. MIYATA, T. KAWASAKI AND Y. NOSE, *Biochem. Biophys. Res. Commun.*, 27 (1967) 601.
2 H. BLUM AND B. DEUS, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 1240.
3 C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
4 A. F. REGA, R. I. WEED, C. F. REED, G. G. BERG AND A. ROTHSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 297.
5 A. W. HOLLDORF, O. E. ZINSSER AND P. OBRECHT, in preparation.
6 B. DEUS, H. E. C. BLUM AND H. HOLZER, *Anal. Biochem.*, 27 (1969) 492.
7 B. DEUS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 18, Academic Press, New York and London, 1970, p. 221.
8 N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York and London, 1955, p. 660.
9 E. NOLTMANN AND F. H. BRUNS, *Hoppe-Seyler's Z. Physiol. Chem.*, 313 (1958) 194.
10 R. GIANETTO AND C. DE DUVE, *Biochem. J.*, 59 (1955) 433.
11 F. APPELMANS, R. WATTIAUX AND C. DE DUVE, *Biochem. J.*, 59 (1955) 438.
12 C. KUTZBACH AND L. JAENICKE, *Ann. Chem.*, 692 (1966) 26.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 G. SMITS AND E. FLORIJN, *Biochim. Biophys. Acta*, 5 (1950) 535.
15 F. LEUTHARDT AND H. NIELSEN, *Helv. Chim. Acta*, 35 (1952) 1196.

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