DISPLACEMENT OF THIAMINE PYROPHOSPHATE FROM SWOLLEN MITOCHONDRIA

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It has been shown in previous publications that swollen mitochondria from fatty liver contain decreased amounts of cytochrome *c*, of pyridine nucleotides and of adenylnucleotides[1-3]. One of the most important reasons for the decrease has been found to be the increased diffusion of these substances from the mitochondria into the surrounding medium.

It seemed interesting to us to investigate the behaviour of another coenzyme, thiamine pyrophosphate (TPP) in mitochondria from fatty liver. The distribution of TPP in liver cells of normal and thiamine-deficient rats has been studied by GOETHART[4]. This author reported that in the normal rat about 30% of the TPP is contained in the mitochondria, while the rest is present mainly in the soluble part of the cytoplasm.

EXPERIMENTAL

Wistar albino rats weighing 100–120 g fed on a standard diet were used. They were killed by decapitation and the livers immediately removed and transferred to the cold room at 2° C. 10% homogenates were prepared in a Potter Elvehjem homogenizer with 0.25*M* sucrose in most cases. In some instances, 0.25*M* sucrose containing polyvinylpyrrolidone, pH 7.6, as described by NOVIKOFF[5], was used. In other experiments, the homogenates were made in water. Differential centrifugation of the homogenates was performed either in the Spinco preparative ultracentrifuge, or in the Zernike refrigerated centrifuge, at 2°. The nuclear fraction, which contained nuclei, unbroken cells, red cells, tissue débris and a small amount of mitochondria, was sedimented at 1,000 *g* for 10 min. The sediment was washed thrice, in order to decrease the mitochondrial contamination. Mitochondria were sedimented from the combined supernatant fluids at 12,000 *g* for 30 min. In order to prevent damage to the particles, the mitochondrial sediment was used without washing. Microsomes were sedimented at 48,000 *g* for 1 h. Since the amount of TPP present in this fraction is very small[4], microsomes were not isolated in most experiments. The fraction consisting of the soluble part of the cytoplasm plus microsomes is reported in the text and in the tables as "supernatant fraction".

TPP was determined by the "one-stage method" of WESTENBRINK AND STEYN PARVÉ[6]. Each determination was performed in triplicate.

The nitrogen content of each homogenate and of each fraction was determined in small samples by the usual micro-kjeldahl technique.

The content of total lipids of each liver was determined by extracting the dry powder of the organ with ether for 3–4 hours in a Kumagawa-Suto apparatus and by determining the difference in weight of the sample before and after the extraction.

Fatty infiltration of the liver was obtained: (1) by injecting daily subcutaneously 0.2 ml of a 20% solution of CCl_4 in peanut oil, during a period of 3 days (2) by feeding the animals for 4 weeks on a diet deficient in choline. The composition of this diet has been reported elsewhere[2]. A group of rats fed on this diet received each day 10 mg choline hydrochloride intraperitoneally; this group was used as a control. The obtained values were analysed for statistical significance

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by calculating the "t" values of Fisher. Only the differences with a "t" value corresponding to a probability $P<0.01$ were accepted as significant. The standard deviation of the mean (σ) is reported in the tables after each average value.

RESULTS

Table I reports the data for the TPP distribution in the homogenate fractions of normal rats. The obtained values were very similar to those reported by GOETHART[4], when the suspension medium was 0.25 M sucrose. The same distribution was found also in the homogenates prepared with 0.88 M sucrose (mitochondria in this case were sedimented at 48,000 g for 30 min), or with 0.25 M sucrose containing polyvinylpyrrolidone. When the homogenates were prepared with distilled water, the distribution of TPP in the cytoplasm fractions was however very different. In fact, the amount of TPP contained in the mitochondria was strongly decreased under these conditions, while a corresponding increase was observed in the supernatant fraction.

Some experiments were made in order to study the influence of hypotonic treatment of mitochondria prepared in 0.25 M sucrose on their TPP content. Mitochondria isolated from 1 g normal liver with 0.25 M sucrose were suspended again in 0.25 M sucrose, divided into two identical fractions and resedimented at 18,000 g for 30 min. The two mitochondrial pellets were then suspended in 0.25 M sucrose and in water respectively, and the suspensions incubated at 18° C for times varying from 0 to 30 min. In some experiments the incubation temperature was 4° C.

TABLE I

CONTENT AND DISTRIBUTION OF TPP IN CYTOPLASM FRACTION OF NORMAL RATS

	*Homogenates in 0.25 M sucrose**			*Homogenates in water***		
	μg/g liver	*μg/mg N*	%	*μg/g liver*	*μg/mg N*	%
Homogenate	14.0 ± 1.9	0.45		13.4 ± 0.9	0.42	
Nuclear fraction	0.9 ± 0.3	0.19	7.1 ± 2.9	0.55 ± 0.16	0.13	4.6 ± 1.3
Mitochondria	4.85 ± 1.14	0.61	36.1 ± 5.2	2.76 ± 0.29	0.32	23.1 ± 2.1
Supernatant	7.58 ± 1.23	0.45	56.8 ± 4.1	8.62 ± 0.71	0.50	72.3 ± 2.4
Recovery %	94.2 ± 2.5			89.0 ± 2.2		

* 10 experiments.
** 6 experiments.

After incubation, the mitochondria were sedimented again at 18,000 g for 30 min. TPP was then determined both in the sediment and in the supernatant fluid. As is shown in Table II, more than 80% of the TPP was still contained in the sediment after 10 min incubation at 18° C when the suspension fluid was 0.25 M sucrose. When the suspension fluid was water, only 35% of the TPP was still contained in the sediment after this time. The total amount of TPP recovered was higher in 0.25 M sucrose than in water. This means that a considerable part of TPP is destroyed as a result of incubation of mitochondria in water. This fact agrees with the finding by BERTHET *et al.*[7] that the activity of the acid phosphatase, present in the lysosomes, increases strongly upon suspending in water.

When the incubation time was more than 10 min, the amount of TPP present in the sediment was strongly decreased also in mitochondria suspended in 0.25 M

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TABLE II

RELEASE OF TPP FROM MITOCHONDRIA AFTER INCUBATION IN DIFFERENT SUSPENSION MEDIA

The values represent the mean of 3–4 experiments.

Incubation time in minutes	*Incubation temperature*	*Suspension medium*	*TPP recovered in the sediment*		*TPP recovered in the supernatant*		*Total recovery*
			μg	*% of total*	*μg*	*% of total*	*μg*
10	18° C	0.25*M* sucrose	5.00 ± 0.20	85.9 ± 6.3	0.82 ± 0.05	14.1 ± 4.6	5.82
		water	1.12 ± 0.09	35.2 ± 5.5	2.06 ± 0.42	64.8 ± 5.8	3.18
20	18° C	0.25*M* sucrose	1.39 ± 0.19	42.7 ± 4.8	1.86 ± 0.25	57.3 ± 4.3	3.25
		water	0.40 ± 0.08	16.2 ± 2.5	2.06 ± 0.38	83.8 ± 3.9	2.46
30	18° C	0.25*M* sucrose	0.82 ± 0.15	30.1 ± 2.9	1.91 ± 0.24	69.9 ± 2.7	2.73
		water	0.37 ± 0.12	23.8 ± 2.2	1.20 ± 0.25	76.2 ± 4.7	1.57
20	4° C	0.25*M* sucrose	4.10 ± 0.23	95.3 ± 1.4	0.20 ± 0.05	4.7 ± 1.3	4.30
		water	1.55 ± 0.35	39.3 ± 2.8	2.40 ± 0.19	60.7 ± 2.6	3.95
20	18° C	0.88*M* sucrose	2.72 ± 0.13	79.5 ± 2.8	0.70 ± 0.23	20.5 ± 2.4	3.42
		water	0.37 ± 0.11	16.4 ± 0.9	1.89 ± 0.23	83.6 ± 0.9	2.26

sucrose, while a corresponding increase was found in the supernatant. After an incubation time of 20 to 30 minutes, mitochondria are considerably swollen in 0.25*M* sucrose also. Thus it seems highly probable that loss of TPP from the particles occurring under these conditions is concerned with the loss of their structural integrity.

When the incubation temperature was 4° C, practically no difference in total TPP recovery was found between mitochondria incubated in 0.25*M* sucrose and those suspended in water. The difference in distribution between sediment and supernatant remained, however, consistent. This means that the decrease of TPP in mitochondria suspended in water is the result of at least two phenomena, *viz.* destruction, probably through phosphatase action, and displacement by diffusion. Since the temperature during the fractionation procedure of the homogenates was 2° C, it seems improbable that the activation of phosphatase has played a role in decreasing the percentage of TPP contained in the mitochondria isolated from water homogenates (Table I). This is shown also by the fact that the % recovery of TPP after fractionation was about the same for both the homogenates prepared with 0.25*M* sucrose and those prepared with water.

TABLE III

CONTENT AND DISTRIBUTION OF TPP IN FATTY LIVER

	Fatty liver by CCl_4			*Fatty liver by feeding on the diet deficient in choline*		
	μg/g liver	*μg/mg N*	*%*	*μg/g liver*	*μg/mg N*	*%*
Homogenate	12.18 ± 3.43	0.42		8.23 ± 1.92	0.31	
Nuclear fraction	1.16 ± 0.6	0.24	10.1 ± 4.6	0.47 ± 0.19	0.09	6.0 ± 2.3
Mitochondria	2.41 ± 0.75	0.30	21.0 ± 3.2	2.04 ± 0.53	0.27	25.8 ± 1.9
Supernatant	7.90 ± 2.57	0.52	68.9 ± 5.3	5.36 ± 1.15	0.39	68.2 ± 2.8
Recovery %	94.2 ± 3.5			94.1 ± 3.8		
Number of experiments	8			8		
Total fats in the liver (mg/g)	58.8 ± 6.9			86.0 ± 8.7		

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Table III reports the values for TPP distribution in fatty liver. A strong decrease of TPP content of the mitochondria as compared to the content of mitochondria from normal livers was recorded in both types of fatty livers studied. The amount contained in the supernatant was increased on a % basis. The TPP distribution in fatty liver thus resembles that found for water homogenates of normal livers.

Rats fed on a diet deficient in choline, in contrast to those injected with CCl_4, also showed a decrease in total TPP per g liver. This was not entirely dependent upon partial replacement of protein by fat since the amount of TPP per mg N was also decreased.

Rats fed on the same diet for 5 weeks, and injected with 10 mg choline every day, showed neither a significant change of total TPP content per g liver, nor a modification of the TPP distribution in the cytoplasm fractions. Total lipid content of the livers of these animals was normal.

DISCUSSION

The experiments described in this paper show that TPP is displaced from the mitochondria into the surrounding medium as a consequence of hypotonic treatment. This displacement is already observed with swollen mitochondria from fatty livers that have not undergone hypotonic treatment. This seems to be another case of a coenzyme that is displaced from mitochondria as a consequence of morphological damage.

The amount of TPP contained in fatty liver obtained by feeding rats on the diet deficient in choline was considerably decreased. This result would be expected on the basis of the existing knowledge. In fact, increase of acid phosphatase activity occurs in fatty livers[8], and it may produce increased destruction of TPP. In addition, ATP concentration in fatty liver is less than normal[3], and this may be the cause of decreased synthesis of TPP through the phosphorylation of thiamine.

The fact that the decrease of TPP did not occur in fatty liver produced by CCl_4 is however difficult to reconcile with these hypotheses. In fact, both phosphatase activation and decrease of ATP occur also in this type of liver injury[8]. The reason for the different behaviour of TPP in the two types of fatty liver infiltration studied, may lie perhaps in the difference in the length of time for which the treatment was applied; this was 4 weeks in the case of rats fed on the diet deficient in choline, and only 3 days in that of animals injected with CCl_4. In addition, a considerable amount of regeneration always occurs in the liver following treatment with CCl_4, and this may have complicated the results. It is at present impossible to say whether the displacement of TPP from mitochondria cooperates in the production of decreased oxidation of pyruvate, which has been observed in fatty liver (DIANZANI[2], FISCHER[9], FISCHER AND RECANT[10]). It is known that the pyruvate oxidizing system requires the presence of several other factors, some of which (DPN, DIANZANI[2]; CoA, SEVERI AND FONNESU[11]) are also decreased in fatty liver. The

Rats fed on this diet and treated with 10 mg choline daily		
µg/g liver	*µg/mg N*	%
12.0 ± 0.80	0.41	
0.9 ± 0.2	0.15	7.8 ± 1.5
4.0 ± 0.2	0.52	36.2 ± 0.9
6.2 ± 0.3	0.41	56.0 ± 1.8
93.3 ± 2.6		
6		
40.5 ± 2.8		

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addition of sufficient amounts of DPN to mitochondrial preparations restores almost completely pyruvate oxidation under aerobic conditions[2]. It may be remembered, with regard to the last point, that the effect of the addition of TPP on the oxidation of pyruvate by oxygen is also very small in homogenates of breast and heart muscles of thiamine-deficient pigeons (FRANKEN AND STAPERT[12]).

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SUMMARY

The distribution of TPP in cytoplasm fractions of rat liver is studied. When the homogenate was prepared with 0.25*M* sucrose, about 30% of TPP was present in mitochondria. When the homogenization medium was distilled water, the amount of TPP present in this fraction was markedly decreased, while that present in the supernatant fluid was increased. Mitochondria suspended in 0.25*M* sucrose and incubated at 18° C for 10 min release in the suspension fluid about 20% of their TPP; under the same conditions, mitochondria suspended in water release about 65% of their TPP. An increased destruction of TPP was observed as a result of incubation of mitochondria in water at 18° C.

The distribution of TPP in fatty liver homogenates prepared in 0.25 *M* sucrose resembles strongly that observed for homogenates of normal rat liver prepared in distilled water; TPP was decreased in the mitochondrial fraction and correspondingly increased in the supernatant. Total TPP is decreased in fatty liver obtained by feeding rats on a diet deficient in choline. It remains practically unchanged in fatty liver produced by CCl_4.

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THE EFFECT OF EDTA ON THE INTERACTION BETWEEN ACTOMYOSIN AND ADENOSINE TRIPHOSPHATE*

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It has been well established by FRIESS[1] and BOWEN AND KERWIN[2] that EDTA is a striking activator of myosin ATPase in the presence of high concentration of KCl.

* The following abbreviations will be used; ATP adenosine triphosphate, ATPase adenosine-triphosphatase, and EDTA ethylenediaminetetraacetic acid.

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