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Thiamine Phosphates and Phosphate Transport in Liver Mitochondria

KARL-HEINZ KIESSLING

Institute of Zoophysiology, Uppsala, Sweden

Incubation of mitochondria with radioactive inorganic phosphate results in an incorporation of ^{32}P into the intramitochondrial thiamine diphosphate. The incorporation is localized mainly to the terminal phosphate group (βP), and is higher with, than without pyruvate as a substrate.

Synthetic thiamine compounds (thiamine, thiamine mono-, di-, tri-, and tetraphosphate) added to mitochondria do not, however, take part in the phosphate turnover in the same degree as the endogenous compound. No further synthesis from the added compounds could be found, nor did the thiamine phosphates transfer their phosphate directly to the adenylic system at any appreciable rate. Only a very slight incorporation of ^{32}P into the added thiamine phosphates could be obtained.

In animal tissues thiamine occurs mainly as thiamine diphosphate and only to a smaller extent as thiamine. The occurrence of thiamine triphosphate * in rat liver has been reported by Rossi-Fanelli, Siliprandi, and Fasella [1].

Only few investigations dealing with the role of thiamine phosphates in the phosphate transport have been published. Kiessling and Lindahl [2] found a turnover of the phosphorus of TDP in a cyclophorase system isolated from guinea pig liver when incubated with inorganic ^{32}P, and the turnover rate was higher when pyruvate is metabolized than without added "sparker". No synthetic TDP was added, *i. e.* the only TDP present during the incubation was that initially existing in the liver (endogenous TDP) **.

* The following abbreviations have been used: T thiamine, TMP thiamine monophosphate, TDP thiamine diphosphate, TTP thiamine triphosphate, TTeP thiamine tetraphosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, G-6-P glucose-6-phosphate, P_i inorganic phosphate, ^{32}P radioactive phosphate, TCA trichloroacetic acid, P/O ratio of number of micromoles of esterified phosphate to the number of microatoms of oxygen consumed.

** Endogenous thiamine phosphate: thiamine phosphate originating from the tissue. In this paper two types of endogenous thiamine phosphate have been distinguished between, *viz.* intra- and extramitochondrial thiamine phosphate.

Synthetic thiamine phosphate: thiamine phosphate added to the samples and hence not originating from the tissue.

Bartley [3] on the other hand was able to demonstrate only a rather low turnover of phosphate in TDP, when adding this substance to a kidney cyclophorase system with pyruvate or α-ketoglutarate as a substrate.

However, using ^{32}P-labelled synthetic TDP, Ogata, Nohara, Morita, and Kawai [4] found a direct transport of phosphate from TD^{32}P to ADP with a liver cyclophorase system.

The first part of this paper deals with the incorporation of radioactive phosphorus into endogenous TDP in mitochondria. In the second part the participation in the phosphate transport of synthetic thiamine phosphates added to mitochondria has been studied (Fig. 1).

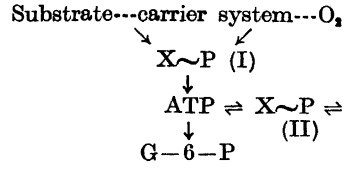

Fig. 1.

Two possibilities for the participation of thiamine compounds in the phosphate metabolism are proposed in Fig. 1. X~P indicates a thiamine phosphate. This thiamine phosphate can either be involved in the phosphate transport from the substrate-carrier level to glucose (I), or be in equilibrium with ATP in a side reaction (II). The second part of the investigation has been carried out along these alternative possibilities.

MATERIALS AND METHODS

Mitochondria were prepared from rat and guinea pig liver mainly according to the method of Schneider and Hogeboom [5]. The medium for isolation was 0.25 M sucrose, and after two washings the mitochondria were suspended in 0.25 M sucrose. The pH of the suspension was adjusted to 7.4 with TRIS-buffer or potassium phosphate buffer to a final concentration of 0.03 M.

In the experiments with rat liver, mitochondria from 10 g of liver were suspended in the buffers mentioned, and made up with sucrose to a final volume of 20 ml. To each Warburg vessel 1 ml of the mitochondrial suspension was added together with 1.2 ml of other solutions (*cf.* below); 0.2 ml KOH 2 M was added to the center well.

When isolated from guinea pig liver, large quantities of mitochondria were used in each sample (mitochondria from 5—10 g of liver). For this reason these incubations were performed in Fernbach flasks. The size of the flasks was about 200 ml. 3 ml from each sample were transferred to Warburg vessels for respiration measurements. At the end of the experiment 0.2 ml H_2SO_4 5 M was added to the Warburg vessels, and TCA to a concentration of 9 % to the Fernbach flasks in order to stop the reaction. The precipitate formed was centrifuged down and pH adjusted to 3.

From the Warburg incubations with rat mitochondria the thiamine phosphates were separated by paper chromatography [13] directly after pH had been adjusted to 3. From the Fernbach incubations with guinea pig mitochondria TDP was isolated, except in one case, by adsorption on Fuller's earth, eluated with pyridine, and purified by paper chromatography as described by Kiessling [6]. Finally a second paper chromatographic solvent (Siliprandi and Siliprandi [7]) was used in both cases as an additional purification of the thiamine phosphates. With the two solvents used the R_F values of the thiamine phosphates and of P_i differ greatly, and after the application of the two methods no inorganic phosphate could be found to contaminate the thiamine phosphates.

In the experiments with guinea pig mitochondria only endogenous TDP was present during the incubations. To facilitate the isolation synthetic TDP (2–5 mg) was added after the proteins were detached, and pH adjusted to 3. The TDP isolated in this way was a mixture of endogenous and synthetic TDP. According to Goethart [8] mitochondria from 1 g of liver contain about 4 μg TDP. Hence a rough estimate of the radioactivity of the intramitochondrial TDP can be obtained by multiplication with a dilution factor.

ATP+ADP was precipitated with Lohmann's reagent [9], and isolated by means of paper chromatography [10]. In the experiments with hexokinase present the amount of ATP was low. The specific radioactivity therefore was determined on hydrolyzable phosphate from ATP+ADP isolated from the samples.

The radioactivity was measured in an M 6 standard liquid counter tube from 20th Century Electronics Ltd. The radioactivity figures always refer to counts per 5 min.

Phosphorus was determined according to Berenblum and Chain [11]. TDP supplied by Roche Products Ltd. contained only traces of TMP. ATP and ADP in the form of the sodium salt were purchased from Sigma Chemical Company.

In my experiments I needed TDP, TTP, and TTeP labelled only in their hydrolyzable phosphate groups. By heating thiamine and unhydrous phosphoric acid Viscontini *et al.*[12] had obtained a mixture of thiamine phosphates from which they could separate TMP, TDP, and TTP by means of paper chromatography. Using another solvent I had been able to isolate from this mixture, besides the compounds mentioned, also TTeP and four additional thiamine phosphates with more than four phosphate groups [13]. By heating TDP (or TMP) with a mixture of anhydrous phosphoric acid and radioactive inorganic phosphate I obtained a mixture of thiamine phosphates labelled in the desired way. They could be separated by paper chromatography as described for the non-labelled compounds above [13]. The radioactivity of the hydrolyzable and non-hydrolyzable phosphate (the latter value in brackets) was for TDP 70 700 (600), TTP 31 600 (208), and TTeP 10 600 (160). These figures are intended only to give an idea of the relative distribution of ^{32}P within the molecules *.

The yeast hexokinase was prepared at the Wenner Gren Institute in Stockholm according to Berger *et al.*[14], except for the cytolysis in toluene, which was performed as described by Bailey and Webb [15]. The purification was followed up to step five.

RESULTS

Incorporation of ^{32}P into endogenous TDP in liver mitochondria

The mitochondria from a guinea pig liver (20 g liver) were isolated, and distributed on two Fernbach flasks, one with and one without substrate. The supernatant obtained at the preparation of the mitochondria from 10 g of liver was added to a third flask together with substrate. The substrate was pyruvate together with small amounts of α-ketoglutarate (*cf.* Table 1). To all flasks ATP, glucose, hexokinase, cytochrome c and cozymase were added. Besides these additions each flask contained radioactive inorganic phosphate. The final volume was 45 ml. From each sample 3.0 ml were transferred to Warburg vessels for respiration measurements. The oxygen consumption is shown in Fig. 2. 5 mg synthetic TDP was added to each sample to facilitate the isolation of endogenous TDP.

From the figures in Table 1 it appears that the highest specific radioactivity of TDP has been obtained in the sample with mitochondria and substrate (1.6×10^3 counts/100 μg hydrolyzable P). In accordance with the calculations

* Ogata *et al.*[4] have prepared radioactive TDP in which, however, the αP and βP were equally labelled.

Table 1.

Incubation	Radioactivity of endogenous + synt. TDP	Radioactivity of endogenous TDP	Radioactivity of ATP—ADP	Radioactivity of inorg. P
Mitoch. + substrate	1.6×10^3	2.00×10^5	4.20×10^5	1.34×10^6
Supernatant + substrate	0.30×10^3	0.30×10^5	1.20×10^5	1.05×10^6
Mitoch. without substrate	0.46×10^3	0.57×10^5	0.53×10^5	1.09×10^6

Radioactivity of TDP, inorganic and organic phosphate after incubation of mitochondria and supernatant from guinea pig liver with radioactive inorganic phosphate.

No synthetic TDP was added to the samples until the incubation was terminated, and then only to make possible the isolation of endogenous TDP. The figures given in the table for TDP refer to 100 μg synthetic + endogenous TDP, or to 100 μg endogenous TDP only.

The vessels contain 10 mg ATP, 0.5 ml cytochrome c 0.1 %, 0.5 ml DPN (0.3 mg/ml), 0.3 ml α-ketoglutarate 0.3 M, 1.0 ml pyruvate 0.3 M, 1.0 ml $MgCl_2$ 0.1 M, 75 mg glucose, hexokinase in excess, non-radioactive and radioactive inorganic phosphate. Mitochondria or supernatant was added as is seen in the table, and the volume made up to 45 ml with sucrose 0.25 M. In the third flask α-ketoglutarate and pyruvate were omitted. The figures refer to 100 μg phosphorus (for TDP and ATP this means hydrolyzable phosphorus). Time of incubation 20 min.

mentioned above 100 μg hydrolyzable intramitochondrial TDP phosphorus gives 2.0×10^5 counts, *i. e.* nearly half that of ATP—ADP (4.2×10^5 counts/100 μg hydrolyzable P).

In the sample with supernatant and substrate the TDP exhibited radioactivity, possibly depending on synthesis of TDP from thiamine as the synthesis of TDP is mainly localized to the supernatant according to Leuthardt and Nielsen [16]. The radioactivity was about 1/5 of that of intramitochondrial

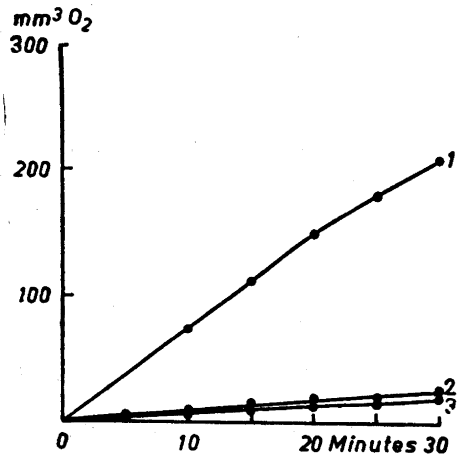

Fig. 2. Oxygen consumption in mitochondria and supernatant from guinea pig liver. 3 ml from each Fernbach flask (Table 1) were added to Warburg vessels. The center wells contained 0.2 ml KOH 2 M, and the gas phase was air.
1. mitochondria + substrate.
2. supernatant + substrate.
3. mitochondria without substrate.

TDP. Thus the radioactivity of the intramitochondrial TDP cannot, to any great extent, come from TDP synthesized in the mitochondria during the incubation.

A modification of the previous type of experiment was carried out as a control. The mitochondria were incubated with substrate as described above. The vessel contained mitochondria from 5 g of liver, the final volume being 20 ml. The radioactivity of the added phosphate was six times stronger than in the experiment above. After 20 min at 30°C the sample was rapidly cooled down to 0°C, the mitochondria centrifuged down, and washed once with sucrose. By this procedure most of the radioactive inorganic phosphate and other additions were removed, and in this way a small extract could be obtained for isolation of TDP. TCA was added to the washed mitochondria to a final volume of 5 ml giving a concentration of 9 %. After removal of the precipitated proteins and adjustment of pH to 3, 2 mg synthetic TDP were added. One ml of the sample was put as a line on a Whatman No. 1 paper, and TDP isolated by chromatography [13].

The radioactivity of the hydrolyzable phosphate, referred to 100 μg of endogenous TDP-phosphate was 2.87×10^6 counts and 4.3×10^6 for ATP—ADP. The inorganic phosphate gave 7.6×10^6 counts per 100 μg P.

In a third type of experiment the ratio between the amount of mitochondria present and the radioactive labelling of the intramitochondrial TDP was examined. Mitochondria from 10 g of liver were added to vessel 1, and from 5 g to vessel 2. Otherwise the additions were the same to both vessels. The same amount of radioactive inorganic phosphate was added to the two vessels, and the final volume of both made up to 33 ml with 0.25 M sucrose. After incubation the proteins were removed with TCA, 2 mg synthetic TDP added, and the synthetic + intramitochondrial TDP isolated by adsorption on Fuller's earth and by paper chromatography as described above. The results are given in Table 2.

Table 2.

Sample	Oxygen consumption	Radioactivity of endogenous + synt. TDP	Radioactivity of endogenous TDP	Radioactivity of ATP—ADP	Radioactivity of inorg. P
1. (10 g liver)	121	3.30×10^4	1.66×10^6	2.26×10^6	4.23×10^6
2. (5 g liver)	54	1.77×10^4	1.77×10^6	3.11×10^6	5.68×10^6

Results from an experiment with various amounts of mitochondria.
The oxygen consumption is given as mm^3/20 min/3 ml sample. The radioactivity of TDP and ATP—ADP is expressed as counts/100 μg hydrolyzable P, and of inorganic phosphate as counts/100 μg P. Apart from mitochondria the Fernbach flasks contained 10 mg ADP, 1 mg cytochrome c, 0.3 mg cozymase, 0.3 ml α-ketoglutarate 0.3 M, 1.5 ml pyruvate 0.3 M, 1.0 ml Mg^{2+} 0.1 M, 75 mg glucose, hexokinase and radioactive inorganic phosphate. Mitochondria from 10 g of liver suspended in 20 ml sucrose-phosphate medium pH 7.4 were added to sample 1 and from 5 g to sample 2, and the volume made up to 33 ml with 0.25 M sucrose. The samples were incubated at 30°C for 20 min.

The figures show that the specific radioactivity of the total TDP (synthetic + intramitochondrial) from sample 1 (10 g of liver) is about twice that of sample 2 (5 g of liver). The specific radioactivity is the same in both samples as far as only the intramitochondrial TDP is concerned.

Comparing the radioactivity in TDP of the βP with that of the αP it appears that mainly the βP becomes labelled. The ratio between the radioactivity of βP and αP varies from 8—14 in the three types of experiments reported above.

The figures from the experiments show that the intramitochondrial TDP incorporates ^{32}P, and mainly in the hydrolyzable phosphate (βP). This incorporation is more rapid when a substrate as pyruvate is metabolized than when no substrate is added.

In the following experiments the role of added synthetic thiamine phosphates in a mitochondrial system is studied.

Synthetic thiamine derivatives as phosphate acceptors in mitochondria

Rat liver mitochondria in a 0.01 M phosphate buffer in 0.25 M sucrose were incubated with T, TMP, TDP, or TTP. One mg of the thiamine compound in 0.5 ml water was added to each vessel. Besides, the vessels contained 0.8 mg ATP, 0.1 ml 0.3 M glutamate, hexokinase, 4 mg glucose, 0.05 ml Mg^{2+} 0.1 M, and 0.05 mC radioactive inorganic phosphate. The samples were made isotonic by addition of 1 M sucrose. Finally 1 ml mitochondria suspension was added. After 20 min incubation at 30°C 0.2 ml of 5 M H_2SO_4 was added, samples were taken for determination of organic and inorganic ^{32}P and, after neutralization, the thiamine phosphates were isolated on paper chromatograms. The presence of synthetic thiamine compounds did not affect the P/O value which was about 2. No spots could be found on the chromatograms suggesting any further phosphorylation of the thiamine compounds during the incubation. On the contrary a slight hydrolysis of the thiamine phosphates had taken place. TTeP had partly been hydrolyzed to TTP, and TTP to about 20 % to TDP.

TTeP did not contain any radioactivity at all. The hydrolyzable phosphate of TTP gave 600 counts/100 μg hydrolyzable P and of TDP 520 counts/100 μg hydrolyzable P.

The results show that added thiamine or thiamine phosphates do not at all or only at a very low rate act as phosphate acceptors in a respirating mitochondria system. The incorporation of ^{32}P into TDP is slow compared with that of the mitochondrial TDP, described above.

Possibly thiamine phosphates are in equilibrium with ATP in a side reaction (Fig. 1, alternative II). In the presence of glucose and hexokinase, however, no AT^{32}P would accumulate and, as a consequence, no formation of thiamine phosphates would occur to give rise to radioactive thiamine phosphates. An accumulation of AT^{32}P could experimentally be effected by excluding hexokinase. Incubation of TMP and TDP with ADP in the same way as described above but without hexokinase did, however, not give rise to TTP from TDP or TDP from TMP, nor was a higher incorporation of ^{32}P obtained compared with that found in the experiments with hexokinase present. Neither

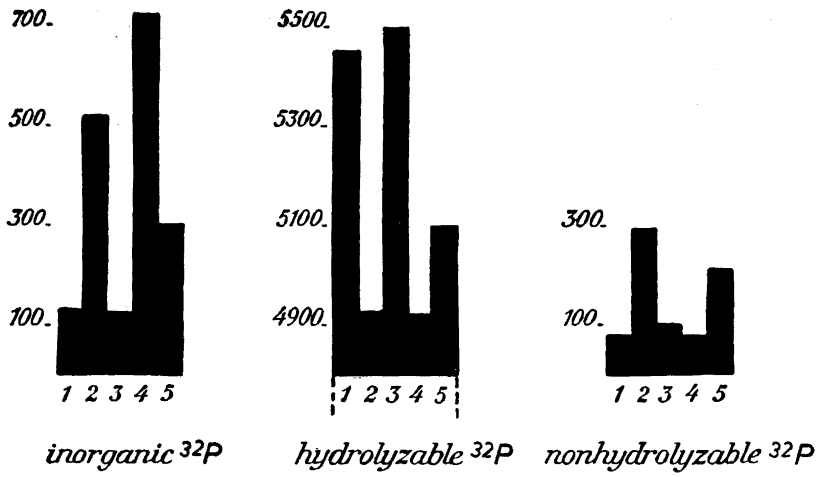

Fig. 3. The radioactivity in the inorganic, hydrolyzable, and non-hydrolyzable phosphate fraction from a mitochondria experiment with labelled TDP.

1. mitochondria + ADP + $TD^{32}P$ + hexokinase + glucose (0 min)
2. mitochondria + ADP + $TD^{32}P$ + hexokinase + glucose (20 min)
3. mitochondria + ADP + $TD^{32}P$ (20 min)
4. ADP + $TD^{32}P$ + hexokinase + glucose (20 min)
5. mitochondria + ADP + $TD^{32}P$ + hexokinase + glucose + inorganic non-radioactive phosphate (50 μM) + 0.1 ml glutamate 0.3 M (20 min)

The rat mitochondria were suspended in a TRIS-sucrose medium pH 7.4. 0.4 ml $TD^{32}P$ (1 000 μg/ml) was added to each flask together with 1 mg ADP, hexokinase, and 4 mg glucose (except in flask 3). To vessels 1—3 and 5 was added 1 ml mitochondria, and the volumes of all samples were made up to 2.2 ml with sucrose. Incubation time 20 min at 30°C. The oxygen consumption in sample 5 was 108 mm^3 in 20 min. The radioactivity was measured on 0.1 ml of the samples.

did exclusion of substrate influence upon the radioactivity in the added thiamine phosphates.

In the experiments without hexokinase no hydrolysis of the thiamine phosphates was observed. It therefore seems that the hexokinase preparation, purified to this special stage, still contains a phosphatase which attacks thiamine phosphates.

Synthetic thiamine phosphates as phosphate donors in mitochondria

From the above results it is obvious that added thiamine phosphates do not take part in the phosphate transport from the substrate-carrier level *via* ATP to glucose in the system used (Fig. 1, alternative I). Neither was the supposition confirmed that thiamine phosphates are in equilibrium with ATP in a side reaction (Fig. 1, alternative II), as it was impossible to transfer ^{32}P to the thiamine phosphates when $AT^{32}P$ was accumulated by excluding hexokinase.

This indicates that no equilibrium exists between added thiamine phosphates and ATP in a side reaction, or that the equilibrium is displaced far towards ATP.

In order to examine the second alternative thiamine phosphates were prepared containing radioactive phosphorus only in their hydrolyzable phosphate groups. If a direct phosphate transfer exsists from thiamine phosphates to ADP, the ATP formed would be radioactive. The γP should be trapped off by the hexokinase and the glucose, and rediscovered as non-hydrolyzable glucose-6-phosphate. Thus a decrease in the hydrolyzable phosphate and an increase in the non-hydrolyzable phosphate would be easy to follow without previously isolating the thiamine phosphates or the phosphate acceptor.

The results of an experiment are seen in Fig. 3.

As was pointed out earlier the hexokinase preparation used is contaminated with a phosphatase which slowly hydrolysis thiamine phosphates. When hexokinase is present the phosphatase splits off phosphate from TDP, and this gives rise to the radioactivity in the inorganic fraction in samples 2 and 4 (Fig. 3). With hexokinase present but no mitochondria (sample 4) the radioactivity in the inorganic fraction is higher than when mitochondria have been added. Very likely this is effected by ADP being phosphorylated to ATP in the presence of mitochondria, as these may contain traces of substrate. In the sample without mitochondria (sample 4) no inorganic phosphate is eliminated in this way. Therefore the radioactivity of the inorganic fraction in this sample is somewhat higher than in sample 2.

The radioactivity in the non-hydrolyzable fraction rises in the sample where both mitochondria and hexokinase are present (sample 2). This rise is, however, not larger than what corresponds to the decrease in the inorganic fraction in sample 2, which fraction can be assumed initially to have been as high as in sample 4.

In the fifth sample the components present were the same as in samples 1 and 2. In addition glutamate and inorganic non-radioactive phosphate were added. With a substrate and inorganic phosphate present TDP was not hydrolyzed to the same degree as without these additions as is seen in Fig. 3. The radioactivity peak of the hydrolyzable phosphate in sample 5 is somewhat higher than in 2 and 4, and the radioactivity of the inorganic fraction is lower than in 2. The radioactivity of the non-hydrolyzable fraction is even lower than the inorganic fraction. This indicates that the phosphate of the glucose-6-phosphate can not originate directly from the highly labelled TDP-phosphate. It must have been diluted primarily with inorganic non-labelled phosphate before being transferred *via* ATP to glucose. From this it may be concluded that intact mitochondria do not contain an enzyme system which transfers phosphate from added TDP to ADP without previous hydrolysis to inorganic phosphate, or if such an enzyme system exists, the rate of transfer must be very low.

The same results were obtained by using TTeP or TTP instead of TDP. With TMP no liberation of inorganic phosphate took place, nor could any rise in the radioactivity of the non-hydrolyzable fraction be observed.

DISCUSSION

In this paper the incorporation of radioactive phosphorus into intramitochondrial TDP has been studied as well as the extent to which synthetic thiamine phosphates added to mitochondria take part in the phosphate metabolism.

The results show that intramitochondrial TDP and added synthetic thiamine phosphates do not take part in the phosphate metabolism to the same extent.

The specific radioactivity of the intramitochondrial TDP was high after incubation (1/2—2/3 of that of ATP—ADP), whereas the added synthetic thiamine phosphates neither incorporated ^{32}P in any appreciable degree, nor supplied phosphate to ADP.

In a previous note [2] an incorporation of ^{32}P into TDP was found to take place in a cyclophorase system from guinea pig liver. Only the endogenous TDP was present during the incubation. The incorporation into TDP depended on the metabolization of a substrate, in this case pyruvate, and was proportional to the oxygen consumption.

Bartley could not verify these results [3]. He used a kidney cyclophorase system to which synthetic thiamine phosphates were added. The specific radioactivity of the added thiamine phosphates was fairly low. These investigations on incorporation of ^{32}P into thiamine phosphates by a cyclophorase system are in keeping with the results obtained with mitochondria in the present paper which show that intramitochondrial TDP incorporates ^{32}P in a much higher degree than added synthetic thiamine phosphates.

From Bartley's experiments it is not possible to decide to what extent the thiamine phosphates furnish ADP with phosphate, and then appear as phosphate compounds with a reduced number of phosphate groups, as he only determines the incorporation of ^{32}P into the added thiamine compounds. The transfer of phosphate from added synthetic TDP to ADP in a cyclophorase system has been examined by Ogata *et al.*[4]. They found a direct transfer of phosphate from TD^{32}P to ADP. My experiments, however, supply no evidence for the existence of an active enzyme system in intact mitochondria which catalyses phosphate transport from added thiamine phosphates to ADP.

These discrepancies may depend on the fact that no intact mitochondria exist in a cyclophorase system. TDP added to the system might easily come in contact with the actual enzyme. In so doing it forms a short-lived and unstable combination and, after delivering one phosphate, leaves room for another TDP. In the intact mitochondria the intramitochondrial TDP would be much stronger bound in the enzyme. Hence it would not be split off after delivering phosphate, but the generated TMP would be rephosphorylated to TDP.

To explain the differences as depending exclusively on difficulties of TDP to penetrate the membrane of the intact mitochondria is not convincing, as TDP in the tissues is mainly synthesized outside the mitochondria [16], and thus must penetrate the membrane to reach its apoenzyme.

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