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RAPID FORMATION OF THIAMINE TRIPHOSPHATE IN HEART MUSCLE AFTER ADMINISTRATION OF DISULFIDE DERIVATIVES OF THIAMINE

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Abstract—Massive doses of ^{35}S-labeled disulfide derivatives of thiamine and ^{35}S-labeled thiamine-HCl were administered to rats, and a comparative study was made of the formation of phosphorylated thiamine in the heart and liver by means of starch block electrophoretic techniques. It was noted that thiamine propyl disulfide and thiamine tetrahydrofurfuryl disulfide, when compared with thiamine-HCl, transferred far more rapidly into heart muscle cells and subsequently were converted into thiamine phosphates. It was also shown that the thiamine triphosphate fraction in the extract of heart tissues showed higher amounts of radioactivity than the thiamine diphosphate fraction. It was conjectured that thiamine triphosphate might play an important role in the contraction processes of the heart.

ALTHOUGH thiamine and its derivatives have been used clinically as therapeutic agents in large quantities, particularly in Japan and Germany, pharmacological evaluation of thiamine so far has uncovered no pharmacodynamic activity likely to serve as a basis for therapeutic uses.[1] It is a well-known fact that in accordance with the size of dosage, substances can be metabolized into different products. Such a reaction must be considered when high doses of thiamine or its derivatives are administered intravenously.

In addition to thiamine diphosphate (TDP), Rossi-Fanelli *et al.*[2] and other more recent authors[3] have pointed out that thiamine triphosphate (TTP) is present in organs such as liver, heart, kidney, and brain and also in peripheral nerves. Although some data[4] suggesting that TTP is involved in the energy metabolism are available, there is no direct evidence that TTP may play a specific role in the energy metabolism that is independent of the metabolic role of TDP in carbohydrate metabolism.

In recent years a considerable amount of evidence has been accumulated, primarily as a result of studies by VON MURALT,[5] which suggests that thiamine may play a vital part in peripheral neurophysiology which is independent of its classical role as a coenzyme.[6, 7]

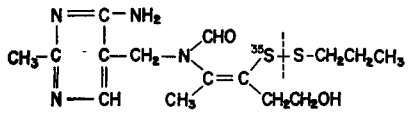
Another interesting development was that a maximal amount of ^{35}S-thiamine in the liver and heart was found by Khmelevskii[8] in the TTP fraction, there being slightly less in the TDP fraction, after intravenous administration of ^{35}S-labeled thiamine to rats. On the other hand, Wiss and Brubacher[9] reported that TDP was the only thiamine form detected after the administration of physiological doses of ^{35}S-labeled thiamine to thiamine-depleted rats.

In a number of investigations[10–14] it is proposed that disulfide derivatives of thiamine, such as thiamine propyl disulfide and thiamine tetrahydrofurfuryl disulfide, are readily absorbed by the organs and are reduced rapidly to liberate thiamine in the cell, whereas thiamine-HCl enters the cell rather slowly. The following experiments were undertaken to determine whether tissue accumulation of TTP could be produced by the administration of high doses of thiamine or its derivatives.

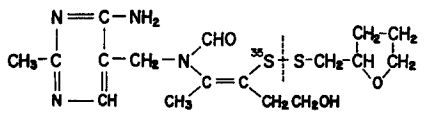
METHODS

Administration of drugs

For this experiment, 88 Wistar male rats weighing from 100 to 150 g were used. The rats were fed a stock diet (Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum*. The drugs used were ^{35}S-labeled thiamine tetrahydrofurfuryl disulfide (TTFD), thiamine propyl disulfide (TPD), and thiamine-HCl obtained as a gift from the Takeda Chemical Industries, Ltd., Osaka, Japan (Fig. 1).



Thiamine Propyl Disulfide (TPD)



Thiamine Tetrahydrofurfuryl Disulfide (TTFD)

FIG. 1. Chemical structure of thiamine propyl disulfide (TPD) and thiamine tetrahydrofurfuryl disulfide (TTFD). ^{35}S: labeled at this S atom.

TTFD and TPD labeled with ^{35}S at the inner sulfur of S—S bonds were dissolved in dilute hydrochloric acid (pH 4·5); thiamine-HCl was dissolved in distilled water. The concentrations of each drug were so adjusted that no more than 2 ml of drug solutions was administered. The rats were lightly anesthetized with ether and the drug injected into a lateral tail vein in doses which varied from 2 mg to 50 mg per kg. The rats were sacrificed by a blow on the head at fixed time intervals ranging from 30 min to 48 hr after the drug administrations.

Method for thiamine phosphates

The assay of thiamine phosphates in the heart and liver was carried out by Khmelevskii's method, with minor modifications.[8] The assay was based on a separation of ^{35}S-thiamine phosphates by starch block electrophoresis, and their content in the respective fractions of the starch block was determined radiometrically. The heart was the primary organ used for examinations; the liver was also studied. The heart and liver were removed and washed quickly in water, then blotted dry and weighed, after which the whole heart or liver tissue (100 mg wet weight) was placed in a glass homogenizer and ground with 5 ml of 0·25 N HCl. The homogenate was heated for

5 min in a boiling water bath in order to extract thiamine phosphates. After standing for 5 min, the pH was adjusted to 6·2 with 10% NaOH. This was then centrifuged for 5 min at 3000 rev/min. NaOH (1%) was added to the extract until pH 6·8 was attained. Crystalline trypsin was added to the tissue extract, and the mixture was incubated for 3 hr at 37°. The precipitate that resulted was removed by centrifugation. The supernatant was then concentrated by freeze-drying. The extract so obtained was starch-blocked and subjected to electrophoresis. The inside measurements of the cell were 55 × 5 × 2 cm. Current strength for electrophoresis in two parallel cells was 30 mA; potential 380 V; duration of electrophoresis 21 hr. The electrode was immersed in a tray containing 2% KCl solution. For the buffer solution, a 0·5 M solution of formic acid was used.

At the conclusion of an experiment the starch block was removed from the cell and cut into 14 zones, each 2 cm wide. Each fraction of the starch block was placed in a separate test tube and stirred into 8 ml distilled water. After standing for 2 hr, the supernatant was removed from each test tube. About 90 per cent of the TTP and TDP subjected to electrophoresis was recovered from the starch by this procedure. The eluate so obtained was concentrated to a suitable volume by heating, after which the concentrated eluate was placed in an aluminium foil dish, 25 mm in diameter, for radioactivity determination, which was performed on a windowless gas-flow counter. In order to determine the position of the TTP, TDP, thiamine monophosphate (TMP), and thiamine in the starch block, a thiochrome technique was used. Molybdate reagent was also used for the detection of thiamine phosphates. For identification, synthetic TTP, TDP, TMP, and thiamine-HCl were used.

RESULTS

Preliminary studies with ^{35}S-labeled TTFD indicated that when labeled TTFD was administered in moderate doses (below 2 mg/kg), the radioactivity in the thiamine phosphate fractions of the extract of heart tissues was too low for detection by the present method. Thus, TTFD was injected i.v. in doses of about 10 mg/kg body weight, corresponding to 30 μc ^{35}S/kg. Figure 2 shows electrophoregrams of the extract of heart tissues 30 min after the i.v. injection of 10 mg ^{35}S-labeled TTFD/kg. As will be

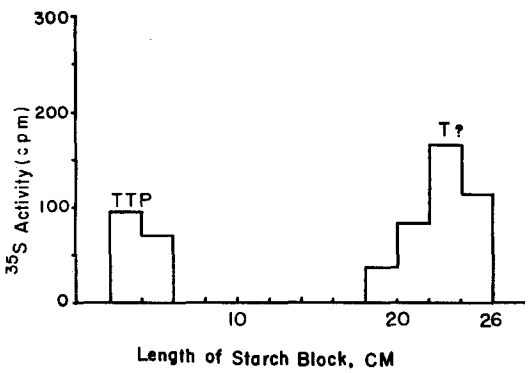


FIG. 2. Electrophoretic fractionation of the heart tissue extract, 30 min after i.v. injection of ^{35}S-labeled thiamine tetrahydrofurfuryl disulfide (10 mg/kg).

seen from the figure, most of the radioactivity was found in the thiamine (T) fraction and in the TTP fraction, with negligible radioactivity in the TDP and TMP fractions. Though there was a large amount of radioactivity in the T fraction, this might be in part due to the ^{35}S-labeled TTFD itself in the tissue, because TTFD was not well separated from thiamine by the present method.

As shown in Fig. 3, 1 hr after TTFD injection a maximal amount of ^{35}S-thiamine was always found in the TTP fraction, with a lesser amount in the TDP fraction. The radioactivity in the TTP fraction was about 60% (8387 $\pm$ 559 counts/min/g fresh tissue) of the total radioactivity of the ^{35}S-thiamine found in the whole heart. However, the quantity of thiamine in the TTP fraction was considered to be far smaller, when compared with that in the TDP fraction, to judge from the intensity of the color with the thiochrome reaction. In some liver samples the radioactivity in the TDP fraction exceeded that of the TTP fraction (Fig. 4). In this case the individual variation from test to test was greater. The fact that the radioactivity in the T fraction was low in both tissues may be due not only to the low level of free ^{35}S-thiamine in the tissues but also to its adsorption during the course of starch-block electrophoresis, as described by Khmelevskii.

Two hours after TTFD injection, no large differences were apparent in the electrophoregram of the heart tissue extract, when compared with that obtained 1 hr after TTFD injection. Therefore, the amount of ^{35}S-thiamine phosphate in the heart

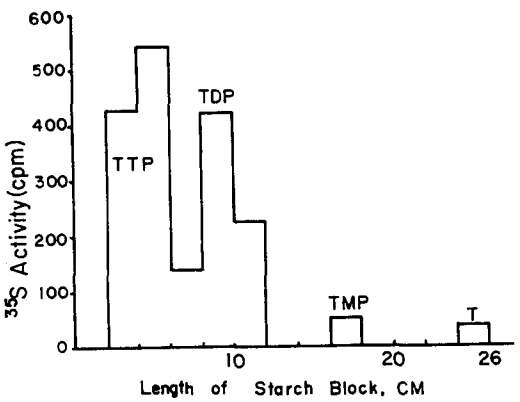


FIG. 3. Electrophoretic fractionation of the heart tissue extract 1 hr after injection of ^{35}S-labeled thiamine tetrahydrofurfuryl disulfide (10 mg/kg).

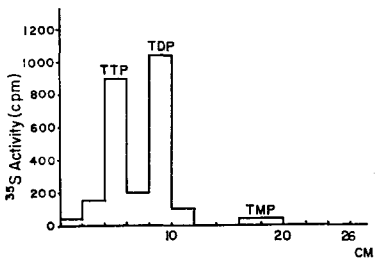


FIG. 4. Electrophoretic fractionation of the liver tissue extract 1 hr after injection of ^{35}S-labeled thiamine tetrahydrofurfuryl disulfide (10 mg/kg).

tissue appeared to reach a steady state as early as 1 hr after the injection of 10 mg TTFD/kg. However, the time at which the maximal TTP level occurred was dependent on the dose of TTFD injected. Although an increase in the radioactivity in the TTP fraction in accordance with an increase in the dose of TTFD administered (up to about 30 mg/kg) was seen, there was no further increase with a higher dose.

Only 0·5% of the total radioactivity of ^{35}S-labeled TTFD administered was recovered as ^{35}S-thiamine phosphates from the whole heart 1 hr after injection, and 7% from the whole liver. Similar experiments were performed in which ^{35}S-labeled thiamine propyl disulfide was administered to 12 rats. The results obtained were essentially identical with those found in the experiment with TTFD.

Figure 5 shows the comparison of the results obtained in the experiments with TTFD and thiamine-HCl. The radioactivity in the TTP fraction of the extract of heart tissues 1 hr after TTFD injection was about 4·8 times greater than the radioactivity in experiments in which thiamine was injected in the same doses and with the same specific activity. However, there were no qualitative or quantitative differences in the electrophoregrams of the extract of liver tissues after thiamine injection, in comparison with those after TTFD injection.

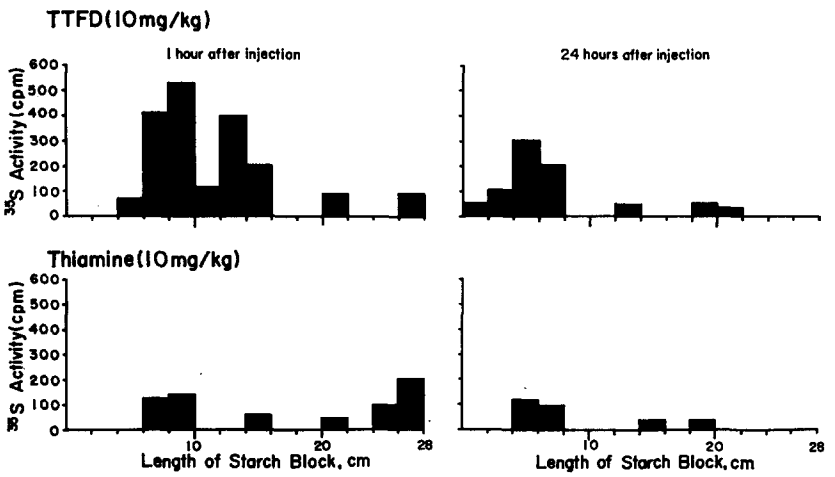


FIG. 5. Comparison of the electrophoretic fractionations of the heart tissue extract after administration of thiamine tetrahydrofurfuryl disulfide and thiamine-HCl.

Twenty-four hours after the administration of TTFD (10 mg/kg) there was a considerable decrease in the total amount of ^{35}S-thiamine (free and phosphorylated) in the heart tissue. Such diminution may possibly be due to the breakdown of ^{35}S-thiamine and/or its transport to other organs. In contrast, a maximal radioactivity in the TTP fraction was found 24 hr after thiamine injection; but even at this time the radioactivity in the TTP fraction was less than that observed in the experiment with TTFD. Even at 48 hr after TTFD injection, a significant amount of radioactivity was still found in the TTP fraction of the extract of heart tissues.

DISCUSSION

Existing methods for the determination of thiamine phosphates are not sufficiently sensitive for the analysis of the small amounts of TTP, TDP, and TMP found in biological material. However, the method described by Khmelevskii[8] permitted determination of the relative content of labeled thiamine phosphates in the heart and liver tissues of rats injected with ^{35}S-labeled disulfide derivatives of thiamine, such as thiamine propyl disulfide and thiamine tetrafurfuryl disulfide, and ^{35}S-labeled thiamine-HCl.

The data obtained in the present work as presented in Fig. 3 indicate that 1 hr after the injection of TTFD, the TTP fraction of the extract of heart tissues showed a higher amount of radioactivity than the TDP fraction. These results with disulfide derivatives of thiamine are in general agreement with those of Khmelevskii in that the maximal amount of ^{35}S-thiamine in the liver and heart was found in the TTP fraction after the injection of ^{35}S-thiamine to normal rats.

A problem that remains unsolved is whether this thiamine metabolite with the same electrophoretic mobility as TTP isolated in the present experiments is TTP or not. This substance was clearly distinguishable from TDP, but the quantity of this TTP-like substance in the TTP fraction was insufficient for detailed studies. However, it is likely that this metabolite is TTP.

According to Khmelevskii, the periods corresponding to the maximal level of labeled thiamine were 2 hr after the injection of thiamine in the liver and 24–48 hr after injection in the heart. However, it was shown in the present work that the time at which the maximal TTP level occurs may be dependent on the dose of thiamine injected. Figure 5 shows that 1 hr after the administration of TTFD in doses of 10 mg/kg, a maximal amount of ^{35}S-thiamine was found in the TTP fraction not only in the liver but also in the heart. Under the conditions of the present experiments, there were no significant differences in the electrophoregrams of the extract of liver tissues after TTFD injection, in comparison with those after thiamine injection. On the other hand, the radioactivity in the TTP fraction of the extract of heart tissues 1 hr after TTFD injection was about 4·8 times greater than the radioactivity in experiments in which thiamine was injected in the same doses and same specific activity. The results presented above suggest that, in contrast with thiamine, there was a marked and rapid formation of TTP in the heart muscle after the injection of a large dose of thiamine alkyl disulfide.

A probable explanation of this marked and rapid formation of TTP in the heart muscle is as follows. As compared with thiamine, disulfide derivatives of thiamine may possibly be transferred more rapidly into cells, and the disulfide bond of these drugs may be reductively split (Fig. 1). Although the phosphorylation of thiamine takes place chiefly in the liver,[15] there may well be an unusually high rate of TTP synthesis also in the heart muscle after the injection of a large dose of thiamine alkyl disulfide. However, whether the accumulation of TTP in heart is due to enhanced synthesis of TTP in the heart muscle cannot be determined clearly from these experiments.

In contrast with thiamine, which is known to be inert or exerts only a negative inotropic effect in high concentrations, Kanno[16] and Nakazawa and Ueno[17] have demonstrated that both TPD and TTFD in concentrations from 10^{-5} to 10^{-3} M exerted a marked positive inotropic and negative chronotropic effect on the isolated atria

of guinea pig and rat. With regard to the cardiac action of TTP, Plotka and Jequier[18] reported that TTP in concentrations from 10^{-6} to 10^{-5} M had two unique pharmacological actions on the heart: positive inotropic action and antiarrhythmic action. More recently, Furukawa *et al.*[19] showed that TTP exerted a marked positive inotropic effect on the papillary muscle of the guinea pig heart, in which a hypodynamic condition was induced by dinitrophenol or *p*-chloromercuribenzoic acid.

From the present experiment, no direct indication as to the mechanism of an inotropic effect of disulfide derivatives of thiamine can be found. However, from the data presented in this report and those available in the literature, it was surmised that the cardiac action of disulfide derivatives of thiamine on isolated atria appears to be indirect and may probably be mediated through the action of TTP which is presumed to have been rapidly formed from these drugs in the heart. Further work is necessary however, to establish the validity of this speculation.

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