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# EFFECTS OF THIAMIN ANTAGONISTS ON DRUG HYDROXYLATION AND PROPERTIES OF CYTOCHROME P-450 IN THE RAT

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**Abstract**—The administration of small amounts of thiamin (0·3 μg/day or more, i.p., for 21 days) depressed microsomal cytochrome P-450 content and the $V_{max}$ of aniline hydroxylase when compared to values obtained from rats fed a thiamin-deficient diet (approximately 0·1 μg of thiamine/day in basal diet). The concurrent administration of neopyrithiamin (50 μg/day, i.p.) eliminated the depressant effect of 10·0 μg of thiamin/day, but was without significant effect in rats receiving more than 100 μg of thiamin/day. In contrast, 100 μg of oxythiamin/day had no thiamin-opposing effect on cytochrome P-450 content and only partially counteracted the effects of 1 μg of thiamin/day on aniline hydroxylase activity. These treatments were without significant effect on the $K_m$ for this reaction. Using ethyl isocyanide as the ligand, there appears to be a qualitative change induced in the cytochrome P-450 from thiamin-deficient rats. The absorption peak height ratios indicate that cytochrome $P_1$-450 is increased in a manner analogous to that produced by the administration of 3-methylcholanthrene. This was supported by the fact that aniline binding, as evidenced by increased $\Delta A_{max}$, is enchanced in microsomes from thiamin-deficient animals, whereas the hexobarbital spectral shift was unaltered.

DIETARY INGESTION of high levels of thiamin hydrochloride has been reported to depress the metabolism of aniline,[1] heptachlor,[1] zoxazolamine[2] and aminopyrine[2] in male rats without significantly altering hexobarbital oxidation. Associated with the decreased rates of metabolism of these substrates are a decrease in the content of cytochrome P-450 per milligram of microsomal protein,[2] a decrease in activity of cytochrome *c* reductase, an increase in liver weight per unit of body weight, an increase in zoxazolamine paralysis time and increased glucose 6-phosphate dehydrogenase activity.[2]

The thiamin antagonists, oxythiamin and neopyrithiamin, have been reported to block the coenzyme functions of thiamin. Neopyrithiamin reduces the activity of pyruvate dehydrogenase and produces polyneuritis analogous to that of thiamin deficiency; however, the administration of oxythiamin reduces the activity of pyruvate decarboxylase but does not induce polyneuritis. It is felt that neopyrithiamin–PP inhibits thiamin pyrophosphokinase competitively, whereas oxythiamine–PP may selectively interfere with thiamin–PP binding to the apoenzyme. The antagonistic effects of neopyrithiamin are readily reversed with thiamin, while the loss of activity

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due to oxythiamin is reversed poorly.[3] Rats were administered these inhibitors along with varying quantities of thiamin in an attempt to discern whether they were capable of reversing the effects of thiamin on hepatic drug hydroxylation reactions.

## MATERIALS AND METHODS

*Animals.* Fifty-seven white, male Sprague–Dawley rats (Holtzman Co., Madison, Wis.), weighing approximately 50 g each, were fed a standard laboratory test diet consisting of casein (16%), sucrose (73%), non-nutrient cellulose (4%), Jones–Foster salt mix (4%) and corn oil (3%). Except for thiamin, all vitamins necessary for rat growth were provided to all groups.[4] Unless otherwise stated, thiamin HCl solutions were prepared daily and administered in doses ranging from 0·1 to 1000 μg/day by i.p. injection. One group received no antagonist, one group received 50 μg of neopyrithiamin/day (i.p.) and one group received 100 μg of oxythiamin/day (i.p.).

*Enzyme preparation.* After 17–19 days of treatment, the rats were decapitated and their livers quickly removed and homogenized with two volumes of cold 0·15 M KCl using a motor-driven Teflon–glass tissue homogenizer. The microsomes recovered, after centrifuging the 9000 *g* supernatant at 105,000 *g* for 60 min, were suspended in 0·15 M KCl and recentrifuged at 105,000 *g* for 60 min. Protein content of this resuspended pellet was analyzed by the method of Gornall *et al.*[5]

*Enzyme assays.* Washed microsomes equal to 5 mg of microsomal protein were added to incubation flasks containing nicotinamide adenine dinucleotide (NADP), 5 μmoles; glucose 6-phosphate (G-6-P), 25 μmoles; magnesium sulfate, 25 μmoles; glucose 6-phosphate dehydrogenase, 2 enzyme units; aniline, 0·5 ml and enough 0·1 M phosphate buffer, pH 7·4, to make 5·0 ml. Four concentrations of aniline were used: 0·5 mM; 0·125 mM; 0·05 mM and 0·025 mM. The mixtures were incubated for 20 min at 37° under air in a Dubnoff metabolic shaker at 120 oscillations/min. Reactions were stopped by swirling the flasks in ice-cold water.

The aromatic hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol by the method of Kato and Gillette.[6] The ability of microsomes to bind aniline and hexobarbital was determined[7] using microsomes diluted to 2 mg of protein/ml with 0·3 M phosphate buffer, pH 7·4. Seven serial 1-μl additions of substrate to a cuvette containing 3·0 ml of microsomes were made using a Hamilton syringe and repeating dispenser. After each addition, the difference in absorbance between the two predetermined wavelengths was measured using the Aminco–Chance spectrophotometer in the dual wavelength mode. Apparent Michaelis–Menten constant ($K_m$) and $V_{max}$ of aniline metabolism as well as spectral dissociation constant ($K_s$) and maximal spectral shift ($\Delta A_{max}$) were calculated by the method of Wilkinson[8] using an Olivetti–Underwood programma 101 computer.

Cytochrome P-450 content was measured by the method of Omura and Sato[9] using 2·0 mg of protein/ml of microsomes. The ethyl isocyanide difference spectra were determined by the method of Omura and Sato[9] except that the microsomes were diluted to 2·0 mg/ml with 1·5 M phosphate buffer, pH 7·5, to prevent changes in pH which affect relative sizes of absorption maxima. To 3·0 ml of diluted microsomes, 15 μl of 5% ethyl isocyanide was added, and the difference in absorbance between 430 and 490 nm and between 455 and 490 nm was determined using the Aminco–Chance spectrophotometer in the dual wavelength mode.

TABLE 1. EFFECT OF THIAMIN INHIBITORS ON GROWTH RATE, LIVER WEIGHT AND HEPATIC MICROSOMAL PROTEIN IN RATS ADMINISTERED VARIOUS LEVELS OF THIAMIN*

| Thiamin injected (μg/day, i.p.) | Average total weight gain (g) | | | Liver weight (g) | | | Microsomal protein (mg/g liver) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Oxythiamin† | Neopyrithiamin‡ | Control | Oxythiamin | Neopyrithiamin | Control | Oxythiamin | Neopyrithiamin |
| 0·1 | 10·3 (3) | | | 3·02 | | | 25·3 | | |
| 0·3 | 19·8 (3) | | | 3·33 | | | 18·9 | | |
| 1·0 | 26·5 (3) | 0·3 (3) | | 3·80 | 2·63 | | 22·5 | 14·4 | |
| 3·0 | 42·3 (6) | 7·0 (4) | | 4·37 | 3·37 | | 30·0 | 23·3 | |
| 10·0 | 75·0 (3) | 45·2 (6) | 22·7 (2) | 6·55 | 5·76 | 3·40 | 34·2 | 37·4 | 30·8 |
| 30·0 | 102·2 (3) | 30·3 (3) | 81·2 (3) | 8·07 | 7·70 | 7·45 | 29·1 | 33·6 | 29·1 |
| 100·0 | 99·2 (3) | 95·0 (2) | 76·5 (3) | 7·83 | 7·60 | 7·77 | 46·8 | 39·0 | 44·4 |
| 1000·0 | | 81·5 (3) | 93·3 (3) | | 7·33 | 7·42 | | 36·9 | 33·9 |

* Values in parentheses are number of animals.
† Oxythiamin administered at dose of 100 mg/day.
‡ Neopyrithiamin administered at dose to 50 mg/day.

TABLE 2. EFFECT OF THIAMIN INHIBITORS ON THE KINETICS OF ANILINE HYDROXYLASE OF LIVER MICROSOMES FROM RATS ADMINISTERED VARIOUS LEVELS OF THIAMIN

| Thiamin injected ($\mu$g/day, i.p.) | Inhibitor: None | | Oxythiamin | | Neopyrithiamin | |
|---|---|---|---|---|---|---|
| | $V_{max}$* | $K_m$ (mM) | $V_{max}$* | $K_m$ (mM) | $V_{max}$* | $K_m$ (mM) |
| 0·1 | 100·6 ± 1·4 | 0·017 ± 0·001 | | | | |
| 0·3 | 42·6 ± 1·3 | 0·017 ± 0·003 | | | | |
| 1·0 | 35·5 ± 1·6 | 0·024 ± 0·004 | 60·9 ± 1·1† | 0·015 ± 0·001 | | |
| 3·0 | 32·7 ± 0·9 | 0·013 ± 0·002 | 32·7 ± 0·9 | 0·030 ± 0·003† | | |
| 10·0 | 42·0 ± 1·2 | 0·010 ± 0·002 | 21·5 ± 1·4† | 0·036 ± 0·008† | 130·2 ± 14·7† | 0·023 ± 0·011 |
| 30·0 | 18·5 ± 0·2 | 0·021 ± 0·001 | 16·8 ± 0·8 | 0·022 ± 0·005 | 34·9 ± 0·9† | 0·012 ± 0·002† |
| 100·0 | 26·5 ± 1·2 | 0·011 ± 0·003 | 20·2 ± 0·8† | 0·010 ± 0·003 | 28·7 ± 2·2 | 0·013 ± 0·006 |
| 1000·0 | | | 21·0 ± 0·2 | 0·031 ± 0·001 | 23·9 ± 1·6 | 0·016 ± 0·005 |

* nM *p*-aminophenol formed per milligram of protein per hr.
† Significantly different from controls given same quantity of thiamin ($P < 0·01$).

TABLE 3. EFFECT OF THIAMIN INHIBITORS ON MICROSOMAL CYTOCHROME P-450 LEVELS AND ETHYL ISOCYANIDE DIFFERENCE SPECTRA*

| Thiamine injected ($\mu$g/day, i.p.) | With no inhibitor | | | | With oxythiamin | | | | With neopyrithiamin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P-450 level† | Ethyl isocyanide (Δ absorbance) 430–490 | 455–490 | Ratio | P-450 level† | Ethyl isocyanide (Δ absorbance) 430–490 | 455–490 | Ratio | P-450 level† | Ethyl isocyanide (Δ absorbance) 430–490 | 455–490 | Ratio |
| 0·1 | 1·044 | 0·053 | 0·077 | 1·45 | | | | | | | | |
| 0·3 | 0·885 | 0·044 | 0·058 | 1·32 | | | | | | | | |
| 1·0 | 0·797 | 0·031 | 0·040 | 1·29 | 0·703 | 0·034 | 0·043 | 1·26 | | | | |
| 3·0 | 0·626 | 0·033 | 0·037 | 1·12 | 0·561 | 0·033 | 0·039 | 1·18 | | | | |
| 10·0 | 0·742 | 0·044 | 0·045 | 1·02 | 0·478 | 0·028 | 0·028 | 1·00 | 1·098 | 0·065 | 0·073 | 1·12 |
| 30·0 | 0·429 | 0·028 | 0·024 | 0·86 | 0·467 | 0·031 | 0·028 | 0·90 | 0·484 | 0·028 | 0·027 | 0·97 |
| 100·0 | 0·511 | 0·035 | 0·030 | 0·86 | 0·440 | 0·022 | 0·022 | 1·00 | 0·412 | 0·032 | 0·028 | 0·88 |
| 1000·0 | | | | | 0·429 | 0·028 | 0·026 | 0·93 | 0·351 | 0·025 | 0·023 | 0·92 |

* All values are means of two to six animals as noted in Table 1.
† Micromoles of cytochrome P-450 per milligram of microsomal protein.

## RESULTS

The effects of the thiamin inhibitors on growth rate, liver weight and microsomal protein content are shown in Table 1. At each level of thiamin administered, both oxythiamin (100 $\mu$g/day) and neopyrithiamin (50 $\mu$g/day) depressed growth rate. Maximum growth rate in control animals was observed in rats receiving 30 $\mu$g of thiamin/day. In order to obtain maximum growth rate in animals receiving neopyrithiamin, 1000 $\mu$g/day of thiamin was required, and in animals administered oxythiamin, 100 $\mu$g of thiamin/day was required. Maximal liver weight was attained with injections of 30 $\mu$g of thiamin/day regardless of the inhibitor present, although depression in liver weight was evident at lower levels of thiamin in the presence of inhibitors. Microsomal protein appeared to be unaffected by inhibitors except at low thiamin levels in rats given oxythiamin.

In general, the $V_{max}$ for aniline hydroxylase activity in control animals varied inversely with the dose of thiamin. The greatest depression in $V_{max}$ occurred as thiamin intake rose from 0·1 to 0·3 $\mu$g/day (Table 2). Neopyrithiamin administered at a dose of 50 $\mu$g/day eliminated the depressant effect of 10·0 $\mu$g thiamin/day on aniline $V_{max}$, but was not effective in opposing thiamin in doses greater than 30 $\mu$g/day. In contrast, 100 $\mu$g of oxythiamin only partially counteracted the effect of 1·0 $\mu$g of thiamin/day and had no thiamin-opposing effect on the $V_{max}$ of aniline hydroxylase activity of microsomes from rats receiving 3·0 $\mu$g or more of thiamin/day.

There was no apparent trend in the alterations in $K_m$ of aniline metabolism induced by varying the dosage of thiamin or by the administration of thiamin inhibitors.

With increasing doses of thiamin, the concentration of cytochrome P-450 progressively decreased (log $B_1$ ingested to P-450; $r = -0{\cdot}92$). Neopyrithiamin reversed the effect of 10·0 $\mu$g of thiamin/day but had no effect on cytochrome P-450 content of microsomes of rats receiving 30 $\mu$g or more of thiamin/day. At thiamin intakes greater than 1 $\mu$g/day, oxythiamin produced no reversal of the depressant effect of thiamin on cytochrome P-450 content and may in fact have caused further depression.

As noted in Table 3, marked changes in the ethyl isocyanide difference spectra occurred as a result of altering thiamin intake (log $B_1$ injected to ethyl isocyanide ratio; $r = -0{\cdot}984$). The administration of oxythiamin did not appreciably affect these changes. The addition of aniline to microsomes from thiamin-deficient rats resulted in a significantly greater spectral shift than when added to microsomes from rats given

TABLE 4. EFFECT OF DIETARY THIAMIN DEFICIENCY ON THE APPARENT SPECTRAL DISSOCIATION CONSTANT ($K_s$) AND MAXIMAL SPECTRAL SHIFT ($A_{max}$) FOR HEXOBARBITAL AND ANILINE BINDING

| Diet | $K_s$* (mM ± S.E.) | | $A_{max}$* ($\Delta A$/mg protein × $10^3$ ±S.E.) | |
|---|---|---|---|---|
| | Hexobarbital | Aniline | Hexobarbital | Aniline |
| Thiamin deficient | 0·1597 ± 0·0155 | 0·2487 ± 0·0424 | 26·4 ± 0·7 | 20·7 ± 1·4† |
| High thiamin‡ | 0·1566 ± 0·0061 | 0·2003 ± 0·0186 | 25·0 ± 0·3 | 15·0 ± 0·5 |

* Values were determined from seven points on Lineweaver–Burke plots using pooled livers of six rats per group.

† Different from group fed high thiamin ($P < 0{\cdot}01$).

‡ This diet provided the rats with approximately 2000 $\mu$g of thiamin/day.

high levels of thiamin (Table 4). No changes in the apparent spectral constants ($K_s$) were observed with either aniline or hexobarbital nor was there a change in the maximal spectral shift when hexobarbital was used as a substrate.

## DISCUSSION

Increasing doses of thiamin counteracted the effects of oxythiamin and neopyrithiamin on growth rate and aniline hydroxylase activity. These findings are consistent with the idea that these compounds are competitive antagonists of thiamin.

In rats receiving no inhibitors, the concentration of cytochrome P-450 per unit of microsomal protein decreased with increasing daily doses of thiamin. Not only is there a change in cytochrome P-450 content but there is also a qualitative alteration in the cytochrome as evidenced by altered ethyl isocyanide difference spectra (Table 3). Sladek and Mannering[10] reported that the administration of certain polycyclic hydrocarbons such as 3-methylcholanthrene (MC) induced the synthesis of a new species of cytochrome P-450 which they designated as cytochrome $P_1$-450. This new species could be detected by its characteristic absorption spectrum when complexed with ethyl isocyanide and performed differently in the metabolism of drugs. Whereas the normal ratio of ethyl isocyanide absorption peak heights (Δ A455–A490 to ΔA430–A490) is about 0·5 to 0·7, that obtained following MC pretreatment is approximately 1·4 to 1·8.[10,11] The ratio of ethyl isocyanide absorption peak heights from rats receiving high doses of thiamin was 0·86 when calculated by this procedure, whereas the ratio from rats receiving the thiamin-deficient diet was 1·45. Peak height ratios were inversely related to the dose of thiamin injected. If the ethyl isocyanide absorption peak ratio is a true indicator of the relative amount of cytochrome $P_1$-450 of microsomes, these data show that microsomes from thiamin-deficient animals have considerably more of this cytochrome species than those from rats receiving high levels of thiamin. It has been shown previously that enzyme induction by MC increases total cytochrome P-450 and enhances aniline hydroxylase activity without increasing hexobarbital metabolism. Enhanced aniline binding to these microsomes was also demonstrated.[10,11] Microsomes from rats fed a thiamin-deficient diet metabolize aniline at a significantly higher rate than those from rats given increasing levels of thiamin. Hexobarbital metabolism in the male rat is not uniformly altered by the level of dietary thiamin.[1,2]

It appears that thiamin deficiency alters cytochrome P-450 qualitatively in a manner which may be analogous to that produced by the administration of MC. Since total cytochrome is also increased, it appears that thiamin lack does not induce a preferential lability of cytochrome P-450, but rather induces a condition favoring synthesis of a new species analogous to the $P_1$-450 of Sladek and Mannering.[10] Further evidence supporting this conclusion was provided by a subsequent experiment in which microsomes from rats fed a thiamin-deficient or high thiamin diet were used in substrate binding studies. Hexobarbital added serially to microsomes from rats fed a thiamin-deficient diet resulted in $K_s$ and $A_{max}$ values that were statistically indistinguishable from values observed using microsomes from rats fed the high thiamin diet. On the other hand, when aniline was added to microsomes from rats fed the thiamin-deficient diet, the apparent $\Delta A_{max}$ was significantly higher than that observed when aniline was similarly added to microsomes from rats fed the high thiamin diet. There was no alteration in $K_s$. These data support the concept that the concentration of cytochrome

P-450 is similar in both dietary groups whereas the $P_1$-450 is elevated in microsomes from rats fed the deficient diet.

Although oxythiamin had little effect on the ethyl isocyanide absorption peak ratio at various doses of thiamin, it appears to have a thiamin-potentiating effect on cytochrome P-450 content and the $V_{max}$ of aniline hydroxylase at high thiamin doses. Neopyrithiamin appeared to induce a higher 455/430 ratio in animals receiving less than 100 $\mu$g of thiamin/day and also increased the quantity of cytochrome P-450. Thus, it appears that neopyrithiamin is capable of producing effects on the drug hydroxylation enzymes of rat liver analogous to that of thiamin deficiency, whereas oxythiamin may produce further depression of aniline hydroxylation in animals given large doses of thiamin.

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