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EFFECT OF *N*-METHYL-THIOTETRAZOLE ON RAT LIVER MICROSOMAL VITAMIN K-DEPENDENT CARBOXYLATION*

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Abstract—The use of a number of antibiotics which contain an *N*-methyl-thiotetrazole (NMTT) side chain has been reported to be associated with an increased incidence of hypoprothrombinemia. The suggested role of NMTT as an inhibitor of the liver microsomal vitamin K-dependent carboxylase has been investigated. In standard incubations, NMTT had no effect on carboxylation when vitamin KH_2 was a substrate but was a weak inhibitor when [vitamin K + NADH] was a substrate. Microsomal vitamin K reductases, however, were not inhibited by NMTT. Preincubation of the incubation mixture with NADH and NMTT resulted in inhibition of carboxylase activity when either vitamin KH_2 or [vitamin K + NADH] was the substrate. A fraction of the microsomal membrane which was not readily solubilized by dilute detergent protected the enzyme from this inhibition. The data suggest that NMTT is metabolized to an active inhibitor or is able to covalently inactivate the enzyme in the presence of NMTT. The vitamin K responsiveness of the clinically observed hypoprothrombinemia suggests that it is not related to this *in vitro* inhibition of the vitamin K-dependent carboxylase.

An increased incidence of a vitamin K reversible hypoprothrombinemia has been noted following administration of several new β-lactam antibiotics which contain an *N*-methyl-thiotetrazole (NMTT) side chain [1–5]. Antibiotic-induced hypoprothrombinemia has been a long-recognized response to the administration of a large number of antibiotics and has usually been ascribed to decreased synthesis of the bacterial forms of vitamin K, the menaquinones [6]. More recently, Smith and Lipsky [7] have suggested that menaquinones present in the gut are not utilized for clotting factor synthesis by the human, and Lipsky [8] has presented data interpreted as evidence that the hypoprothrombinemic effect of this class of oxalactam and cephalosporin antibiotics is due to an inhibition of the vitamin K-dependent γ-carboxylation of glutamic acid by free NMTT released during metabolism of these antibiotics. This carboxylation reaction is essential for the formation of biologically-active prothrombin and clotting factors VII, IX, and X.

The vitamin K-dependent carboxylase utilizes the reduced form of vitamin K (vitamin KH_2) as a substrate, and the crude microsomal preparation used to study this membrane contains a number of NAD(P)H-dependent reductases which will reduce vitamin K quinone [9, 10]. The initial report [8] of NMTT inhibition of the vitamin K-dependent carboxylase utilized [vitamin K + NADH] as a cofactor for the enzyme, and other workers [11, 12] have been unable to achieve the same degree of inhibition. A more recent report [13] has shown that preincubation of the crude enzyme preparation with NMTT enhances inhibition, that glutathione protects against inhibition, and that the disulfide dimer of NMTT is a more potent inhibitor. As the inhibition observed [13] could have been due to an effect of NMTT on either the carboxylase or a microsomal quinone reductase, we have investigated the variables responsible for NMTT inhibition of the vitamin K-dependent carboxylase in more detail.

MATERIALS AND METHODS

Vitamin K-dependent carboxylation was determined in microsomes prepared from the livers of male Holtzman strain rats fed a vitamin K-deficient diet [14] and maintained in coprophagy-preventing cages [15]. Microsomal pellets obtained as previously described [16] were suspended in SIK buffer A (0.25 M sucrose, 25 mM imidazole at pH 7.4, 0.5 M KCl) containing 1.5% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride (PMSF). In some experiments, SIK buffer B (0.1 M KCl) was used; and, in some cases, this suspension was centrifuged at 105,000 *g* for 60 min and the supernatant fraction was used as a source of enzyme. Incubation mixtures contained 300 μl of this suspension or supernatant in a final volume of 530 μl. The carboxylase substrate t-BuOCO-Glu-Glu-Leu-OMe (final concentration 0.5 mM), NADH and inhibitors were added in SIK buffer. Ten microcuries of sodium [^{14}C]bicarbonate (55 mCi/mmole) was added, and the reaction was started by the addition of vitamin K or vitamin KH_2 in 10 μl of ethanol. Samples were incubated at 17° for 30 min and stopped by the addition of 1 ml of 10% trichloroacetic acid. After centrifugation, the supernatant fraction was gassed with CO_2 for 10 min, and the radioactivity, fixed in a 200-μl aliquot, was determined by liquid scintillation spectrometry [16].

Concentration of vitamin KH_2 in incubation mixtures was determined by injection of 50 μl of the incubation mixture into a vial containing 0.5 ml of methanol. This vial was purged with O_2-free N_2, shaken on a Vortex mixer, and centrifuged, and 50 μl of the supernatant fraction was injected onto a duPont Zorbax ODS column (250 × 4.6 mm) which was developed with a mobile phase of methanol–H_2O (96:4) delivered by a Waters 6000A pump. Vitamin KH_2 was detected with a Perkin–Elmer 650-10LC fluorescence spectrophotometer set at 340 nm emission and 430 nm excitation with a 10-mm slit.

The substrate t-BuOCO-Glu-Glu-Leu-OMe was obtained from Bachem, Inc. (Torrance, CA) and sodium [^{14}C]bicarbonate from Amersham/Searle (Arlington Heights, IL). Disulfiram, PMSF, NADH, and vitamin K were obtained from the Sigma Chemical Co. (St. Louis, MO), and vitamin KH_2 was prepared as previously described [17]. Samples of *N*-methyl-thiotetrazole were obtained from the Eli Lilly Co. (Indianapolis, IN) and from Dr. James Lipsky.

RESULTS

The effects of NMTT on vitamin KH_2-dependent and [vitamin K + NADH]-dependent carboxylation are shown in Fig. 1. The results were variable from day to day, but about a 40% inhibition of the reaction was obtained in the presence of 5 mM NMTT when NADH was used as a reductant. Varying the vitamin K concentration between 30 and 90 μg/ml, altering the peptide substrate concentration from 0.5 to 3.0 mM, omission of PMSF, preincubation of the assay mixture in the absence of NMTT, or utilization of a different source of NMTT were assessed but did not lead to a consistent increase in inhibition of this system by NMTT, nor to the degree of inhibition previously reported [8]. In a total of forty separate experiments with numerous variations in incubation conditions, a 50% inhibition of carboxylation by 1 mM NMTT was observed in one experiment but could not be repeated subsequently under identical conditions. In contrast to the inhibition noted at high concentrations of NMTT when [vitamin K + NADH] was used, the data in Fig. 1 clearly indicate that, when the coenzyme active form of the vitamin, vitamin KH_2, was used, no dose-dependent inhibition of the carboxylase was observed.

Disulfiram, tetraethylthioram disulfide, has been demonstrated to be an inhibitor of the vitamin K-dependent carboxylase [13], and an analogy has been drawn between the action of a NMTT dimer and disulfiram. As disulfiram is a known inhibitor of pyridine nucleotide-dependent reductase [18], inhibition of an NADH-dependent quinone reductase by NMTT and disulfiram rather than the carboxylase itself was a possible mechanism of inhibition of the crude microsomal carboxylase system. The data in Fig. 2 indicate that this is not the case. Both disulfiram and NMTT at the concentration used severely inhibited the [vitamin K + NADH]-dependent car-

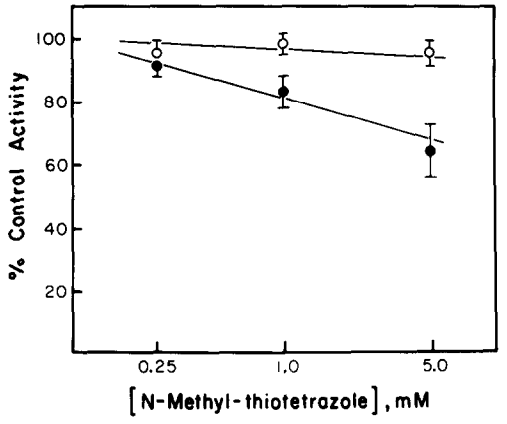

Fig. 1. Effect of NMTT on vitamin K-dependent carboxylase activity. Mixtures containing 60 μg/ml vitamin K and 2 mM NADH (—●—) or 90 μg/ml vitamin KH_2 (—○—) were incubated as described in Materials and Methods in SIK buffer A; the microsomal detergent suspension was not centrifuged before it was used. Control incubation mixtures for [vitamin K + NADH] averaged 1000 cpm after subtraction of a 500 cpm no-vitamin blank. Control incubation mixtures for vitamin KH_2 averaged 4000 cpm after subtraction of a 200 cpm no-vitamin blank. Duplicate incubation mixtures were averaged for each experiment; values plotted are mean ± S.E.M. for six (—●—) or four (—○—) experiments.

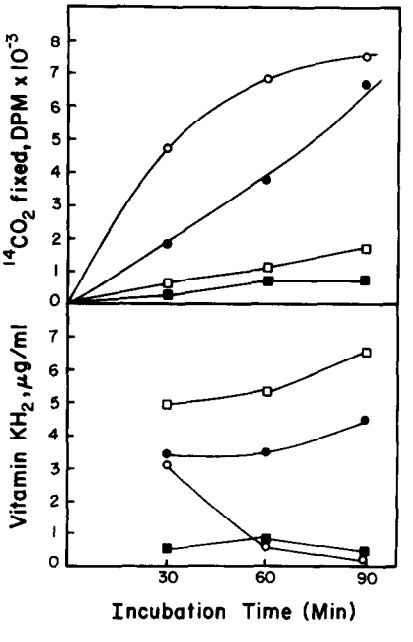

Fig. 2. Effects of NMTT and disulfiram on vitamin K-dependent carboxylase activity and vitamin KH_2 concentrations. Incubation mixtures were prepared in SIK buffer B as described in Materials and Methods and contained 100 μg/ml vitamin K and 2 mM NADH or 100 μg/ml vitamin KH_2. The solubilized microsomes had been frozen, lyophilized, stored at −20°, and thawed; the detergent suspension was centrifuged at 105,000 *g* for 60 min before being used. Carboxylase activity and vitamin KH_2 concentration were determined as described in Materials and Methods. Values for fixed $^{14}CO_2$ represent a 33-μl aliquot of the incubation. Key: (○—○) Vitamin KH_2; (●—●) [vitamin K + NADH]; (□—□) [vitamin K + NADH] + 2 mM NMTT; and (■—■) [vitamin K + NADH] + 0.3 mM disulfiram.

Table 1. Effects of NMTT, NADH, and centrifugation on Vitamin KH_2-dependent carboxylation

Additions	$^{14}CO_2$ fixed, dpm × 10^{-3}	
	Suspension	Supernatant
None	39.3	49.0
5 mM NMTT	37.9	69.1
2 mM NADH	37.3	76.2
NMTT + NADH	34.7	7.9

Microsomes were prepared and solubilized in SIK buffer B and Triton X-100 as described in Materials and Methods. This suspension, or a supernatant fraction prepared by centrifugation of this suspension for 60 min at 105,000 *g*, was used as a source of enzyme. Incubation mixtures were preincubated at 17° for 30 min in the presence or absence of NMTT or NADH, and carboxylase activity was initiated by the addition of vitamin KH_2 and $H^{14}CO_3^-$. Incubation was stopped after 30 min, and fixed $^{14}CO_2$ was determined as described in Materials and Methods. Values are for total fixed CO_2 in the incubation mixtures and are means of duplicates. Values for fixation of $^{14}CO_2$ in the absence of vitamin K were 5×10^2 dpm for incubation mixtures without NADH and 5×10^3 dpm in the presence of NADH; these have been subtracted from the values in the table.

boxylation. The low concentration of vitamin KH_2 in the reaction mixture in the presence of disulfiram and the decrease in rate of vitamin KH_2-dependent carboxylation seen when the concentration of the active coenzyme fell below 1 μg/ml are consistent with an inhibition of a quinone reductase by disulfiram. However, the addition of NMTT to the incubation was associated with a slight increase in vitamin KH_2 concentration, and inhibition of reductase activity cannot explain inhibition by NMTT.

The significant inhibition of carboxylation by NMTT in Fig. 2 compared to Fig. 1 suggested some basic difference in the reaction conditions. The original description of the solubilized microsomal vitamin K-dependent carboxylase preparation [19] and the preparation described in the studies of Lipsky [8, 13] used the supernatant fraction from centrifuged detergent-solubilized microsomes as a source of enzyme activity. As removal of the non-solubilized material was found to have little influence on carboxylase activity, this step was not routinely used in our laboratory. Preliminary experiments suggested that it was the centrifugation of the detergent suspension, as was done to obtain the data shown in Fig. 2, that was responsible for the increased inhibition by NMTT. These experiments also indicated it was the presence of NADH, not of [NADH + vitamin K], that was essential for inhibition. The data in Table 1 clearly illustrate that, in a centrifuged preparation, the vitamin KH_2-dependent carboxylase activity was inhibited by NMTT in the presence, but not the absence, of NADH, and that if the non-soluble fraction of the crude microsomal preparation was not removed, no inhibition by NMTT was observed. Other experiments (data not shown) demonstrated that the effect demonstrated in Table 1 was not

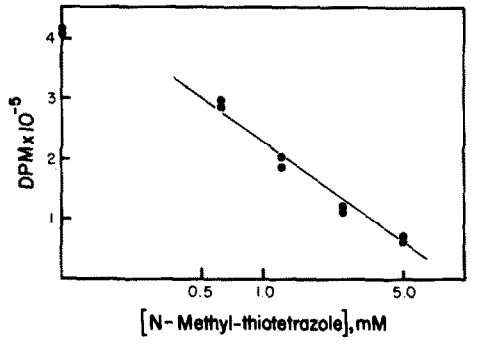

Fig. 3. Effect of NMTT on vitamin K-dependent carboxylase in a centrifuged, solubilized, microsomal preparation. The supernatant preparation described in Table 1 was used as a source of enzyme and preincubated at 17° in the presence of 2 mM NADH and the indicated concentration of NMTT for 30 min, after which 100 μg/ml vitamin KH_2 and $H^{14}CO_3$ were added, and incubation was continued for another 30 min. Carboxylase activity was assayed as described in Materials and Methods.

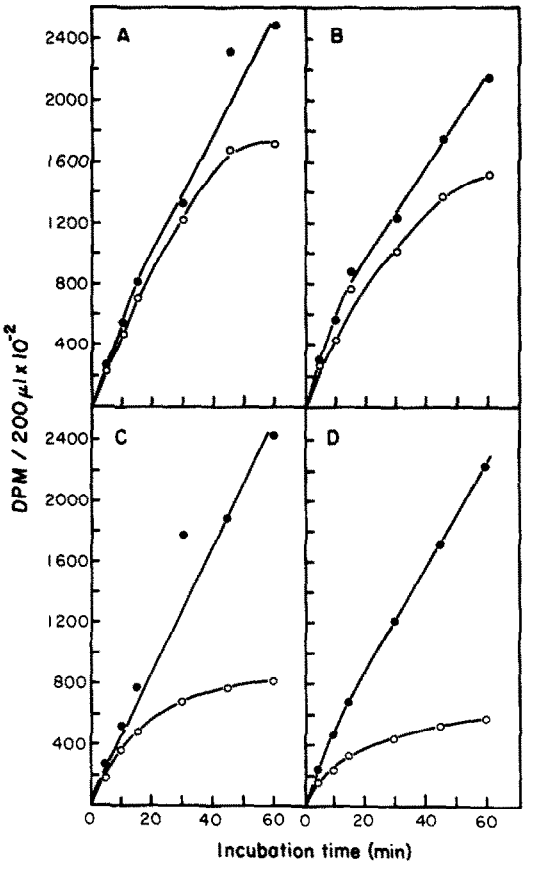

Fig. 4. Effect of preincubation times on NMTT inhibition of vitamin K-dependent carboxylase. The supernatant preparation described in Table 1 was used as a source of enzyme. Mixtures that were five times the standard volume were prepared and incubated in the presence of 2 mM NADH and the presence or absence of 5 mM NMTT for various times at 17°. Assays were initiated by the addition of 100 μg/ml vitamin KH_2 and $H^{14}CO_3$, and aliquots, were taken at various times for determination of carboxylase activity as described in Materials and Methods. Panel A, no preincubation; panel B, 10-min preincubation; panel C, 20-min preincubation; and panel D, 40-min preincubation.

readily seen when the high-salt SIK buffer A was used.

The data in Fig. 3 illustrate the sensitivity of the vitamin K-dependent carboxylase to NMTT under the inhibition conditions used in Table 1. As NMTT inhibition is time dependent, the extent of inhibition observed at any given NMTT concentration is a function of the fixed time point chosen for the assay. The data in Fig. 4 indicate that the uninhibited control activity was essentially linear over a 50-min incubation period and that the addition of NMTT had little effect on the initial rate of the reaction. However, preincubation of the preparation with NMTT and NADH for various periods resulted in a decrease in the duration of time before the rate of $^{14}CO_2$ fixation in the NMTT-inhibited preparations approached zero.

DISCUSSION

These data provide confirmation of the reports of Lipsky [8, 13] that NMTT inhibits the vitamin K-dependent carboxylase *in vitro* and elucidate some of the factors involved. A high level of inhibition has been shown to be dependent on preincubation time, NADH concentration, salt concentration, and the type of solubilized microsomal preparation used. The lack of significant inhibition previously observed in our laboratory and others [11, 12] is undoubtedly related to variations in these or other unknown variables. The lack of a decrease of steady-state levels of vitamin KH_2 by NMTT when [vitamin K + NADH] was used as a source of vitamin indicates that the inhibitory effect of NMTT is on the carboxylase rather than on an associated quinone reductase and suggests that the proposed similarity of action of an NMTT dimer and disulfiram [13] is fortuitous rather than the result of a common mechanism of action. Significant NMTT inhibition of the enzyme depends on a number of factors and is best explained by an NADH-dependent, relatively slow, inactivation of the enzyme. Whether this inhibition involves the production of a more inhibitory metabolite of NMTT or a derivatization of the enzyme is not clear from the available data, but the underlying process is apparently blocked by the presence of enzymes in the more difficultly solubilized fraction of the membrane.

Whether or not the demonstrable *in vitro* inhibition of the vitamin K-dependent carboxylase by NMTT under rather specific incubation conditions has any relationship to the hypoprothrombinemia observed in patients treated with antibiotics that contain this side chain cannot be assessed from the available data. The antibiotics themselves do not inhibit the carboxylase [20]. The antibiotic-induced hypoprothrombinemia has been reported to respond to vitamin K administration [1, 2, 4, 21], and these antibiotics do not produce hypoprothrombinemia in healthy vitamin K-sufficient volunteers [22]. NMTT itself will enhance the hypoprothrombinemia observed in vitamin K-deficient rats [23] but will not produce hypoprothrombinemia in vitamin K-sufficient rats or dogs [24].

In contrast to the apparent involvement with vitamin K status seen in the antibiotic-induced hypoprothrombinemia, the inhibition of the carboxylase by NMTT *in vitro* was not found in this or previous [13] studies to be influenced by the concentration of vitamin K in the incubation mixture. The vitamin K responsiveness of the antibiotic-induced hypoprothrombinemia is, however, similar [25] to that of the hypoprothrombinemia produced by 4-hydroxycoumarin oral anticoagulants. These drugs are thought to inhibit the conversion of vitamin K epoxide to vitamin K and vitamin KH_2 [9, 26, 27]. The enzyme responsible for this conversion is inactive in the detergent-solubilized preparation that investigators have used to study the influence of NMTT on carboxylation [10], and inhibition of this enzyme cannot be responsible for any of the effects observed on *in vitro* carboxylation. Bechtold *et al.* [21] have observed an increase in plasma vitamin K epoxide in cephalosporin-treated patients which is similar to that seen in oral anticoagulant therapy. These data strongly suggest not only that the clinical problem is unrelated to the *in vitro* effect of NMTT on the vitamin K-dependent carboxylase, but also that it is most likely due to an inhibition of the epoxide reductase.

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