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XENOBIOTIC AND ENDOBIOTIC INHIBITORS OF CYTOCHROME P-450dbl FUNCTION, THE TARGET OF THE DEBRISOQUINE/SPARTEINE TYPE POLYMORPHISM*

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Abstract—Five to 10% of Caucasians are poor metabolizers (PM) of debrisoquine, sparteine, bufuralol and numerous other drugs. A deficiency in cytochrome P-450dbl (P-450dbl) function is the cause of this polymorphism of drug oxidation with autosomal recessive inheritance. In the present study, inhibition of bufuralol-1′-hydroxylase in human liver microsomes by drugs and chemicals was performed in a search for potential new substrates for this polymorphic enzyme. Among the 80 alkaloids and drugs tested, 25 were competitive inhibitors. *In vitro* competitive inhibition of bufuralol oxidation by a substance indicates that this compound is able to bind to the same enzymatic site as bufuralol. This may mean that the competing drug also is metabolized by P-450dbl and that its metabolism is subject to the same genetic variation as the oxidation of bufuralol. However, some of these competitive inhibitors are not oxidized by P-450dbl. In this case, however, they may interfere with the *in vivo* phenotyping procedure by inhibiting the formation of metabolites of test drugs such as debrisoquine, sparteine, metoprolol or dextrometorphan.

One of the main sources of interindividual differences in drug response is the variation in drug metabolizing capacity [1–2]. Genetic polymorphisms of human cytochrome P-450 isozymes are one of the recently discovered reasons for such variability in drug metabolism. One of the best studied examples of a genetically-determined polymorphism, is the debrisoquine–sparteine type polymorphism. Five to 10% of Caucasians exhibit the "poor metabolizer" phenotype (PM) and develop exaggerated responses not only towards debrisoquine and sparteine but towards an increasing list of drugs including several β-blocking agents such as bufuralol, metoprolol, propranolol, or antidepressants like nortryptiline and desipramine, and other drugs such as encainide, propafenone, *N*-propylajmaline, 4-methoxyamphetamine and dextromethorphan [3, 4]. The impaired metabolism of debrisoquine and sparteine has been shown to follow Mendelian inheritance, PM-subjects being homozygous for an autosomal recessive gene [3, 5]. The data obtained in our laboratory provide convincing evidence for the absence of the specific isozyme P-450dbl (P-450IID1, formerly also called P-450 BufI) in the PM subjects [6]. Boobis *et al.* [7, 8] have shown that substrates for this enzyme system are potent mutually competitive inhibitors of their metabolism. Drugs showing a decreased clearance in PM subjects are also competitive inhibitors of the 4-hydroxylation of debrisoquine *in vitro*. This has been described for sparteine, guanoxan, phenformin and bufuralol [7–13]. In contrast drugs such as antipyrine and acetanilide, the metabolism of which is independent of the debrisoquine–sparteine polymorphism [14, 15] are weak and non-competitive inhibitors of debrisoquine 4-hydroxylation [7]. The absence of inhibition can be used to predict which drugs will most likely not be subjected to this polymorphism. It must be emphasized that competitive inhibition may indicate merely that a drug is able to bind to the active site of the enzyme catalysing the inhibited reaction and is consistent with but does not prove that this drug is oxidized by the same enzyme.

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MATERIALS AND METHODS

Materials. Bufuralol and 1′-hydroxybufuralol, debrisoquine and 4-hydroxy-debrisoquine were gifts from Hoffman La Roche (Basel, Switzerland). *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate ($MPDP^+$), 1-methyl-4-phenylpyridinium iodide (MPP^+) were purchased from Chemogen (Konstanz, F.R.G.). 4-Phenyl-1,2,3,6-tetrahydropyridine hydrochloride (PTP) and vincristine sulfate were from Aldrich (Steinheim, F.R.G.). The 4-(4-′-hydroxy)-phenyl-1,2,3,6-tetrahydropyridine (4-OH-MPTP) was a gift of A. Brossi, National Institute of Health (Bethesda, MD). Domperidone, trifluperidol, spiperone, droperidol and benperidol were all purchased from Anawa Trading (Wangen, Switzerland). Some alkaloids such as conine hydrochloride, gelsemine hydrochloride, ajmalicine hydrochloride, ajmaline hydrochloride dihydrate, gramine, ibogaine hydrochloride, mimosine, sempervirine nitrage, serpentine tartrate, α-lobeline hydrochloride, homarine hydrochloride were provided by Sarget Laboratoires S.A. (Merignac, France). Reticuline was a generous gift of Prof. Dr. M. H. Zenk (University of Munich, F.R.G.). Budipine, prodipine, medipine and the 4-hydroxy

derivative of budipine were supplied by Byk Gulden (Konstanz, F.R.G.). All other compounds were purchased from Sigma A.G. (Deisenhofen, F.R.G.).

Human liver samples and preparation of microsomes. Human liver samples were obtained from kidney transplant donors [Kidney donor livers (KDL) 19, 20 and 28]. Liver pieces were frozen in liquid nitrogen and stored at −80° prior to use. Microsomes were prepared using a modified method of that previously described by P. J. Meier *et al.* [16]. Small pieces of frozen liver were thawed in a beaker containing approximately 4 vol. of 0.15 M KCl. Using a Polytron 20S homogenizer (Kinematica GmbH, Kriens, Switzerland) set at 5000 rpm for 30 sec, the pieces (5–10 g wet weight) were immediately homogenized in 4 vol. of 1 mM EDTA, 1 mM DTT, 0.02 mM BHT and 0.1 mM PMSF in 0.15 M KCl. After filtration through 2 layers of gauze to remove the connective tissue, the filtrate was again homogenized in a glass–Teflon Potter–Elvehjem homogenizer set at 800 rpm (pestle tube clearance 0.18–0.24 mm). The homogenate was then centrifuged at 12,000 *g* for 15 min, and the resulting supernatant was submitted to a second centrifugation (27,000 *g* for 15 min). The microsomal fraction was obtained after a centrifugation step at 105,000 *g* for 1 hr. The pellets were washed, resuspended and homogenized with a Dounce homogenizer in a 1 mM EDTA-0.1 M sodium pyrophosphate buffer, pH 7.25. The centrifugation was repeated and the pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.4, to reach an approximate protein concentration of 20 mg/ml. The microsomes were then frozen in liquid nitrogen and stored at −80° until further utilisation. The bufuralol-1′-hydroxylase activity in the liver microsomes of the three livers used (KDL 19, KDL 20, KDL 28) was within the range of liver biopsies of *in vivo* phenotyped EM-subjects (0.681–2.14 nmol · min^{-1} · mg $protein^{-1}$).

Assay for bufuralol 1′-hydroxylation in microsomes. Bufuralol 1′-hydroxylase activity was measured using the peroxygenase function of P-450dbl (U. M. Zanger *et al.*, submitted). Five μg of microsomal protein were incubated in a final volume of 150 μl of 0.1 M sodium phosphate buffer, pH 7.4. The inhibitors and the substrate (+)-bufuralol (5, 20, 100 μM) were mixed with the phosphate solution, then the microsomes were added and the reaction was started by the addition of cumene hydroperoxide (125 or 200 μM). After 10 min incubation at 25° the reaction was stopped by adding 15 μl of 60% $HClO_4$ (w/v). Detection of the product 1′-hydroxy-bufuralol was by reversed-phase HPLC as described previously [17].

Assay for debrisoquine 4-hydroxylation in microsomes. Fifty micrograms of microsomal protein were incubated in the presence of 60 μM debrisoquine and different inhibitor concentrations for 60 min at 37° in a final volume of 150 μl of 0.1 M sodium phosphate buffer, pH 7.4. The reaction was started by the addition of 1 mM NADPH supplemented by a regenerating system consisting of 5 mM isocitrate, 5 mM $MgCl_2$, 1 U/ml isocitrate dehydrogenase. The incubation was stopped by 15 μl of 60% $HClO_4$ (w/v). The analysis of the product was performed as described in [17].

Other methods. Protein concentrations were determined by the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

RESULTS

The drugs and chemicals listed in Table 1 were tested for their ability to inhibit bufuralol 1′-hydroxylation. Reticuline and tetrahydro-β-carboline showed interferences with the chromatography of the bufuralol-1′-hydroxylase assay so that we had to follow their inhibitory potencies on debrisoquine 4-hydroxylase (Table 2). In a first approach we determined an IC_{50} value (concentration of inhibitor causing 50% inhibition of the enzymatic activity) for each compound. For those drugs showing inhibition, Dixon plots [18] and Cornish-Bowden plots [19] were used to calculate apparent K_i. Figures 1 and 2 illustrate a Dixon plot and a Cornish-Bowden plot for MPTP and harmaline, respectively.

We then defined the nature of the inhibition and the affinity of the different molecules for the enzyme more precisely using the Michaelis–Menten equation by submitting the data to kinetic analysis by non-linear least-squares fitting program [20].

DISCUSSION

Known substrates of P-450dbl include alkaloids such as sparteine, *N*-propylajmaline, related compounds such as dextrometorphan and numerous synthetic drugs acting in the central or peripheral or autonomic nervous system such as tricyclic antidepressants, β-adrenergic receptor blocking agents and methoxyamphetamine, or antiarrhythmic drugs such as perhexiline and propafenone [3, 4]. All of these substances contain at least one strong basic nitrogen which presumably serves to anchor the pharmacophore to a negative interaction site of the enzyme, and a lipophilic domain with the oxidation site at or near that domain. Within this context the present study was undertaken to search for potential new drug substrates or inhibitors and possible endogenous compounds which may inhibit or be metabolized by the P-450dbl enzyme. Inhibition by itself is not informative, only competitive inhibition of bufuralol 1′-hydroxylase indicates that the drug binds to the active site of P-450dbl. Moreover, competitive inhibition does not mean that all these inhibitors will also be metabolized by P-450dbl. Only further experiments will show if the transformation of these substances is catalyzed by P-450dbl or if poor metabolizers of debrisoquine show impaired metabolism of these inhibitors. However, as has been shown previously [21], some competitive inhibitors, even if not metabolized by P-450dbl can cause *in vivo* drug interactions which convert an extensive metabolizer of debrisoquine into a subject with poor metabolizer phenotype, falsifying results of population studies which try to assess the incidence of the genetic deficiency. For instance, quinidine is a potent competitive inhibitor of P-450dbl *in vitro* (K_i of 186 nM) but is not a substrate of the P-450dbl enzyme [22]. We have shown that a single-dose treatment of quinidine produces a long-lasting and powerful inhibition of metoprolol metabolism *in vivo* and a shift of the EM to the PM phenotype [21].

Table 1. Effect of different compounds on 1'-hydroxy-bufuralol formation by human liver microsomes

Substances (therapeutic class)	Highest conc. tested	IC_{50}	$K_i \pm SE$	Nature of inhibition
Alkaloids				
Ajmaline (antihypertensive, tranquilizer)	1 μM	656 nM	783 ± 7 nM	Comp.
Ajmalicine (antihypertensive, tranquilizer)	25 nM	3.64 nM	3.3 ± 0.4 nM	Comp.
Ibogaine (antidepressant)	10 μM	357 nM	416 ± 37 nM	Comp.
α-Lobeline (respiratory stimulant)	10 μM	113 nM	123 ± 5 nM	Comp.
Vincamine (vasodilator)	100 μM	60 μM	ND	ND
Vinblastine (antineoplastic)	227 μM	130 μM	ND	ND
Coniine	50 μM	10.8 μM	ND	ND
Sempervirine	50 μM	3.9 μM	ND	ND
Gramine	50 μM	66 μM	ND	ND
Ergotamine (vasoconstrictor)	100 μM	137 μM	ND	ND
Digitoxin (cardiotonic)	250 μM	No inhibition		
Gelsemine	50 μM	No inhibition		
Vincristine (antineoplastic)	100 μM	No inhibition		
Reserpine (antihypertensive, tranquilizer)	25 μM	No inhibition		
Homarine	50 μM	No inhibition		
Mimosine	50 μM	No inhibition		
Tetrahydropapaverine (norlaudanosine)	100 μM	39 μM	26.1 ± 1.2 μM	Comp.
Laudanosine	80 μM	33 μM	28.6 ± 1.6 μM	Comp.
Berberine	80 μM	47 μM	30.9 ± 2.5 μM	Comp.
Harmaline	40 μM	14 μM	7.43 ± 1.15 μM	Comp.
Drugs				
Trifluperidol (neuroleptic)	4 μM	0.5 μM	169 ± 15 nM	Comp.
Haloperidol (neuroleptic)	60 μM	4.9 μM	1.23 ± 0.12 μM	Comp.
Fluphenazine (neuroleptic)	5 μM	1.9 μM	1.03 ± 0.11 μM	Comp.
Spiperone (neuroleptic)	100 μM	11.2 μM	ND	ND
Droperidol (neuroleptic)	50 μM	35.6 μM	ND	ND
Benperidol (neuroleptic)	50 μM	67 μM	ND	ND
Domperidone (anti-emetic)	50 μM	14.9 μM	ND	ND
Sulpiride (neuroleptic)	50 μM	No inhibition		
Metoclopramide (anti-emetic)	100 μM	49.6 μM	26.6 ± 2.9 μM	Comp.
Apomorphine (emetic)	80 μM	30.4 μM	15.5 ± 2.3 μM	Comp.
Phenelzine (antidepressant)	100 μM	170 μM	ND	ND
Phenylcyclopropylamine (antidepressant)	60 μM	16.7 μM	30.7 ± 2 μM	Non-comp.
Pargyline (monoamine oxydase inhibitor)	100 μM	No inhibition		
Diphenhydramine (antihistaminic)	496 nM	4.2 μM	462 ± 30 nM	Comp.
Nicardipine (vasolidator)	8 μM	2.2 μM	876 ± 152 nM	Comp.
Octopamine (sympathomimetic)	50 μM	No inhibition		
Synephrine (sympathomimetic)	50 μM	No inhibition		

continued

Table 1. (*continued*)

Substances (therapeutic class)	Highest conc. tested	IC_{50}	$K_i \pm SE$	Nature of inhibition
Budipine (1-*t*-butyl-4,4-diphenylpiperidine) (Parkinson's disease treatment)	100 nM	19.6 nM	15.4 ± 1.6 nM	Comp.
Prodipine (1-isopropyl-4,4-diphenylpiperidine)	208 nM	ND	4.8 ± 0.2 nM	Comp.
Medipine (1-methyl-4,4-diphenylpiperidine)	36.4 μM	4 μM	3.58 ± 0.38 μM	Comp.
4,4-Diphenylpiperidine	53 μM	7.3 μM	3.42 ± 0.4 μM	Comp.
1-*t*-butyl-4-hydroxyphenyl-4-phenylpiperidine	442 nM	247 nM	221.7 ± 19 nM	Comp.
MPTP	50 μM	36.6 μM	16.1 ± 2 μM	Comp.
$MPDP^+$	50 μM	40.4 μM	32 ± 3.9 μM	Comp.
MPP^+	125 μM	124 μM	42.2 ± 6.2 μM	Comp.
PTP	50 μM	12.2 μM	7.4 ± 0.7 μM	Comp.
4-OH-MPTP	100 μM	No inhibition		
Bromocryptine (lactation inhibitor)	100 μM	No inhibition		
Disopyramide (anti-arrhythmic)	100 μM	No inhibition		
Endogenous substances/steroids				
Tryptamine	500 μM	138 μM	ND	ND
Progesterone	500 μM	194 μM	ND	ND
Androstene-3,17-dione	50 μM	84 μM	ND	ND
Norethindrone acetate	100 μM	100 μM	61.6 ± 12 μM	Comp.
L-Dopa	500 μM	No inhibition		
D-Dopa	500 μM	No inhibition		
Norepinephrine	500 μM	No inhibition		
Epinephrine	500 μM	No inhibition		
Metanephrine	100 μM	No inhibition		
Normetanephrine	100 μM	No inhibition		
Dopamine	100 μM	No inhibition		
Serotonin	500 μM	No inhibition		
Melatonine	500 μM	No inhibition		
Salsolinol	500 μM	No inhibition		
Endogenous substances				
Estriol	500 μM	No inhibition		
Estrone	100 μM	No inhibition		
17-α-OH-Progesterone	500 μM	No inhibition		
Cholesterol	100 μM	No inhibition		
β-Estradiol	500 μM	No inhibition		

The IC_{50}s were determined using 5 μM or 10 μM (+)-bufuralol. The inhibition studies were performed using 5, 20, 100 μM (+)-bufuralol. For each substrate concentration bufuralol 1'-hydroxylase was evaluated for 6 concentrations of each inhibitor, ranging from 0 to 2 or 3 times the approximate K_i determined in previous experiments. All points were determined in duplicate incubations.

In previous *in vitro* studies microsomal bufuralol 1'-hydroxylase was assessed by its monooxygenase function. In fact the overall activity measured by that method was due to at least two P-450 isozymes. Both enzymes, P-450dbl (formerly called P-450 BufI, high affinity, high capacity) and P-450 BufII (low affinity, low capacity), respectively, contribute to human liver microsomal bufuralol-1'-hydroxylation [23]. Recently, U. Zanger *et al.*, in our laboratory, have developed a novel assay system using the peroxygenase function of P-450dbl which allows the specific assessment of the function of P-450dbl in microsomes without major interferences by other isoenzymes, and enabled us to conduct the present study [22].

Table 2. IC_{50} values for inhibition of debrisoquine 4-hydroxylase activity of human liver microsomes

Inhibitor	IC_{50} (μM)
Reticuline	4
Tetrahydro-β-carboline	50

The IC_{50}s were determined using 60 μM debrisoquine. Inhibitions were performed using four different inhibitor concentrations. All points were determined in duplicate.

Data from other laboratories suggested that chlopromazine, nortryptiline and haloperidol bind to the same cytochrome P-450 active site as debrisoquine and sparteine [24, 25]. Recently, a significantly higher mean debrisoquine metabolic ratio (MR, debrisoquine/4-OH-debrisoquine excretion in the urine) has been described in patients treated with thioridazine and levopromazine as compared to patients without neuroleptic treatment [26]. Some authors have reported that a combination of neuro-

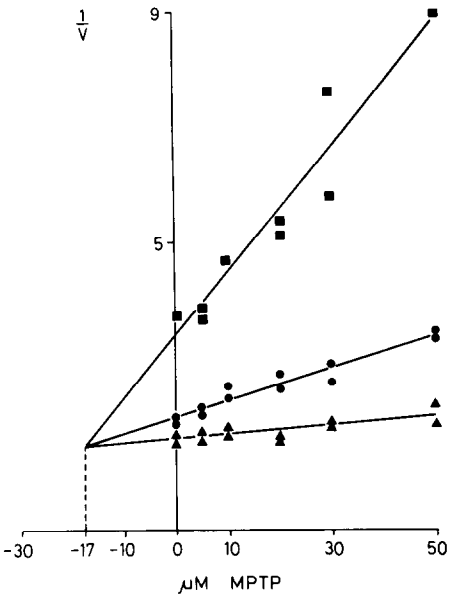

Fig. 1. Dixon plot illustrating the effect of various concentrations of MPTP on bufuralol-1′-hydroxylase activity. The rate of appearance (V, nmol·min^{-1}·mg protein^{-1}) of 1-OH-bufuralol following a 10 min incubation with (+)-bufuralol (—■— 5, —●— 20, —▲— 100 μM) in human liver microsomes (KDL 19). The lines correspond to the best fit of the data.

leptic and tricyclic antidepressant drugs influences the transformation of the last compound and produces elevated tricyclic antidepressant concentrations in blood [27, 28]. We observed that several additional neuroleptics are able to bind to P-450dbl. The two most potent ones are the butyrophenone trifluperidol (K_i of 169 nM) and the phenothiazine fluphenazine (K_i of 1 μM). This observation may be of clinical importance, as these compounds have a high affinity and therefore could block the active site of P-450dbl as long as their *in vivo* concentrations in the liver remain sufficiently above the K_i concentration. The competitive inhibition alone, therefore, has potential clinical significance whether or not these compounds are metabolized by P-450dbl. The parenteral administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces a persistent Parkinsonism-like syndrome in human drug addicts due to selective destruction of the dopamine-containing cells of the substantia nigra in brain [29, 30]. 1-*t*-Butyl-4,4-diphenyl-piperidine (budipine) is a novel agent in the therapy of Parkinson's disease [31]. This compound and its analogs showed strong affinity for P-450dbl. Budipine is known to mitigate MPTP toxicity as has been described for pargyline and deprenyl [32]. Interestingly, also, MPTP and several of its analogs were competitive inhibitors of P-450dbl, in contrast to pargyline. Compounds similar to MPTP may be present in the environment. One of them is the harmala-alkaloid tetrahydro-β-carboline, which has been proposed as a naturally occurring toxicant causing Parkinson's disease [33, 34]. It also inhibits P-450dbl activity. All present-day neuroleptic drugs selectively act on dopamine receptors throughout the brain and the body. The fundamental molecular explanation for the selective dopamine receptor-blocking action of neuroleptics is that all these drugs can adopt a conformation or shape in which certain portions or domains of the molecule are identical to the corresponding features of dopamine [35]. This observation and the fact that we were able to detect P-450dbl in human brain [36] led us to check the possibility of P-450dbl involvement in the biosynthetic pathway of dopamine. We tested a series of possible endogenous candidates, but, as can be seen in Table 1, none of them were inhibitory. Recently, antihistaminic compounds of the orphenadrine–diphenhydramine type were described as influencing the urinary metabolic ratio of debrisoquine both in patients suffering from Parkinson's disease and in controls [37]. In fact, diphenhydramine competitively inhibits P-450dbl (K_i: 462 nM) *in vitro* which could explain that the hydroxylation of debrisoquine indeed is hindered in patients under this drug. Many drug and combustion products are derived from plants or are similar in chemical structure to plant metabolites. It has been proposed that some P-450 gene families (including the one of P-450dbl) have evolved and diverged in animals due to their exposure to plant metabolites and decayed plant products during the last billion years [38]. In fact, several of the naturally occurring plant alkaloids have been shown to competitively inhibit sparteine and bufuralol oxidation [24, 25]. As depicted in Table 1, vincristine, reserpine, mimosine, digitoxine, gelsemine, homarine did not inhibit bufuralol 1′-hydroxylation. For all other compounds tested, the affinity for the enzyme varied from the low to high micromolar range. Of interest is the strong inhibitory potency of ajmalicine because this compound is still used in some countries as an antihypertensive agent or as a tranquilizer. Interestingly, reticuline, one of the most widespread alkaloids in the plant kingdom, a precursor of many aporphine

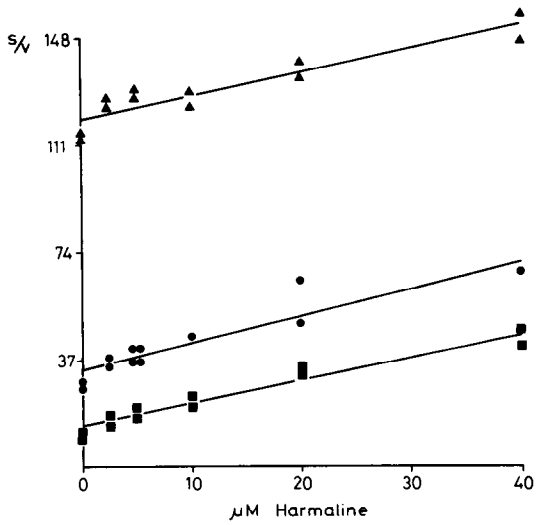

Fig. 2. Effects of harmaline on the 1′-hydroxylation of bufuralol in human liver microsomes (KDL 19). (+)-bufuralol (—■— 5, —●— 20, —▲— 100 μM). The solid line corresponds to the program-generated best fit.

and morphine type alkaloids, inhibits debrisoquine 4-hydroxylation.

The increasing list of alkaloids inhibiting P-450dbl and being substrates of this enzyme substantiates the hypothesis that the debrisoquine gene locus may have evolved to deal metabolically with toxic food constituents, particularly plant alkaloids. These *in vitro* inhibition studies of human liver microsomal bufuralol-1′-hydroxylase constitute a simple and fast experimental approach to analyse the inhibiting capacity of different substances. This study should serve as a guide for further experiments in which the metabolism of these inhibitors has to be examined.

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