adenylic acid in the present experiments; see Table 1). Preliminary treatment with chlorgyline under conditions when this inhibitor largely blocked type A MAO activity (to judge from the deamination of 5-HT), but left type B MAO activity almost unchanged (tested by deamination of β-phenylethylamine), prevented the appearance of ability to deaminate histamine or AMP under these experimental conditions (Table 1). Conversely, deprenil, a type B MAO inhibitor, did not prevent the appearance of ability to catalyze these reactions in mitochondria incubated under aerobic conditions in the presence of Cu^{2+} cations. The results indicate that the ability to undergo transformation of catalytic activity under conditions favoring oxidation of SH groups is a feature of type A MAO but not of type B. It is known that MAO deaminates the most important of the neuromediators [5] and that transformation is particularly readily undergone by the MAO of brain tissue [4].

LITERATURE CITED

1. V. Z. Gorkin, Zh. Vses. Khim. Obshch. im. Mendeleeva, No. 2, 181 (1876).
2. J. P. Johnston, Biochem. Pharmacol., 17, 1285 (1968).
3. J. Knoll and K. Magyar, Adv. Biochem. Psychopharmacol., 5, 393 (1972).
4. T. A. Moskvitina et al., J. Neurochem., 26, 209 (1976).
5. N. H. Neff and H.-Y. T. Yang, Life Sci., 14, 2061 (1974).
6. I. V. Verevkina (Veryovkina) et al., Biochim. Biophys. Acta, 258, 56 (1972).

INTERACTION BETWEEN 2,6-DIMETHYL-3,5-DICARBETHOXY-1,4-DIHYDROPYRIDINE AND ENZYMES OF THE NADPH-SPECIFIC ELECTRON TRANSPORT CHAIN OF RAT LIVER MICROSOMES

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2,6-Dimethyl-3,5-dicarbethoxy-1,4-dihydropyridine interacts with the NADPH-dependent electron transport system of rat liver microsomes: It forms a type 1 complex with the terminal oxidase (cytochrome P-450) and also definitely inhibits the activity of NADPH-cytochrome c reductase and methindione demethylase. In experiments in vivo repeated administration of the compound had no inducing action on microsomal enzymes.

KEY WORDS: liver microsomes; electron transport system; 2,6-dimethyl-3,5-dicarbethoxy-1,4-dihydropyridine; antioxidants.

The compound 2,6-dimethyl-3,5-dicarbethoxy-1,4-dihydropyridine (DHP) possesses marked antioxidant and antiradical activity [1, 2, 6]. By oxidation of DHP in vivo 2,6-dimethyl-3,5-dicarbethoxypyridine is formed.

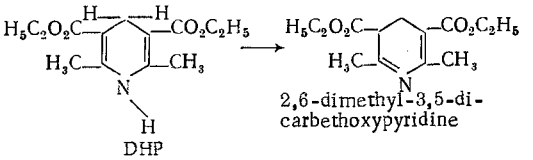

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TABLE 1. Effect of DHP and Its Oxidized Form on NADPH-Cytochrome c Reductase Activity in Rat Liver Microsomes (M ± m)

Experimental conditions	Cytochrome c reductase activity, nmoles/min/mg protein	Activity, % of control
Microsomes + NADH (2.5 mM) (control)	319±8,4 (5)	100
Microsomes + NADH + oxidized form of DHP (20 mM)	322±5.6 (5)	100
Microsomes + NADPH + DHP (20 mM)	182±4,1* (5)	57
Microsomes + NADPH + DHP (60 mM)	115±1,8* (5)	36

Legend: 1) Protein content of sample 1.67 mg. 2) Number of experiments shown in parentheses (also in Table 2). 3) Asterisk indicates that difference from control is statistically significant, $P < 0.01$.

TABLE 2. Effect of DHP on Content of Protein and Cytochromes P-450 and b_5 and on Aniline Hydroxylase Activity in Rat Liver Microsomes (M ± m)

Experimental conditions	Content of microsomal protein in liver, mg/g	Content of cytochrome P-450 nmoles/mg protein	Content of cytochrome b_5, nmoles/mg protein	Aniline hydroxylase activity, nmoles 4-aminophenol/mg protein/30 min
Control	20,74±1,44 (11) 100%	0,42±0,01 (6) 100%	0,20±0,02 (6)	1,05±0,025 (15) 100%
After addition of DHP (days):				
1	15,08±0,61* (4) 73%	0,61±0,04* (6) 145%	0,25±0,03 (6)	1,10±0,027 (5) 104%
2	27,16±1,25* (5) 131%	0,41±0,01 (5)	0,15±0,002* (5)	1,41±0,024 (5) 134%
3	18,44±0,37 (6)	0,44±0,004 (5)	0,20±0,004 (5)	1,41±0,027 (5) 134%
4	20,90±0,34 (6)	0,43±0,01 (5)	0,16±0,001 (5)	0,85±0,013 (5) 81%
6	20,09±0,32 (5)	0,30±0,001* (5) 71%	0,15±0,00* (5)	—
10	22,52±0,85 (6)	0,37±0,004* (6) 88%	0,17±0,001 (6)	0,86±0,000 (5) 82%

* Changes (in %) significant ($P < 0.05$) relative to control.

The antioxidant property of DHP can be used clinically for the treatment of various pathological states [4, 5]. The mechanism of the antioxidant effect of chemical compounds is known to be definitely linked with the function of the NADPH-dependent electron transport system of the liver microsomes [13].

It was accordingly decided to study interaction between DHP and certain components of the liver microsomes.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male rats weighing 180-200 g. The liver microsomes were isolated by the method of Cinti et al. [9]. Interaction between DHP and enzymes of the NADPH-dependent electron transport system of the microsomes was studied by spectrophotometric methods. The binding of cytochrome P-450 with substrates was recorded on the Specord UV VIS (East Germany) spectrophotometer by the method of Schenkman et al. [12].

Microsomal cytochromes were determined quantitatively by the method of Omura and Sato [11]. The following extinction coefficients were used: for cytochrome b_5 163 $cm^{-1} \cdot mM^{-1}$ for the difference between absorption spectra at 424 and 409 nm, for cytochrome P-450 91 $mm^{-1} \cdot mM^{-1}$ for the difference between absorption at 450 and 490 nm.

Activity of NADPH-cytochrome c reductase was studied by the method of Torrielli and Slater [13]. The demethylation of methindione was determined from the quantity of formaldehyde formed during oxidative demethylation of the substance [3]. Activity of aniline hydroxylase was determined by the phenolindophenol method from the formation of 4-aminophenol [8]. Protein was determined by the biuret reaction [10].

EXPERIMENTAL RESULTS

Investigation of the effect of DHP on the first part of the microsomal electron transport chain (NADPH-cytochrome c reductase) showed that the compound definitely inhibits the activity of that enzyme in vitro; moreover, the inhibitory effect increases with an increase in the concentration of the antioxidant. Like the other known antioxidant, pyrogallate, perhaps DHP binds the radical semiquinone form of the flavoprotein and thereby blocks electron transport in the first part of the microsomal chain [13]. Oxidized DHP (3,5-dicarbethoxy-2,6-dimethylpyridine), which has lost its antiradical properties, has no inhibitory effect on NADPH-specific flavoprotein (Table 1).

The compound DHP also interacts with the terminal part of the chain, cytochrome P-450. On binding with cytochrome P-450, DHP induces type 1 spectral changes. The absorption maximum of the differential spectrum of type 1 preparations lies in the 390-nm region; DHP itself absorbs in that region. For that reason the type 1 spectrum of the preparation was identified from the absorption minimum in the 420-nm region and its deepening in the presence of an excess of NADPH. 3,5-Dicarbethoxy-2,6-dimethylpyridine also forms a type 1 complex with cytochrome P-450.

By forming a complex with cytochrome P-450, DHP may disturb interaction of other xenobiotics with this enzyme. This has been shown by the writers for two different substrates of cytochrome P-450. For instance, DHP was shown to be a competitive inhibitor of the demethylation of methindione, a typical substrate of cytochrome P-450; DHP also prevents interaction between CCl_4 and cytochrome P-450 [7].

The writers showed previously that DHP in vitro inhibits the peroxidation of lipids of unsaturated fatty acids initiated by CCl_4 metabolites in rat liver microsomes [7]. The mechanism of this effect of the antioxidant, taking into account the results described above, can be represented as follows. First, by blocking centers of radical formation (NADPH-cytochrome c reductase and cytochrome P-450), DHP may inhibit the conversion of CCl_4 into the $[\cdot CCl_3]$ radical. Second, since it possesses antiradical properties, DHP can directly bind the $[\cdot CCl_3]$ radical. This leads to inhibition of peroxidation processes.

The ability of DHP to block the activity of the enzymes of the NADPH-dependent electron transport system of the microsomes may thus play a definite role in the mechanism of its antioxidant effect. Antioxidants have been used for a long time as pharmacotherapeutic agents. However, during prolonged administration of DHP induction of microsomal enzymes may be observed, with a consequent diminution of the antioxidant properties. Because of this the effect of DHP, given by repeated oral doses of 200 mg/kg as a suspension in Tween-80, on the quantitative content of cytochromes b_5 and P-450, the concentration of microsomal protein, and aniline hydroxylase activity was studied. As Table 2 shows, during the first 2 days the compound caused definite fluctuations in protein content, which returned to normal during the following day. The level of cytochrome P-450 did not increase until the second day, and the concentration of this enzyme started to fall on the sixth day. The content of cytochrome b_5 and aniline hydroxylase activity showed no significant changes at any time during the study. Repeated administration of DHP thus has no inductive effect on the microsomal enzymes studied.

LITERATURE CITED

1. A. Kh. Velena, "Investigation of the action of antioxidants of the dihydropyridine series on biological membranes and their models," Author's Abstract of Candidate's Dissertation, Riga (1975).
2. G. Ya. Dubur, Ya. R. Uldrikis, A. Ya. Kumerova, et al., Abstracts of Proceedings of the Seventh International Symposium on the Chemistry of Natural Compounds [in Russian], Riga (1970), p. 682.
3. V. Klinger, Farmokol. Toksikol., No. 1, 63 (1972).
4. A. Ya. Maiore, L. A. Zarinya, G. Ya. Dubur, et al., Izv. Akad. Nauk Latv. SSR, No. 7, 107 (1972).
5. M. N. Merzlyak, A. T. Bozhenova, Yu. N. Kaurov, et al., in: Biological Antioxidants [in Russian], Moscow (1975), p. 161.
6. G. D. Tirzitis and G. Ya. Dubur, Khim. Geterotsikl. Soed., No. 1, 133 (1972).
7. A. P. Shtokman, "The role of microsomal enzymes of the liver in metabolism and in the antihepatotoxic effect of methindione," Author's Abstract of Candidate's Dissertation, Riga (1975).
8. R. I. Chabra, T. E. Gram, and J. R. Fouts, Toxicol. Appl. Pharmacol., 22, 50 (1972).

9. D. L. Cinti, P. Moldens, and J. R. Schenkman, Biochem. Pharmacol., 21, 3249 (1972).
10. E. Layne, in: Methods in Enzymology, Vol. 3 (ed. by S. P. Colowick and N. O. Kaplan), Academic Press, New York (1957), p. 451.
11. T. Omura and R. Sato, J. Biol. Chem., 239, 2370 (1964).
12. J. Schenkman, H. Remmer, and R. Estabrook, Mol. Pharmacol., 3, 113 (1967).
13. M. V. Torrielli and T. P. Slater, Biochem. Pharmacol., 20, 2027 (1971).

LATENT CREATINE PHOSPHOKINASE ACTIVITY IN THE LIPOSOMES

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To study the conditions for preparing liposomes with a high internal protein content the uptake of creatine phosphokinase by the liposomes was studied quantitatively under different experimental conditions. Addition of 20% of a positively charged lipid (stearylamine) to the lipid mixture followed by ultrasonic treatment of the liposomes for 1 min gave the highest levels of latent activity of the enzyme. Maximal uptake (6.1%) was obtained by the use of a 300 mM glucose solution, pH 7.0, containing 0.5 mg protein/ml, as the aqueous phase. Washing the preparations of liposomes with physiological saline at 205,000g gave minimal external enzyme activity. When the liposomes were kept for 7 days under argon protection at 4°C there was no decrease in the internal protein content and no increase in external activity.
KEY WORDS: liposomes; creatine phosphokinase.

One way of correcting inborn errors of metabolism is by replacement enzyme therapy. It has been shown that cells can take up proteins in vitro in cultures of mutant fibroblasts [2]. After parenteral administration of purified enzymes to patients a sharp increase in their activity has been found in the liver and cerebrospinal fluid [4, 8]. The absence of a clinical effect following treatment of this sort is attributed to the slow rate of penetration of proteins through the plasma membrane of the cells and to the development of hyperthermic crises and immunological reactions during repeated administration of the enzymes [3].

Recently published investigations have shown that these disadvantages can be overcome by protecting the injected proteins with artificial lipid membranes [10]. These structures, which have been called liposomes [13], are vesicles, surrounded by multilayered membranes, formed by the swelling of phospholipids in an aqueous medium. If the procedure is carried out in a solution of proteins, these can be incorporated into the interlamellar spaces of the liposomes [10].

This paper gives the results of an investigation into the conditions for preparing liposomes with a high internal protein content and minimal external activity, their purification from unbound protein, verification of the integrity of the liposomes, and their keeping properties.

EXPERIMENTAL METHOD

Creatine phosphokinase (CPK; Olaine factory, USSR) was used as the test system. This protein has high stability when kept in solution. Phosphatidylcholine (PCh) was obtained from phospholipids of egg yolks [9].

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