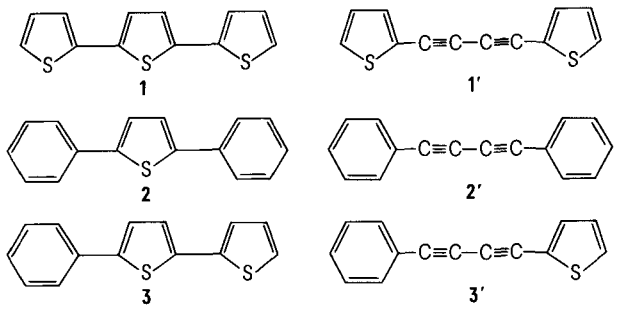
We recently reported that α-terthienyl (**1**)[4a,6] and 1,4-diphenylbutadiyne (**2′**)[7] were the first 2 new type B phototoxic compounds found outside the psoralens. We now disclose that 3 new type B phototoxic molecules have been discovered, namely 1,4-di(2-thienyl)butadiyne (**1′**), 2-phenyl-5-(2-thienyl)thiophene (**3**), and 1-phenyl-4-(2-thienyl)butadiyne (**3′**).

The close relationship between butadiynes and thiophenes in plants[8] suggested that these 2 types of molecules might have similar phototoxic properties. This hypothesis turned out to be incorrect since **2′** was phototoxic and **2** was not. However, the components of the other 2 pairs, **1** and **1′**, and **3** and **3′**, all exhibited type B phototoxicity, as demonstrated by their activity in the presence of UVA against *C. utilis*, and against both *E. coli* B and *S. cerevisiae* under nitrogen.



Because of the convenient screening procedures now available, it appears certain that the number of type B phototoxic molecules which are not furocoumarins will increase rapidly, and a structure-activity relationship will eventually be established. Fowlks et al.[9] suggested that there was a direct relationship between the diameter of the zone of inhibition and the activity of the compound tested. In general, this will not be true since the results will also depend on the light absorption characteristics and on the solubility of the compounds being tested, which cannot be expected to be identical in all compounds.

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Effect of liposomes containing cholesterol on a hepatic cholesterol-7a-hydroxylase and drug oxidation system

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Summary. A statistically significant increase ($p < 0.01$) in the activities of the microsomal enzymes cholesterol-7a-hydroxylase (EC 1.14.13.17), biphenyl-4-hydroxylase, aniline hydroxylase, NADPH-cytochrome-c-reductase (EC 1.6.2.4) and p-nitroanisole-O-demethylase, was shown in cholesterol-loaded rat liver microsomes. The data indicate that profound functional changes are induced in membrane proteins by exogenous cholesterol.

Cholesterol is a common component of biological membrane lipids, playing an important role in the control of the activity of membrane enzymes by modulating their structure[2-5]. Liver microsomal membrane cholesterol, which contributes up to 7% of the total lipid, may have both metabolic and structural functions[6]. Cholesterol from either 'aqueous solutions', or from lipid vesicles (liposomes), can be incorporated into the structure of biomembranes, producing profound functional changes in membrane-associated enzymes[3,4,7]. In the present study the effect of cholesterol on the activity of some microsomal enzyes was examined by incubating liver microsomes with cholesterol-rich liposomes.

Materials and methods. Liposomes of dipalmitoyl phosphatidyl choline (DPL) (Sigma) and cholesterol (Sigma) in the molar ratio 1:2, with (4-^{14}C)-cholesterol (sp. act. 57 mCi/mmole; Amersham, England) as a tracer, were prepared essentially as previously described[8] and suspended in 1.15% KCl-0.1 M potassium phosphate buffer, pH 7.4. Liposomes were extracted with $CHCl_3$:CH_3OH[9] and the extract chromatographed on silica gel G with appropriate standards, using $CHCl_3$:CH_3OH:CH_3COOH:H_2O (20:12:3:1 v/v) as the developing system. The content of lipid phosphorus was measured[10] in the scraped spots previously digested in 70% $HClO_4$ at 180 °C, and the cholesterol content by gas chromatography using cholestane as an internal standard. Microsomes were isolated from rat livers, according to Burke and Bridges[11], and suspended in 1.15% KCl-0.1 M potassium phosphate buffer, pH 7.4, at a concentration of 10 mg protein per ml. The microsomal lipids were quantified by $CHCl_3$:CH_3OH extraction[10] and the amount of phospholipids and cholesterol was estimated as stated above.

A liposomal suspension (40 ml), corresponding to 1.0 μmole of cholesterol, was incubated with 20 mg microsomal protein with continuous stirring at room temperature (23 °C), in a 1.15% KCl-phosphate buffer, 0.1 M, pH 7.4. Aliquots of the incubation mixture (4.0 ml) were removed at appropriate time intervals and after dilution with the same buffer were centrifuged for 30 min at 100,000 × g. The precipitate was resuspended in 20% (w/V) sucrose-phosphate buffer, 0.1 M, pH 7.4, and layered at the interface between 27% sucrose-phosphate buffer, 0.1 M, pH 7.4, and 1.15% KCl-phosphate buffer, 0.1 M, pH 7.4. The gradient was centrifuged for 2 h at 100,000 × g and the microsomes free of liposomes were recovered as a pellet at

the bottom of the gradient. Microsome used for measurement of ^{14}C-cholesterol uptake were dissolved in 1 ml 1% sodium dodecyl sulfate (SDS) and counted in 10 ml of a dioxanebased scintillator (Bray's solution). Cholesterol uptake is expressed as nmoles of cholesterol incorporated per mg microsomal protein. Cholesterol-7a-hydroxylase assay was based on the method of Shefer et al.[12] Biphenyl-4-hydroxylase activity was determined as described by Prough and Burke[13]; aniline hydroxylase activity as described by Imai et al.[14]; NADPH-cytochrome-c-reductase according to Phillips and Langdom[15] and p-nitroanisole-O-demethylase activity according to Netter and Seidel[16].

Results and discussion. We have recently developed procedures (gradients) for the removal of adherent cholesterol from membranes previously incubated either with cholesterol 'solution' or with liposomes containing cholesterol, which permit the determination of the rate of cholesterol incorporation within the membrane framework. Thus, we have observed that the incorporation of cholesterol follows second order kinetics[17]. In the present experiments the incubation of cholesterol-rich liposomes with liver microsomes resulted in a transfer of cholesterol from liposomes to microsomes, which was linear with time over the 6 h of the study (figure 1). Based on the estimate that the indigenous levels of cholesterol and phospholipids in microsomes are 65±6 nmoles/mg protein and 423±32 nmoles/mg protein, respectively, the fraction incorporated into the membrane during a 6-h incubation was 33.8% of the indigenous cholesterol and was also 6.2% of the tolerable cholesterol overload, assuming that the maximum quantity of overload of a membrane would lead to a limiting 1:1 ratio of cholesterol to phospholipids[5]. To link the structure-modifying effect of cholesterol incorporated within the microsomal membranes to possible modulations of their activity the following procedure was used. As a first step estimations were made of the activities of the cholesterol-7a-hydroxylase (a mixed function oxidase, catalyzing the rate-limiting step in the pathway of bile acid synthesis), and of some enzymes of the drug oxidation system, namely, biphenyl-4-hydroxylase, aniline hydroxylase, p-nitroanisole-O-demethylase and NADPH-cytochrome-c-reductase. As shown in the table, cholesterol causes a statistically significant increase ($p < 0.01$) in the activity of each of the above-mentioned enzymes. Moreover, biphenyl-4-hydroxylase activity, estimated in a time course experiment, increased linearly with the amount of cholesterol incorporated (figure 2).

In separate control experiments microsomal membranes incubated with pure DPL liposomes had no effect on the microsomal enzyme activity. To examine whether the cholesterol effect could be reversed by removing the added cholesterol from the membranes, the following experiment was performed. Microsomal membranes enriched with cholesterol were incubated for 3 h with either 1.15% KCl-0.1 M potassium phosphate buffer, pH 7.4, or with egg-lecithin liposomes which are known to remove cholesterol from biomembranes[18]. A statistically significant decrease ($p < 0.01$) was shown, which clearly indicates that the enzymatic modulations caused by cholesterol are partially reversible (table).

The cholesterol to phospholipids (C/PL) ratio is considered

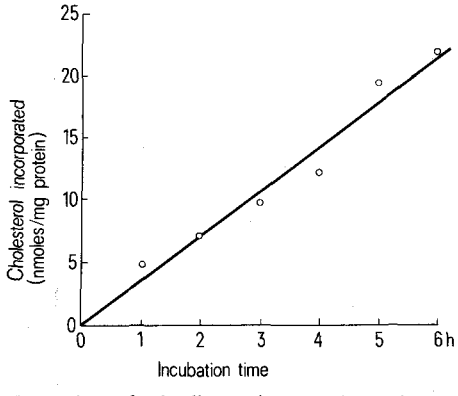

Fig. 1. Cholesterol uptake by liver microsomal membranes during incubation in the presence of cholesterol-rich liposomes. Microsomes (0.5 mg protein/ml) were incubated in the presence of 25 nmoles liposomal cholesterol/ml in a medium consisting of 1.15% KCl-0.1 M phosphate buffer, pH 7.4, 100 U/ml penicilling-G and 100 µg/ml streptomycin sulfate. Computer analysis for curve fitting was performed in a programable Hewlett-Packard HP-97 calculator. The coefficients of determination (r^2) for linear fitting were above 0.95. The zero-time intercept was subtracted from all measurements. Analogous treatment was applied to the biphenyl-4-hydroxylase in which, however, the statistically determined intercept corresponded to the actually determined activity of zero-time preincubation, and was included in the determination of the regression line.

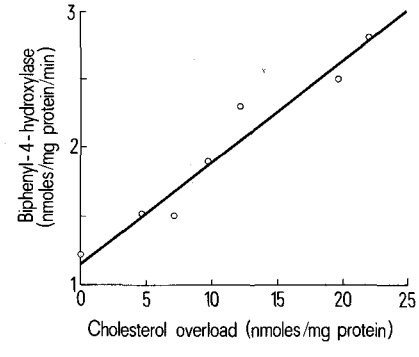

Fig. 2. Biphenyl-4-hydroxylase activity of microsomal membranes, as a function of the cholesterol overload in the membrane (i.e., nmoles cholesterol incorporated per mg microsomal protein).

Effect of liposomes on the activity of certain rat liver microsomal enzymes

Enzymes tested	Untreated microsomes	CH/DPL treated microsomes	CH/DPL treated microsomes incubated with egg-lecithin
Cholesterol-7a-hydroxylase	0.023±0.003	0.038±0.004*	0.029±0.003**
Biphenyl-4-hydroxylase	1.05 ±0.10	2.8 ±0.15*	1.8 ±0.12**
Aniline hydroxylase	0.40 ±0.03	0.89 ±0.07*	0.59±0.6**
NADPH-cytochrome-c-reductase	51 ±3.3	112 ±4.8*	75 ±4.1**
p-Nitroanisole-0-demethylase	0.32 ±0.02	0.69 ±0.03*	0.48 ±0.02**

Enzyme activities were estimated as: nmoles 7a-hydroxycholesterol/mg protein/min; nmoles 4-hydroxybiphenyl/mg protein/min; nmoles p-aminophenol/mg protein/min; nmoles cytochrome-c reduced/mg protein/min. * Statistically significant compared with untreated microsomes ($p < 0.01$); ** statistically significant compared with untreated and CH/DPL treated microsomes ($p < 0.01$). Values given are mean± SD for 4 samples. CH/DPL, cholesterol/dipalmitoylphosphatidylcholine.

to be the main determinant of fluidity in liposomes[18] and biomembranes, so that membrane enzyme activities may increase or decrease, as dictated by the optimal fluidity level[19]. The present results reveal a high degree of sensitivity of the microsomal membranes to cholesterol, as small changes of the ratio C/PL can produce considerable changes in the microsomal activity.

We recently reported[20] the existence of 2 different cytochromes P_{450} for cholesterol-7a-hydroxylase and for drug oxidation. This was based on the observed differential effect of oestradiol, in vivo, on the activity of these 2 processes. The present results may indicate that oestradiol changes the activity of the endoplasmic reticulum by a mechanism other than by altering its cholesterol to phospholipids ratio.

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Enhancement by pyridoxine of the action of diazepam on spinal presynaptic inhibition[1]

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Summary. In spinal unanesthetized cats, pyridoxine pretreatment significantly enhanced the diazepam-induced increase in dorsal root potentials and dorsal root reflexes.

The predominant action of diazepam on spinal synaptic processes exhibits itself as a pronounced enhancement of the electrophysiological correlates of presynaptic inhibition. Thus there is an increase in the antidromic discharge across the afferent dorsal spinal roots (the dorsal root reflex) as well as in the electrotonic spread of depolarization (the dorsal root potential)[2]. Because of the proposed mediation of primary afferent depolarization by γ-aminobutyric acid (GABA)[2,3], the relationship between the spinal action of diazepam and pharmacological antagonists of GABA, as well as substances which manipulate GABA levels in the CNS, was investigated. Semicarbazide, which reduces GABA levels, and the GABA antagonist bicuculline were found to block the action of diazepam[4,5], which suggested a GABA link in some of the actions of diazepam, particularly those which may underly its skeletal muscle relaxant and anticonvulsant activity.

Since pyridoxal phosphate is a coenzyme known to be required in the biosynthesis of GABA from glutamic acid[6], it appeared likely that a B_6-induced increase in the availability of GABA could enhance the spinal and anticonvulsant actions of diazepam. The possibility that pyridoxine may potentiate the enhancement of presynaptic inhibition by diazepam is explored in this report.

Experiments were performed on adult cats the spinal cord of which was transected at the atlanto-occipital junction under ether. Immediately following the transection, anesthesia was discontinued and the cats were placed under artificial respiration, with end tidal levels of CO_2 maintained between 3 and 3.5%. Both carotid arteries were ligated and the brain made ischaemic by temporary manual pressure applied to the vertebral arteries.

The lumbosacral spinal cord was exposed by laminectomy, the dura sectioned, and a pool of paraffin oil was prepared to cover nerve tissue and maintained at 37 °C by thermostatic control. A heating pad was placed under the cat to

The increase in DRP and DRR in spinal cats 5 min after diazepam, with and without pretreatment with pyridoxine

	B_6/diazepam	Saline/diazepam	t-test (non-paired)
DRP	33.8± 4 (8)*	15.7± 2.9 (9)	$p<0.01$
DRR	109.1±25 (8)	40.5± 12.6 (7)	$p<0.05$

* Mean percent increase in surface area, followed by the SE and the number of experiments in parentheses.