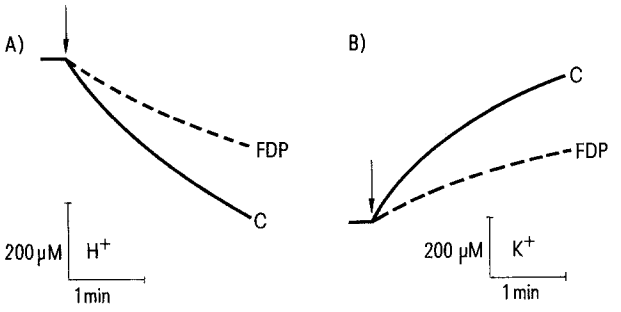

Tracing of H^+ uptake, with disappearance of H^+ from the medium (A) and K^+ ejection, with increase of K^+ in the medium (B), after addition of valinomycin/FCCP (arrow). Control (C) and 4 mM FDP (FDP).

incubation medium of 100 mM NaCl, 30 mM KCl, 5.5 mM glucose and 2.5 mM Tris-HCl pH 8.0; K^+ translocation in a medium of 250 mM sucrose, 5.5 mM glucose and 5 mM Tris-HCl pH 8.0, by adding 1 mM KCl before the addition of valinomycin/FCCP.

Results and discussion. The figure shows typical tracings of H^+ and K^+ movements obtained in the presence and absence of FDP: the inhibitory effect of FDP on both fluxes, as documented in table 1 and table 2, seems rather specific.

A blank with NaCl was carried out to see whether the effect was due to the added Na^+ counterions or to osmotic factors. The samples with added NaCl showed a difference of less than 5% from the controls.

The only sugar with an action comparable to, albeit lower than that of FDP is F2,6P. If one considers the K^+/H^+ ratio (table 3), with an active Na/K-ATPase only FDP lowers the ratio by increasing the external H^+ and decreasing the external K^+. In the presence of Ca/Mg/ouabain, only FDP increases the ratio.

The presence of Ca/Mg/ouabain modifies the homeostasis of K^+ and H^+ fluxes induced by valinomycin/FCCP, and only FDP seems to be able to counteract such a modification.

The demonstrated interaction of FDP with the red cell membrane[2] might be related to this phenomenon and FDP could act as a trigger of a stimulus-response-recovery cycle of the red cell according to Roth et al.[11]. The clinical effects of FDP cannot, in fact, be explained on the basis of a penetration of the compound through the cell membranes, which is not possible, and FDP must therefore exert its intracellular effects by acting from outside. The present data, together with those obtained with human red cells[2] indicate that, by affecting ion translocation, FDP influences phosphofructokinase and hence glycolysis on one side and membrane polarization on the other.

Table 1. Inhibitory effect of 1.5 mM sugars, in the presence and absence of Ca/Mg/ouabain (CaMgOu), on the valinomycin/FCCP-induced K^+ ejection from rat red blood cells

		CaMgOu
Control	79.6 ± 1.1 (100)	44.7 ± 0.8 (100)
FDP	44.3 ± 0.9** (55)	31.5 ± 0.7* (70)
F2, 6P	64.0 ± 0.8* (80)	35.1 ± 0.4* (78)
F6P	67.0 ± 0.6 (84)	40.1 ± 0.6 (90)
F + P	68.5 ± 0.7 (86)	38.0 ± 0.5 (85)
FDPc	69.6 ± 0.8 (87)	37.6 ± 0.4 (84)

Mean ± SE of six experiments. Values expressed as nion/min/mg Hb. Percent in brackets. ** Statistically significant by Student's t-test ($p < 0.01$). * Statistically significant by Student's t-test ($p < 0.05$).

Table 2. Inhibitory effect of 1.5 mM sugars, in the presence and absence of Ca/Mg/ouabain (CaMgOu), on the valinomycin/FCCP-induced H^+ uptake by rat red blood cells

		CaMgOu
Control	46.7 ± 2.1 (100)	58.0 ± 1.8 (100)
FDP	29.0 ± 1.8** (62)	30.0 ± 1.6** (51)
F2, 6P	32.8 ± 1.6* (70)	43.0 ± 1.5* (74)
F6P	40.6 ± 1.4 (87)	56.0 ± 1.8 (96)
F + P	38.3 ± 1.2 (82)	49.8 ± 2.1 (86)
FDPc	38.5 ± 1.6 (83)	49.6 ± 1.3 (85)

Mean ± SE of six experiments. Values expressed as nion/min/mg Hb. Percent in brackets. ** Statistically significant by Student's t-test ($p < 0.01$). * Statistically significant by Student's t-test ($p < 0.05$).

Table 3. K^+/H^+ ratio calculated from tables 1 and 2 data, in the presence and absence of Ca/Mg/ouabain (CaMgOu)

		CaMgOu
Control	1.70	0.77
FDP	1.52	1.05
F2, 6P	1.95	0.81
F6P	1.65	0.71
F + P	1.78	0.76
FDPc	1.80	0.75

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0014-4754/86/020138-02$1.50 + 0.20/0

Differential effect of silybin on the Fe^{2+}-ADP and t-butyl hydroperoxide-induced microsomal lipid peroxidation[1]

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Summary. We have observed a differential effect of silybin dihemisuccinate on rat liver microsonal oxygen consumption and on lipid peroxidation induced by NADPH-Fe^{2+}-ADP and t-butyl hydroperoxide. These results are ascribed to the antioxidant properties of the flavonoid. The differences observed in the effect of the catalysts may be a consequence of the different capacity of silybin to act as a scavenger of free radicals formed by NADPH-Fe^{2+}-ADP or t-butyl hydroperoxide.
Key words. Flavonoids; antioxidants; liver microsomes; lipid peroxidation.

Silymarin is a flavonoid used as a therapeutical agent in many liver diseases[3]. Although its protective mechanism has not been elucidated, it is generally accepted that the flavonoid exerts a membrane stabilizing action preventing or inhibiting lipid peroxidation[4]. The therapeutical use of silymarin has been limited, mainly because of its rather low water solubility and poor enteral absortion. However, a water soluble form of the flavonoid has been developed as a silybin dihemisuccinate disodium salt (silyb). The molecular mechanism (s) of action and therapeutical properties of this new form are currently under study in our laboratory.

Flavonoids are good antioxidants owing to their phenolic structure. Silyb is a potent inhibitor of the linoleate peroxidation induced by iron salts and its effect is comparable to that of antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene[5]. Based on this it has been postulated that the protective action of silymarin (or its soluble form silyb) may be related to its free radical trapping properties.

Liver microsomes are highly sensitive to lipid peroxidation when they are incubated in the presence of NADPH and Fe^{2+}-ADP[6], t-butyl hydroperoxide (TBH)[7] or cumene hydroperoxide[6]. Since the early observation by Hochstein et al.[8] of an enzymatic NADPH-dependent oxidation of microsomal lipids, a number of mechanisms have been proposed involving superoxide radicals ($O_2^{\cdot-}$) and/or hydroxyl radicals ($\cdot OH$) and singlet oxygen ($^1O_2$) as the most important chemicals related to the initiation and/or propagation of the oxidative phenomenon. Therefore, molecules which might act as scavengers or might react with these oxygen-reactive forms could inhibit microsomal peroxidation. In this report we assayed the antioxidant properties of silyb on the liver microsomal lipid peroxidation induced either by NADPH-Fe^{2+}-ADP (NFeA) or by TBH.

Materials and methods. TBH, NADPH and ADP were obtained from Sigma Chemical Co. Silyb was a gift of Dr Madaus and Co. (FRG). Microsomes were prepared according to Auclair et al.[7] from male Wistar rats weighing 180–250 g and anesthetized with sodium barbital (2% i.p.). The microsomal pellet obtained was resuspended in a Krebs-Phosphate buffer (pH 7.4) and its protein concentration was determined according to Lowry[9] and adjusted to 5 mg/ml with Krebs-Phosphate buffer. Microsomal oxygen consumption was followed at 37 °C in a cylindrical glass chamber (vol. 3 ml) equipped with a platinum electrode assembly of the Clark type and recorded with a YSI monitor.

Microsomal lipid peroxidation was assessed by two methods: Determination of the malonaldehyde (MDA) generation as described by Ottolenghi[10] and by the evaluation of the spontaneous chemiluminescence in a Nuclear Chicago Mark I liquid scintillation counter in the out-of-coincidence mode using a 1:100 microsomal suspension prepared in a 5 mM Tris buffer (pH 7.4), 140 mM KCl and 1 mM EDTA as described in Videla[11]. Results were expressed as μmoles of MDA using an $\varepsilon = 1.56 \times 10^5$ l $mole^{-1}$ cm^{-1} at 535 nm and as counts per sec/(cps)/mg protein. Experimental data represent the mean ± SEM and the significance between mean values was assessed by Student's t-test for unpaired results.

Results and discussion. Figure 1 shows tracings of the oxygen consumption obtained from rat liver microsomes in presence of 2 mM NADPH, 20 μM $FeCl_3$ and 5 mM ADP (fig. 1A) or in presence of 0.5 mM (TBH) (fig. 1B). Both inducers produce a rapid increase in the oxygen consumption of microsomes, as was described earlier[6,7]. Addition of silyb leads to a dose-dependent inhibition of the oxygen consumption in both systems. However, in experiments in which TBH was used as the inducer, only low levels of inhibition were obtained even at higher concentrations of the flavonoid. Similar results were obtained when microsomal lipid peroxidation was assessed by the MDA determination (fig. 2). In this assay silyb shows a good inhibitory effect of lipid peroxidation induced by the NFeA system (fig. 2A) with a nearly total supression at a concentration of 5.0 μM of the flavonoid. However, the prooxidant effect of TBH is only poorly suppressed by silyb (fig. 2B) even at concentrations higher than 100 μM (not shown).

Addition of the NFeA system or TBH to the microsomes results in a drastic increase in the low-level chemiluminescence, as is shown in figure 3. In this experimental system, as in the previous one, the inhibitory effect of silyb is clearly different when both prooxidants are compared. While silyb decreases the light emission stimulated by the NFeA system its effect on the TBH induced chemiluminescence does not show any statistical difference.

In view of the wide range of concentrations of the flavonoid used, both in the NFeA system and with the TBH-induced oxygen consumption and lipid peroxidation, it is concluded that under the conditions of our test silyb can only inhibit the stimulatory effect of the NFeA system. Bindolli et al.[6] have obtained a similar inhibitory effect of the flavonoid on the NFeA-induced microsomal oxygen consumption using higher concentrations of silymarin. They discard the possibility that the antioxidative action of silymarin could be ascribed to a chelating effect on Fe^{2+}. In addition, silyb did not produce any change in free Fe^{2+} concentration at concentrations capable of inhibiting the peroxidation of linoleate catalyzed by this metal[5]. Based on this information we propose that silyb may be exerting a free radical scavenging action on the NFeA system.

The rather low inhibitory effect of the flavonoid observed in the TBH-induced peroxidation may be explained by the difference in the free radical species produced during microsomal lipid peroxidation. The presence of $\cdot OH$ has been demostrated in the NFeA-induced lipid peroxidation of microsomes[12], as a product of the dissociation of the perferryl ion[13], but not in the TBH-induced peroxidation. In this latter case the reactive free radical appears to be formed on the structure of the TBH molecule as a terbutoxy radical[14]. Our results could be explained by postulating that silyb acts directly by trapping $\cdot OH$ radicals and not TBH free radicals.

Chemiluminescence is produced by different excited structures related to $2'\Delta G \rightarrow 2^3\Sigma g$ transitions[15], where $^1O_2$ may be the most relevant one. The formation of $^1O_2$ on microsomal lipid peroxidation has been previously demonstrated[7], although this excited structure does not participate in the peroxidation reaction. If $^1O_2$

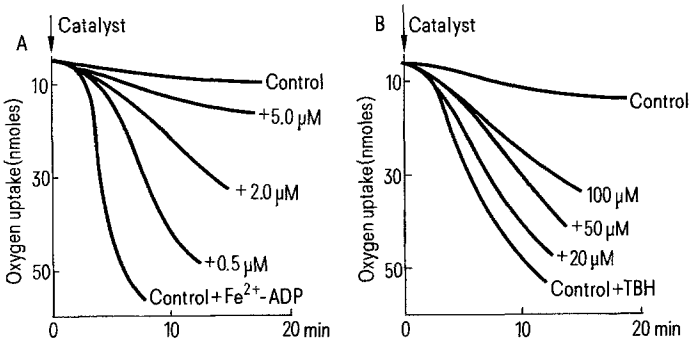

Figure 1. Effect of silybin on the NADPH-Fe^{2+}-ADP (A) and TBH (B) induced oxygen uptake by rat liver microsomes. The assay system contained 3.5 mg/ml of microsomal protein in a final volume of 3 ml. Silybin was added 2 min before the addition of the catalyst (time = 0) to final concentrations indicated in the figure.

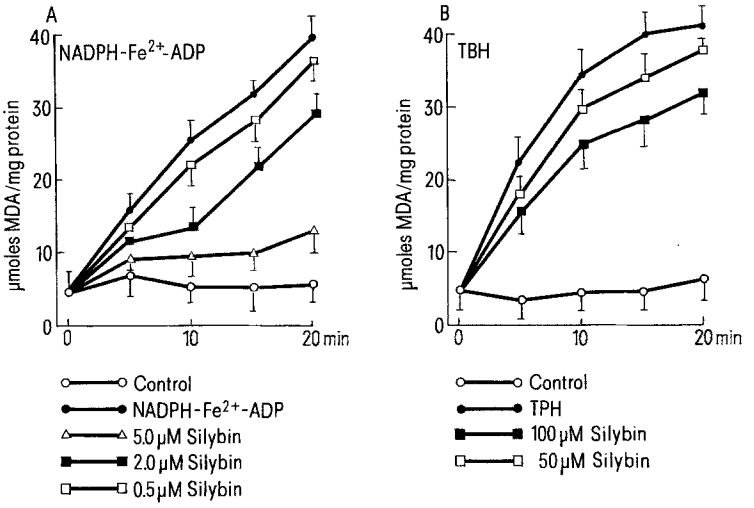

Figure 2. Effect of silybin on the malonaldehyde (MDA) formation induced by NADPH-Fe^{2+}-ADP (A) and TBH (B) on rat liver microsomes. The assay system contained 3.5 mg/ml of microsomal protein in a final volume of 10 ml. Silybin was added 2 min before the addition of the catalyst (time = 0) to final concentrations indicated in the figure. Aliquots of 0.5 ml were taken every 5 min for MDA determination. Each point represents the promedium of five experiments ± SEM.

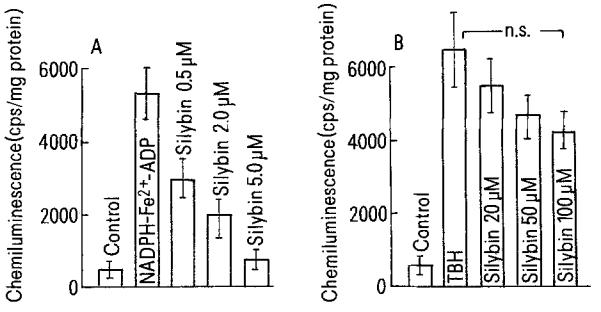

Figure 3. Effect of silybin on the low level chemiluminescence induced by NADPH-Fe^{2+}-ADP (s) and TBH (B) on rat liver microsomes. The assay system contained 3.5 mg/ml of microsomal protein in a final volume of 3 ml. Silybin was added 2 min before the addition of the catalyst (time = 0) to final concentrations indicated in the figure. After 10 min of incubation at 37 °C, 2 ml of a 1:100 dilution was counted in a glass standard vial. Results represent the promedium of six experiments ± SEM. Differences with $p > 0.05$ were considered to be without significance (N.S.) and are indicated in the figure.

is the excited molecule which is most important in the NFeA-induced chemiluminescence, and is only secondary in the TBH-induced mechanism, the differential action of silybin could be explained assuming that this flavonoid acts by trapping preferentially the $^1O_2$ species.

Our results support the free radical scavenger action ascribed to silyb and to silymarin. It is possible that the flavonoid preferentially traps certain structures such as the ·OH free radical and/or the $^1O_2$ excited species. More complex free radical structures such as the t-butoxy radical would not be scavenged by the flavonoid. The membrane stabilizing action ascribed to silymarin and to silyb, as the basis of many of its experimental and therapeutical effects, may well be a consequence of its antioxidant properties and their scavenging actions as proposed here.

1 This research was supported in part by grant B-2019-8412 from Dirección de Investigación y Bibliotecas, Universidad de Chile.
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0014-4754/86/020139-03$1.50 + 0.20/0

Influences of the chemical structure on the activity of new inhibitory compounds of the angiotensin-converting enzyme

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Summary. The ACE inhibitory activity of some perimidines, chinazolinones and amidinohydrazones is described. Relations were found between the chemical structure and the inhibitory activity on the ACE.
Key words. Angiotensin-converting enzyme (ACE); new ACE inhibitory compounds; perimidines; chinazolinones; amidinohydrazones; structure-activity-relations.