findings by assessing its activity against leishmania amastigotes growing *in vitro* in macrophages and against leishmanias in mice. Preliminary results indicate that it shows good effect in the former system but little or none in the latter (unpublished).

It has also been shown that dihydrofolate reductase/thymidylate synthetase is overproduced in *L. major* promastigotes as a resistance mechanism to both the dihydrofolate reductase inhibitor methotrexate and a quinazoline thymidylate synthetase inhibitor [13, 18]. The development of resistance to methotrexate in *L. m. mexicana* promastigotes, however, was not accompanied by the substantial rise in dihydrofolate reductase activity. As the *L. m. mexicana* strain was relatively insensitive to methotrexate before further selection, biochemical changes responsible for the acquisition of resistance might be expected to be small. It may be that the development of further methotrexate resistance in *L. m. mexicana* promastigotes, inexplicable in terms of increased activity or decreased drug sensitivity of the enzyme, could be due to a further reduction in its transport into the parasite, as has recently been reported for methotrexate-resistance in *Crithidia fasciculata* [19], or a further increase in drug metabolism. M&B 35769 is unlikely to require transport across the membrane of *L. m. mexicana*, being lipophilic, but resistance to it may be effected by increased drug metabolism.

In summary, *L. m. mexicana* and *L. donovani* promastigotes were found to be naturally insensitive to methotrexate in culture, growing in the presence of 1 mM of the drug. In contrast, growth of *L. major* promastigotes was inhibited almost totally by 0.02 mM methotrexate. The drug was, however, a potent inhibitor (I_{50}, 2 nM) of *L. m. mexicana* dihydrofolate reductase in cell-free extracts. Further resistance to 1 mM methotrexate was developed in *L. m. mexicana* promastigotes until growth occurred at normal rates. This resistance was not associated with changes in the properties or levels of dihydrofolate reductase activity. Four 2,4-diaminopyrimidines proved more effective than methotrexate against the growth of *L. m. mexicana* promastigotes, although they were less potent inhibitors of dihydrofolate reductase activity. Resistance to one 2,4-diaminopyrimidine was developed in culture until the organism could grow in 10 times the normal lethal concentration. This resistance was also not associated with changes in the properties or levels of the *L. m. mexicana* dihydrofolate reductase.

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Concomitant changes of ethanol partitioning and disordering capacities in rat synaptic membranes

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During the last decade, a great number of investigators have found that membrane disordering and reorganizing could be an essential step in the progression of events leading to intoxication by ethanol and could be involved in the development of tolerance and dependence [1, 2]. Membrane fluidity as membrane ordering index has been extensively studied mainly on brain membranes of experimental animals [3–6]. Membrane molecular order is a result of the complex interactions of multiple membrane entities and can be modulated by compositional and/or structural alterations in these components.

It has been accepted after Meyer and Overton's work [7] that the membrane concentration is more responsible for the observed results than the initial concentration in the

medium. This concentration depends on the partition coefficient [8] between the membrane and the medium. Ethanol membrane: buffer partition coefficient measurements are difficult because of the low partition ccoefficient that was usually calculated by extrapolation [7]. Recently Rubin's group developed a direct method of measure [9] using a double labeling technique and found an impairment of the partition of ethanol (and other anesthetics) into the synaptic and the erythrocyte membranes of alcohol tolerant–dependent animals [9, 10, 11].

We have found [12, 13] that acquisition of this tolerance passed through different steps of membrane disordering, i.e. *in vivo* ethanol administration modulated acute ethanol disordering effect: hyperfluidization after a single administration, "normal" fluidization after short-term alcoholization or *t*-butanol administration, hypofluidization after chronic treatment [4, 12–14]. The question that arises therefore is if this modulation in membrane fluidity is also associated with changes in ethanol partitioning into synaptic membranes.

In order to answer this question, we investigated both ethanol synaptic membrane partitioning and disordering altogether after acute *in vivo* ethanol or *t*-butanol as well as chronic ethanol intoxication. The membrane sensitivity to ethanol was found to be clearly related to the alcohol "binding" capacity and leads to the problem of knowing where in the membrane ethanol is located or exerts its effects [11, 15, 16].

Materials and methods

Alcohol administration. Male Sprague–Dawley rats (Elevage Janvier, 53680 Le Genest. France) weighing 160–180 g were housed individually in temperature-controlled rooms.

Ethanol was administered acutely at a dose of 100 mmol/kg body wt, 18 hr before sacrifice and *t*-butanol at a dosage of 12.5 mmol/kg, 24 hr before sacrifice. Both alcohols were given as a 20% v/v solution in water and an equal volume of water was administered to the corresponding controls rats [14]. Chronically, ethanol was administered during three weeks by gavage as previously [4]. Control rats received starch in a dose equicaloric with that of ethanol and were given the same amount of chow diet (standard laboratory chow U.A.R., 91360 Villemoisson, France) as the pairfed ethanol rats. Blood alcohol levels were controlled using an ADH enzymatic method [17] and the importance of tolerance to ethanol in ethanol-fed animals was assessed by the hypothermic response to a challenge dose of ethanol [4].

Preparation of synaptic membranes. The rats were decapitated without anesthesic; the brains were removed and homogenized using an Elvehjen Potter in 10 vol. of 0.32 M sucrose containing 5 mM Tris–Cl (pH 7.4). Synaptosomes were prepared using the method of Cotman and Matthews [18] described in detail in a previous publication [19]. Protein concentration was measured using Lowry's method [20]. We had previously assessed that the synaptosome fraction was not appreciably contaminated by other subcellular fractions, by determination of Na^+ and K^+-dependent ATPase activity and electron microscopy [4, 19].

Measurement of fluorescence polarization: P. The synaptic suspensions were diluted in 10 mM phosphate buffer (pH 7.4) to a final protein concentration of 20 μg/ml. The fluorescence probe, 1,6 diphenyl-1,3,5 hexatriene (DPH, 1 mM in THF) was then added to give a final concentration of 0.1 μM. The solution was incubated for 1 hr at 37°. Membrane fluidity was evaluated by fluorescence depolarization of DPH (P), according to Shinitzky and Inbar [21] as previously described [4, 14]. After determination of the basal value, aliquots of ethanol (0.35–1.05 M, final concentration) were added and P determined again. The slope of the regression line (P vs ethanol concentration) ΔP was then calculated as a measure of the fluidizing efficacy of ethanol.

Direct determination of the partition of ethanol in synaptosomes. Membrane samples were pooled so that approximately 5–6 of membrane (as protein) were used for the determination of the partition coefficient of ethanol by the equilibrium distribution of isotopic solute between aqueous buffer and sedimented membranes after Rottenberg *et al.* [9]. Tritiated water is added in order to correct for trapped water in the membrane pellet. The membranes suspended in 0.2 M sucrose buffer containing ^3H_2O (0.25 μCi/ml) and ^{14}C ethanol (0.1 μCi/ml) were incubated for 90 min at 25°. They were pelleted (35000 *g* for 30 min), the supernatant was quickly removed and the pellet digested in 0.5 ml of tissue solubilizer (soluene 0.5 N, Packard) at 25° within 15 min. Samples of pellet and supernatant were mixed with a scintillation mixture (Packard Dimilume) and then counted for dual labeling in a Packard Tricarb 3330 scintillation counter. The partition coefficient was derived according to Rottenberg *et al.* [9] from the pellet and supernatant counting ratios. In some cases, as the coefficient was obtained with a tracer concentration of ethanol, extra ethanol (0.1 or 0.6 M) was added to the incubation medium to assess the role of ethanol concentration. The coefficients thus obtained are dimensionless (concentration/concentration).

Expression of results. Differences between the control and the alcohol-treated group were compared by Student's *t*-test for each parameter expressed as means ± SEM. When two parameters were compared, linear regression analysis was used, correlation coefficients calculated and statistical significance checked.

Reagents. DPH was from Aldrich-Europe (Beerse, Belgium), scintillation products from Packard (Rungis, France), ^3H_2O (0.1 mCi/mmole) and [^{14}C]ethanol (55 mCi/mmole) from Amersham (Bucks., U.K.) and blood ethanol determination kits from Sigma (St Louis, Mo. U.S.A.). All other reagents were of analytic grade.

Results and discussion

Eighteen hours after acute ethanol administration when blood alcohol levels returned to zero: 3.7 ± 0.8 mM (5 animals), the basal degree of fluorescence polarization of DPH in synaptic membranes was not different from controls. Nevertheless, synaptic membranes from ethanol-treated animals were more sensitive to further ethanol addition as already shown in a crude synaptic fraction [12, 13]. This fluidizing effect being dose-dependent, the slope of the regression line (ΔP) was calculated in each individual and the difference compared, showing an increase of 30% in ΔP (Table 1). As we have previously discussed (12–14), the acute disordering effect of ethanol is not immediately reversible at ethanol disappearance. A subtle difference persists, as evidenced by the use of the membrane perturbant. Simultaneously, the partition coefficient (K_p) was significantly increased (by 13%) in the alcohol-treated animal membranes (Table 1). The control K_p values were in the same range as those determined previously by Rottenberg [9]. Addition of ethanol *in vitro* to the assays also increased the partition by 5–10% with 0.1 M and 50–60% with 0.6 M but to the same extent in alcohol-treated and control animals confirming, in the alcohol-treated animals, an intrinsic sensitization of the membrane.

Twenty-four hours after *t*-butanol administration, as previously found in a crude synaptic fraction [14] and in contrast to what was found after ethanol administration, no significant differences in sensitivity to ethanol added *in vivo* or in ethanol partitioning were found between control and treated animals (Table 1).

As expected [4, 19] the chronic ethanol regimen produced tolerance to the hypothermic effect of a 65.2 mmol/kg body wt challenge test dose of ethanol. After 19 days, the maximum fall in body temperature was 2.5 ± 0.1° in the control group and 1.6 ± 0.3° in ethanol group (N = 4 per group, $P < 0.001$).

Table 1. Effect of acute administration of ethanol (100 mmol/kg p.o.) or *t*-butanol (12.5 mmol/kg, p.o.) and chronic administration of ethanol on rat synaptic parameters

Animal treatment	P	ΔP	K_p
Controls (5)	0.321 ± 0.003	0.022 ± 0.001	0.76 ± 0.02
Ethanol (5)	0.324 ± 0.002	0.028 ± 0.002†	0.86 ± 0.04*
Controls (5)	0.326 ± 0.003	0.024 ± 0.002	0.76 ± 0.07
t-Butanol (5)	0.329 ± 0.001	0.023 ± 0.001	0.73 ± 0.07
Starch-controls (5)	0.322 ± 0.001	0.031 ± 0.003	0.96 ± 0.01
Ethanol-fed (5)	0.323 ± 0.002	0.014 ± 0.002†	0.48 ± 0.08†

P, Basal degree of fluorescence polarization of DPH.
ΔP, Fluidizing efficacy of ethanol added *in vitro*.
K_p, Basal partition coefficient of ethanol.
Animals were sacrificed by decapitation 18 hr after the single or the last ethanol administration and 24 hr after *t*-butanol administration.
For the chronic intoxication, animals were given daily intubation of ethanol with increasing dosages (62.5 to 130.4 mmol/kg body wt).
The values are means ± SEM with the number of animals in parentheses.
Statistical significance: * P values (ethanol vs control group) $0.02 < P \leq 0.05$. † P values (ethanol vs control group) $0.01 < P \leq 0.02$.

At 21 days of chronic ethanol administration, 18 hr after the last ethanol incubation, when blood alcohol fell to 1.1 ± 0.2 mM (for 5 animals), the degree of synaptosomal fluorescence polarization of DPH remained unaffected (Table 1). Contrasted to the acute ethanol treatment, synaptic membranes were less affected by *in vitro* addition of ethanol. The fluidization efficacy was decreased by more than 50% that was almost as much as the basal partition coefficient (Table 1).

The present data report further documentation of a modulation of the synaptic membrane sensitivity by *in vivo* ethanol administration [4, 12, 14]. In addition this modulation appears clearly related to concomitant changes in alcohol partitioning capacity. Acute ethanol treatment increases the ethanol "binding" to the membrane as well as the membrane sensitivity. Chronic treatment decreases both ethanol "binding" and membrane sensitivity. Finally, *t*-butanol pretreatment which does not affect membrane sensitivity to ethanol, does not change ethanol "binding". Comparing the results of acute and chronic ethanol intoxications, the differences in the partition coefficient of ethanol were strongly correlated ($r = -0.844$ $P < 0.001$, 20 pairs) with the importance of ethanol disordering efficacy (Fig. 1). These findings indicate that the relative sensitization to the *in vitro* effects of ethanol is probably actually dependent on the membrane ability to bind, non-specifically, ethanol.

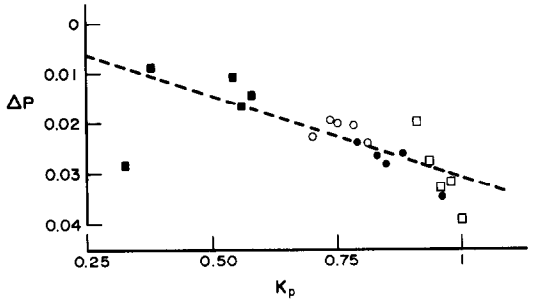

Fig. 1. Ethanol fluidizing efficacy presented as a function of ethanol partition coefficient. Results from acute and chronic ethanol intoxicated animals as well as from controls were plotted altogether. Ethanol fluidizing efficacy was calculated as explained in the results. The slope of the line is 0.0345 with $r = -0,844$. Acute administration: ○, control animals; ●, ethanol-treated animals. Chronic administration: □, starch-control animals; ■, ethanol-fed animals.

These results confirm and extend those of Rottenberg *et al.* [9], Kelly-Murphy *et al.* [10] and Rottenberg [22] who reported a decreased partition coefficient of ethanol in mitochondrial, synaptic and erythrocyte rat membranes as well as reduced protection from hemolysis by ethanol in chronic alcohol intoxication. They also showed, for the first time, a relationship between the importance of ethanol partitioning in membrane and the degree of disordering. The exact mechanism governing this variation in ethanol partitioning is so far unknown and a thorough study of the ethanol binding possibilities in different parts of the membrane appears necessary to explain, at least partly, sensitivity of the membranes to the disordering effect of the drug and acquisition of tolerance to the drug.

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Tumor promoter-induced basophil histamine release: effect of selected flavonoids

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The tumor promoter phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), has been shown to be a potent stimulus of human basophil histamine release [1, 2]. It is active in the absence of extracellular Ca^{2+}, suggesting that it mobilizes intracellular Ca^{2+} which is required for the exocytosis of basophil granules [3]. The extracellular Ca^{2+}-independence of basophil histamine release contrasts with the largely extracellular Ca^{2+}-dependent release of histamine stimulated by other agents such as antigen, anti-IgE, concanavalin A (Con A), the chemoattractant f-Met-Leu-Phe, and the Ca^{2+} ionophore A 23187 [3].

TPA is one of several recognized tumor promoters that act to promote tumor formation (papillomata) in mouse skin treated at first with an "initiator", e.g. 7,12-dimethylbenz[*a*]anthracene. In the two-stage model of carcinogenesis, both an initiator and a promoter are required for tumor development, neither agent alone being sufficient to induce tumorigenesis. Other recently recognized tumor promoters include teleocidin, an indole product of the mycelia of *Streptomyces mediocidicus,* and aplysiatoxin, a polyacetate compound derived from a blue green alga [4, 5]. Teleocidin and aplysiatoxin, like TPA, are known to stimulate rat brain protein kinase C *in vitro* [6, 7], an enzyme believed to be important in histamine release from rat basophilic leukemia cells (RBL) [8] and in the regulation of a variety of cell activation processes [9].

We decided to investigate whether teleocidin and aplysiatoxin, like TPA, were capable of stimulating human basophil histamine release. In addition, we wished to determine whether certain flavonoids known to be effective inhibitors of TPA-induced histamine release [10] would also inhibit teleocidin- and aplysiatoxin-induced histamine release. Our findings form the basis of this report.

Materials and methods

Preparation of leukocyte suspensions for histamine release and determination of histamine. Leukocyte suspensions were prepared as described previously, and histamine was determined in total leukocyte suspensions and supernatant fractions in accordance with earlier reports [10–13]. The Tris buffer contained 0.6 mM Ca^{2+}, 1.0 mM Mg^{2+} and 0.03% human serum albumin for all experiments except for studies of histamine release in the absence of buffer Ca^{2+}; in these experiments Ca^{2+} was eliminated from the Tris buffer.

Chemicals. Quercetin, fisetin and chalcone were obtained from the Aldrich Chemical Co., Milwaukee, WI; and luteolin and apigenin from Sarget Laboratoires, Merignac, France. Nobiletin and tangeretin were provided by Dr. James Tatum, Department of Citrus, Lakeland, FL; TPA, phloretin and taxifolin were from Sigma, St. Louis, MO. Teleocidin and aplysiatoxin were isolated by a procedure described previously [5].

Experimental protocol. The effects of the following flavonoids were examined: quercetin (3′,4′,3,5,7-pentahydroxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone), taxifolin (dihydroquercetin), nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone), fisetin (3′,4′,3,7-tetrahydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), tangeretin (4′,5,6,7,8-pentamethoxyflavone), phloretin (β-(*p*-hydroxyphenyl)-2,4,6-trihydroxypropiophenone) and chalcone (1,3-diphenyl-2-propene-1-one). The flavonoids were dissolved in dimethyl sulfoxide (10–20 mM stock solutions) and were diluted in buffer to provide concentrations between 5 and 50 μM in the final cell suspensions. The final concentration of DMSO did not exceed 0.5%, a concentration which did not affect the histamine release process [12]. As a matter of routine, cell suspensions were preincubated for 10 min with the flavonoids prior to addition of the tumor promoters, and the incubation was then continued for an additional 60 min. The reaction was terminated by centrifugation, and supernatant histamine was measured. The tumor promoters were dissolved in DMSO and diluted in buffer to provide final concentrations in cell suspensions of 1–200 ng/ml. All experiments were conducted at 37° in polypropylene tubes. Histamine was determined by an automated technique [13].

Results

Tumor promoter stimulation of basophil histamine release. As shown in Fig. 1, TPA, teleocidin and aplysiatoxin (1–200 ng/ml) each stimulated basophil histamine release in a concentration-dependent manner. The maximal histamine releasing effect was noted at 10–50 ng/ml. Each tumor promoter stimulated histamine release in the presence or absence of extracellular buffer Ca^{2+} although the