

Reversal of carbon tetrachloride induced changes in microviscosity and lipid composition of liver plasma membrane by colchicine in rats

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Abstract

Colchicine is beneficial in the treatment of cirrhotic patients, it prevents changes in plasma membrane bound enzymes induced by CCl₄ intoxication. In this study, lipid composition and microviscosity were measured in liver plasma membranes isolated from rats given CCl₄. Microviscosity values increased in rats given CCl₄ for six weeks but fell considerably in those given CCl₄ for 10 weeks. Both these changes were absent when colchicine was given with CCl₄. The cholesterol/phospholipid molar ratios and lipid peroxide values increased but plasma membrane phospholipids, the length of fatty acyl chains, and the unsaturation index fell significantly after CCl₄ intoxication. Colchicine treatment also prevented these changes. Changes in the lipid composition of liver plasma membranes were significantly correlated with lipid peroxidation. Colchicine prevents changes in the physicochemical properties of liver plasma membranes induced by longterm CCl₄ treatment, probably by blocking peroxidation of unsaturated fatty acids.

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Preliminary trials of colchicine in animals and patients with liver cirrhosis showed that this drug produces an improvement in liver function, reduces the fibrous content of the liver¹ and may be beneficial in this disease.² More recently, Kershenovich *et al*³ reported the results of a longterm, controlled study and confirmed that colchicine prolongs survival in patients with mild to moderate cirrhosis of the liver and improves liver histology in some cirrhotic patients.

The mechanism of this protective effect is not clear. Colchicine has an extremely wide range of biological actions. It inhibits collagen production,⁴ stimulates collagenase activity,⁵ and blocks mitosis⁶ and some functions of polymorphonuclear leucocytes,⁷ some of which are related to its antimicrotubular effect.⁸ In addition, changes in plasma membrane bound enzymes were prevented and the high cholesterol/phospholipid molar ratio was reversed, when colchicine was given simultaneously with CCl₄ in rats that already had cirrhosis after eight weeks of CCl₄ treatment.⁹

It is well known that a number of membrane bound enzymes may be affected by the lipid environment of the hepatocyte plasma membrane.¹⁰ This membrane consists of a lipid bilayer into which a variety of proteins (struc-

ture, enzymes, receptors, transporters) are inserted.¹¹ It has been shown that the structural order in membrane, determined by its lipid and protein content, can influence a variety of functions and physicochemical properties of plasma membrane, including enzyme activity, transport, fluidity, and microviscosity.¹² Microviscosity (η) of anisotropic bilayer membranes is related to motional resistance and increases parallel to the packing of lipids. In contrast, fluidity, applies to the relative motional freedom of the lipid molecules into the bilayer membrane¹³ and its increase expresses a decreased structural order.

The purpose of the present study was: (1) to investigate the effect of CCl₄ on the physicochemical properties and lipid composition of liver plasma membrane and (2) to evaluate the preventive effect of colchicine on the changes induced in these membranes by longterm administration of CCl₄ in rats.

Methods

Male Wistar rats weighing 200 to 250 g had free access to a diet of Purina chow and water. Animals were divided into six groups. Group I (five rats) received 150 μ l/100 g body weight of corn oil by intraperitoneal injection, three times a week for six weeks. Group II comprised seven rats that received 10 μ g/day of colchicine for five days a week. Colchicine was dissolved in water and given by intragastric tube. In group III (five rats), 150 μ l/100 g body weight of a 1:7 (v/v) solution of CCl₄ in corn oil was given by intraperitoneal injection three times a week for six weeks. In animals of group IV (six rats), CCl₄ administration was maintained for 10 weeks. Animals of group V (eight rats) and VI (seven rats) were given 10 μ g/day of colchicine for five days a week, in addition to CCl₄ as described for animals in the groups III and IV, respectively. This study was approved by the hospital research committee and the standard criteria for the care and use of laboratory animals in research were followed.

PREPARATION OF LIVER PLASMA MEMBRANES

Rat liver plasma membranes were isolated and purified according to the method of Neville,¹⁴ as modified by Emmelot.¹⁵ The purity of the membrane suspensions and the degree of contamination with intracellular organelles were assessed by phase-contrast microscopy and by marker enzymes. The 5'-nucleotidase activity¹⁶ was used as a plasma membrane marker and glucose 6-phosphatase and cytochrome *c* oxidase were

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TABLE I Enzymatic and relative specific activity of marker enzymes in plasma membrane preparations (values median (range))

| Group | Specific activity | | Specific activity ratio* | | |
|-------|--|---------------------|--------------------------|---------------------|---------------------|
| | Membrane ($\mu\text{mol phosphate/h/mg protein}$) | Homogenate | 5'-NT | G-6-P-ase | Cyt. c oxidase |
| I | 67.3 (65.4-69.0) | 2.57 (2.5-2.68) | 26.0 (25.4-26.6) | 0.24 (0.10-0.39) | 0.17 (0.05-0.29) |
| II | 66.7 (59.8-73.2) | 2.53 (2.28-2.70) | 26.2 (25.2-26.9) | 0.25 (0.05-0.43) | 0.17 (0.03-0.36) |
| III | 69.1 (57.0-83.2) | 2.68 (2.15-3.30) | 26.1 (25.3-27.5) | 0.26 (0.09-0.40) | 0.19 (0.05-0.38) |
| V | 64.0 (50.6-78.0) | 2.5 (1.96-3.20) | 26.0 (25.4-26.5) | 0.24 (0.13-0.37) | 0.18 (0.05-0.32) |
| IV | 68.7 (62.0-75.5) | 2.66 (2.38-2.88) | 26.08 (25.6-26.4) | 0.26 (0.14-0.40) | 0.18 (0.06-0.33) |
| VI | 70.2 (59.0-81.1) | 2.70 (1.94-3.40) | 26.1 (25.5-26.5) | 0.28 (0.11-0.41) | 0.17 (0.07-0.33) |

*Enzyme specific activity ratio of purified plasma membrane preparations/homogenates.
5'-NT=5' nucleotidase; G-6-P-ase=glucose-6-phosphatase; Cyt. c oxidase=cytochrome c oxidase.

employed to detect microsomal and mitochondrial contamination, respectively.¹⁷ The 5'-nucleotidase activity of the liver plasma membrane preparations isolated from control and experimental groups was 67.8 (1.9) $\mu\text{mol phosphate/h/mg protein}$, while for liver homogenates this value was 2.6 (0.09) $\mu\text{mol phosphate/h/mg protein}$. Thus, a 26.06 (0.38) fold purification of the enzyme marker was achieved. The corresponding values listed for cytochrome c oxidase and glucose-6-phosphatase, showed a low level of contamination and were essentially identical in the control and experimental groups (Table I).

Fluorescence polarisation

This was measured using 1,6-diphenyl 1,3,5-hexatriene (DPH) as the fluorescent probe, as described elsewhere.¹⁸ The probe was dissolved in tetrahydrofuran (20 mM) and added to 5 mM TRIS-HCl (pH 7.4) in 0.25 M sucrose. The mixture was stirred until a stable dispersion was

obtained. To 1.1 ml of plasma membranes (adjusted to 0.28 optical density at 365 nm, 200 μg of protein), 0.4 ml of DPH was added to get a final concentration of the probe of 9 μM . Samples were incubated at 37°C for 45 minutes. Fluorescence polarisation was measured in a Perkin-Elmer MPF 44E spectrofluorometer equipped with polarisers in both excitation and emission beams. Fluorescence polarisation (FP) was calculated from the equation:

$$FP = (I_{vv} - GI_{vh}) / (I_{vv} + GI_{vh})$$

where I_{vv} and I_{vh} are the fluorescence intensities measured with emission polarisers vertically and horizontally oriented. The emission grating factor (G) corrects for parallel diffraction anomalies introduced by the glass of the phototube and was calculated as $G = I_{hv} / I_{hh}$. The probe was excited at 365 nm and emission measured at 425 nm with slit widths of 6 and 5 nm, respectively, for excitation and emission. Fluorescence polarisation values were corrected for light scattering according to Lentz *et al.*¹⁹ Polarisation measurements were carried out in duplicate in each of the animals of the groups.

Microviscosity parameter

These polarisation values were used to determine the microviscosity parameter ($\bar{\eta}$), defined as $[\frac{r_0}{r} - 1]^{-1}$, where r is the value of the fluorescence anisotropy calculated from the measured fluorescence polarisation, FP, according to the equation: $r = 2FP / (3 - FP)$ ¹³ and r_0 is the upper limit value of r of a medium of infinite viscosity. The value of r_0 for DPH has been reported to be 0.362.¹³

LIPID ANALYSIS

Liver plasma membrane lipids were extracted by the procedure of Bligh and Dyer.²⁰ The cholesterol content was determined by the method of Zlatkis.²¹ Lipid phosphorus measurements were carried out as described by Bartlett.²²

Transmethylation of lipids was performed with a sodium methoxide/methanol reagent (Methanolic Base, Supelco, Bellefonte, PA, USA), according to the method described by Alvaro *et al.*,²³ modified by Schüller *et al.*²⁴ The reagent (1 ml) was added to the dried lipid samples and kept under nitrogen for 90 minutes at 110°C. Fatty acid methyl esters were extracted in a water-hexane (1:2;v/v) system and dried under N_2 in small conical centrifuge tubes. The residue, redissolved in 50 to 100 μl of hexane, was used for gas-liquid chromatography, performed on a Perkin-Elmer Sigma 3B gas chromatograph equipped with a dual flame ionisation detector and an integrator computer Sigma 15. The stationary phase was 5% ethyleneglycol adipate on Supelcoport 80-100 mesh in a 180 cm \times 4 mm column. Methyl pentadecanoate was used as internal standard. The gas chromatograph was calibrated and checked daily with commercial fatty acid standards. The double bond index was calculated by the sum of the products of the percentage of each unsaturated fatty acid and the number of double bonds.²⁵

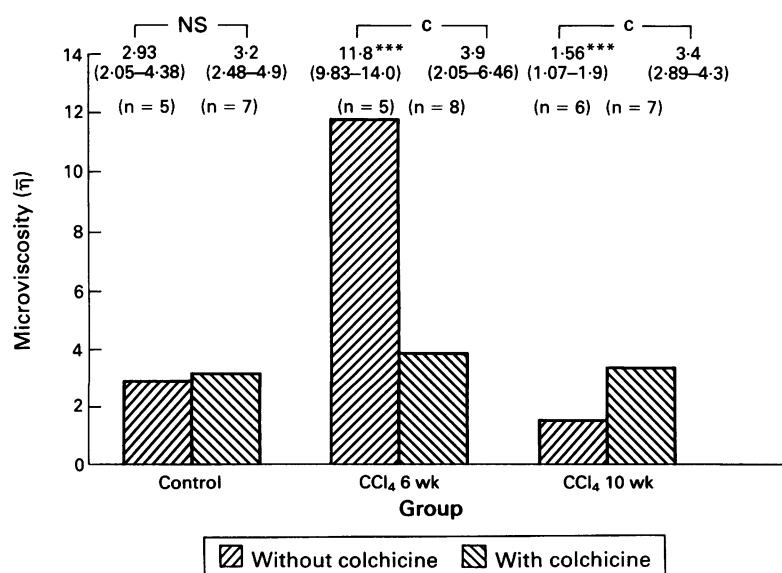


Figure 1: Effect of colchicine on plasma membrane microviscosity in rats exposed to CCl_4 . Data are presented as median (range). NS=not significant, c= $p < 0.001$ between groups with and without colchicine. ***= $p < 0.001$ between control and experimental groups.

TABLE II Effect of colchicine on microviscosity and lipid content in liver plasma membrane of rats chronically intoxicated with carbon tetrachloride (values median (range))

| Group | Cholesterol (nmol/0.1 mg proteins) | Phospholipids | Cholesterol/ phospholipid molar ratio | Microviscosity |
|--|---------------------------------------|------------------|---|-------------------------|
| I (control (n=5)) | 39.6 (22.1-70.0) | 56 (38-100) | 0.69 (0.22-1.1) | 2.93 (2.05-4.38) |
| II (colchicine (n=7)) | 34.3 (24.7-39.9) | 57 (43-100) | 0.61 (0.34-0.85) | 3.20 (2.48-4.9) |
| III (CCl ₄ 6 weeks; n=5) | 54.0 (31-81.9) | 50.0 (30-76) | 1.10* (0.84-1.3) | 11.84*** (9.83-14.0) |
| V (CCl ₄ +colchicine 6 weeks; (n=8)) | 75.0* (48.4-112) | 85.0 (57-104) | 0.81 (0.69-0.86) | 3.90 (2.05-6.46) |
| IV (CCl ₄ 10 weeks; n=6) | 69.5 (30-93) | 40.0 (20-67) | 2.0** (1.35-2.2) | 1.56*** (1.07-1.9) |
| VI (CCl ₄ +colchicine 10 weeks; (n=7)) | 59.1 (46-86.3) | 75.0 (40-97) | 0.95 (0.55-1.2) | 3.4 (2.89-4.33) |

Significance of differences between control and experimental groups: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Significance of differences between groups with and without colchicine: (NS)=non significant; (a)= $p < 0.05$; (b)= $p < 0.01$; (c)= $p < 0.001$.

Lipid peroxidation was estimated by analysing the production of thiobarbituric acid reacting substances (TBARS) using the method described by Ohkawa *et al.*²⁶ Protein content in membrane suspensions was measured according to Bradford.²⁷

STATISTICS

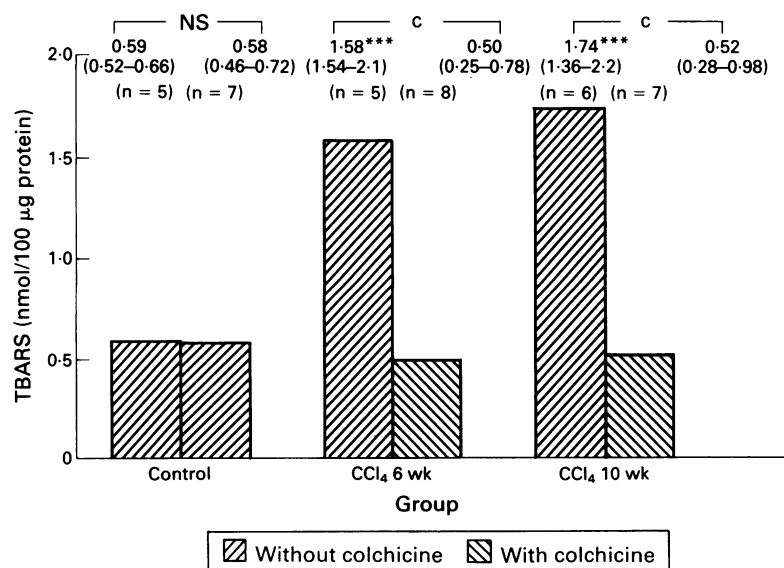
Results were expressed as median (range). Comparison between groups was made using the Mann-Whitney U test for unpaired data, and the significance of correlations was tested using the Spearman rank correlation coefficient (r_s) for non-parametric data. p Values less than 0.05 were considered statistically significant.

Results

PLASMA MEMBRANE MICROVISCOSITY ($\bar{\eta}$)

Microviscosity of liver plasma membranes from

Figure 2: Effect of colchicine on thiobarbituric acid reacting substances (TBARS) in rats given CCl₄. Data are presented as median (range). NS=not significant, c= $p < 0.001$ between groups with and without colchicine. ***= $p < 0.001$ between control and experimental groups.



rats given CCl₄ for six weeks was significantly higher than in control rats (11.84 (9.83-14.0) v 2.93 (2.05-4.38); $p < 0.001$), but was decreased significantly (1.56 (1.07-1.9); $p < 0.001$) in plasma membranes from rats given CCl₄ for 10 weeks. These changes in viscosity values were absent in animals treated either with colchicine in addition to CCl₄ or with colchicine alone (Fig 1, Table II).

LIPID COMPOSITION OF LIVER PLASMA MEMBRANE

The cholesterol content in liver plasma membranes increased and the phospholipid content fell after longterm administration of CCl₄. However, none of these changes reached statistical significance. In spite of this, cholesterol/phospholipid molar ratios increased significantly in these animals, particularly in those exposed to CCl₄ for 10 weeks. This ratio was normal in rats given colchicine in addition to CCl₄ (Table II).

Although lipid peroxides (measured as TBARS) increased steadily during CCl₄ intoxication, the addition of colchicine completely prevented peroxidation of lipids in plasma membranes (Fig 2). The fatty acid patterns of the phospholipids showed that the length and the unsaturation index of fatty acyl chains decreased after six weeks and was particularly noticeable after 10 weeks of intoxication with CCl₄, but these changes were also abolished by the colchicine treatment (Figs 3 and 4).

Lipid peroxidation was significantly and inversely correlated with the index of unsaturation ($r = -0.94$), length of fatty acyl chains ($r = -0.86$), and content of phospholipids ($r = -0.9$) in the liver plasma membranes. In addition, lipid peroxidation was positively correlated with the cholesterol/phospholipid molar ratio in these membranes ($r = 0.80$). There was no significant correlation between microviscosity and the lipid composition of liver plasma membranes.

Discussion

Colchicine was shown to be beneficial in the treatment of patients with mild to moderate cirrhosis of the liver³ and pretreatment of rats with this drug prevented liver damage induced by D-galactosamine²⁸ or CCl₄.²⁹ The mechanism of these effects is not clear. CCl₄ toxicity occurs after cleavage of a carbon-chlorine bond and the generation of trichloromethyl free radicals. These high reactive metabolites initiate peroxidative damage of membrane phospholipids, including plasma cell membrane. Breakdown of these membranes changes their physicochemical properties, and may result in hepatocellular necrosis.

Our study shows that chronic administration of CCl₄ for six weeks induces an increase in liver plasma membrane microviscosity. However, maintaining this treatment for 10 weeks leads to the opposite effect (Fig 1). All these physical changes were prevented by simultaneous administration of colchicine. Studies on the effects of CCl₄ on cell membranes are scanty, but indicate that acute CCl₄ poisoning results in a

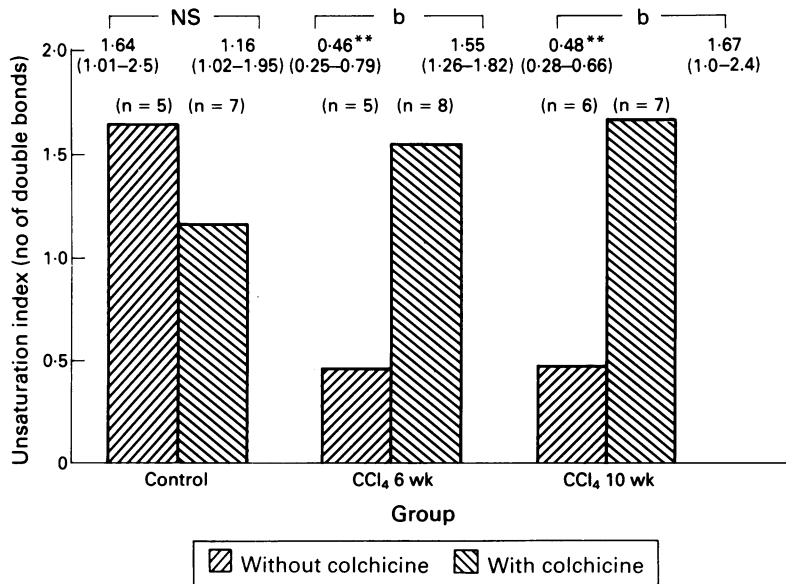


Figure 3: Effect of colchicine on unsaturation index in rats given CCl₄. Data are presented as median (range). NS=not significant, a= $p<0.05$, b= $p<0.01$, c= $p<0.001$ between groups with and without colchicine. **= $p<0.01$ between control and experimental groups.

significant increase in microsomal membrane fluidity.^{32,33}

It is well known that membrane lipid composition is an important factor in determining membrane physicochemical properties.^{10,34} CCl₄ intoxication was associated with a rise in the cholesterol/phospholipid molar ratio and an appreciable increase in lipid peroxidation. In contrast, the phospholipid content decreased after CCl₄ (Table II). The mechanism of action of the latter effect is unclear, however, as the phospholipid value was inversely correlated with lipid peroxides in plasma membranes. Otani *et al.*,³⁰ using a model of lipid peroxidation induced by ischaemia-reperfusion, also reported a significant depletion of membrane phospholipids that was associated with an inhibition of membrane phospholipid synthesis. Some

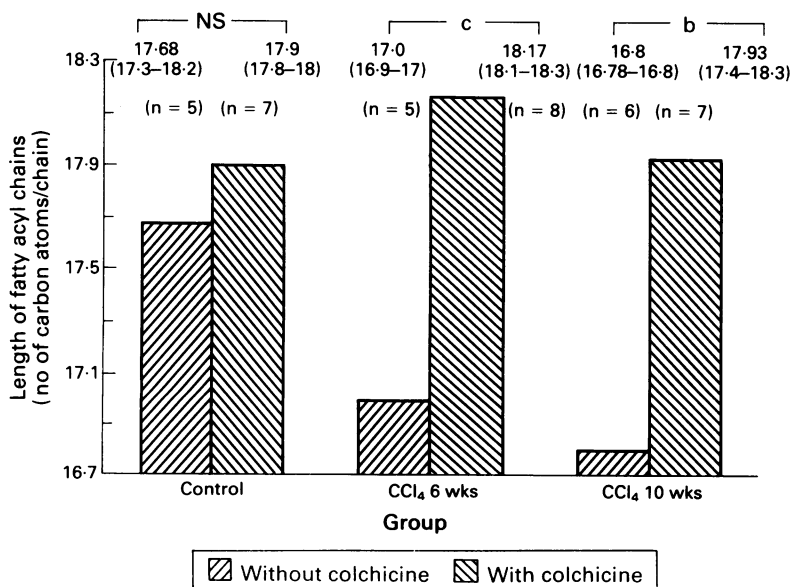


Figure 4: Effect of colchicine on the length of the fatty acyl chains in rats given CCl₄. Data are presented as median (range). NS=not significant, b= $p<0.01$, c= $p<0.001$ between groups with and without colchicine. **= $p<0.01$ between control and experimental groups.

enzymes involved in the regulation of phospholipid biosynthesis are located in the endoplasmic reticulum.³⁵ It is considered that the initial event of liver injury induced by CCl₄ takes place in these membranes, where this agent causes a highly focal lipid peroxidation. We speculate that the CCl₄ induced damage of microsomal membrane is followed by inhibition of phospholipid biosynthesis. Lipid peroxidation induced by CCl₄ may also promote the degradation of phospholipids and may play a part in the reduction of phospholipids in the plasma membranes, since damaged fatty acids are cleaved, presumably as a repair process.³⁶ Our results agree with this mechanism – the length and degree of unsaturation of fatty acyl chains decreased appreciably in rats given CCl₄, and these changes were closely and negatively correlated with lipid peroxide content in plasma membrane. Lipoperoxidative degradation of fatty acyl chains might have been followed by the exclusion of the hydrophilic head of phospholipids from the biomembrane.

The appreciable increase in membrane microviscosity induced by chronic intoxication with CCl₄ for six weeks could be ascribed to the rise in the cholesterol/phospholipid molar ratio.^{10,11} The decrease in microviscosity after 10 weeks of poisoning could be attributed to the peroxidative shortening of the unsaturated fatty acyl chains. These shorter acyl chains decrease chain-chain interactions, reduce microviscosity,¹⁰ and could overcome the effect of the increased cholesterol/phospholipid molar ratio on microviscosity.

With regard to the central part played by the lipid peroxidation in the CCl₄ induced liver injury, the protective effect of colchicine against liver plasma membrane changes in CCl₄ treated rats could be ascribed to its capacity to inhibit peroxidation of fatty acids (Fig 2). Similar effects of colchicine have been previously reported in D-galactosamine induced hepatitis²⁸ and in acute liver damage induced by CCl₄.²⁹ Although the mechanism of this apparently antilipoperoxidant effect is not known, it has been suggested that colchicine may act as a free radical scavenger.³⁷ Other actions at the activation step of CCl₄ by microsomal enzymes or at the level of the propagation of lipid peroxidation cannot, however, be ruled out.²⁹

Many of the biological actions of colchicine are related to its antimicrotubular effect.³⁸ A number of reports have emphasised the relation between microtubules and phospholipids of plasma membranes³⁹ and have shown that lipid-protein interactions within plasma membrane may also modify microviscosity.⁴⁰ Therefore, the protective effect of colchicine against changes in liver plasma membrane could be attributed to its antitubular properties. Exposure of isolated cells to colchicine (5 to 6.3 μ M) results in changes in the physicochemical properties of plasma membranes^{41,42} that can be blocked by some microtubule stabilising agents.⁴¹ Although we cannot exclude this mechanism, it seems to be unlikely at the doses used in our study. Rats given long-term colchicine treatment showed no significant changes in the physicochemical characteristics of plasma membranes (Fig 1) and these results

concur with those reported by Shiba *et al*⁴³ which showed that colchicine did not affect membrane fluidity. Furthermore, the antimicrotubular effects of colchicine are noted with much higher doses than those used in the present study. Cerbon *et al*⁴⁴ showed that the colchicine concentration in rat liver 12 hours after a subcutaneous injection of 10 µg colchicine was less than 5 ng/g of tissue.

In conclusion, longterm administration of CCl₄ to rats resulted in a variety of changes in the physicochemical characteristics of liver plasma membrane, including increased fluidity, lipid peroxidation, and shortening of the fatty acyl chains of phospholipids. All these events were prevented, however, when rats were simultaneously treated with colchicine. This agent may act by hampering lipoperoxidation of fatty acyl chains.

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