Biochemical Pharmacology, Vol. 28, pp. 3467–3470.

0006-2952/79/1201-3467 $02.00/0

Abnormal microviscosity of lamellate cytosomes induced by a diazafluoranthen derivative

(*Received* 26 *March* 1979; *accepted* 20 *June* 1979)

Lamellate cytosomes (LC) are single membrane limited organelles containing concentric membranous material [1]. They may be found in normal cells but are more often drug induced.

Previous studies have shown that the administration of a diazafluoranthen derivative (AC 3579, 2-*N*-methyl-piperazinomethyl-1,3-diazafluoranthen-1-oxide) induces the proliferation of smooth endoplasmic reticulum (SER) and the formation of lamellate cytosomes in rat hepatocytes [2, 3]. This accumulation of proteolipidic material possessing the biochemical characteristics of endoplasmic reticulum [3] is probably the result of a protected lipid degradation [4]. The inhibition of the lipid degradation can be due either to a direct inactivation of lipolytic enzymes (phospholipases) or to the formation of a drug–lipid complex which could hinder the enzymatic cleavage [4, 5].

Since the formation of such a complex would modify the microviscosity ($\bar{\eta}$) of accumulated membranes after AC-3579 treatment, we undertook microviscosity measurements on smooth microsomes and lamellate cytosomes of treated rats after isolation and purification of these subcellular fractions. In an attempt to explain the microviscosity measurement, we determined the cholesterol/phospholipid (PL) and AC-3579/PL ratios of the different membrane fractions. Indeed, it was shown that the cholesterol/phospholipid ratio determined the control of the membrane fluidity [6]. The results support previous reports on the formation of a drug–lipid complex which perturbs the phospholipases activity [4, 7].

Sprague–Dawley rats, 1 month old, received 500 mg/kg of tritiated AC-3579 (sp. radioact. 140 μCi/mg) suspended in distilled water (50 mg/ml) daily by gastric intubation. Animals were killed after a 4 days treatment; drug administration and feeding were stopped 24 hr before death.

Liver was perfused via the portal vein with 0.9% NaCl for 1 min, excised and homogenized in 2.5 vol of 0.25 M sucrose buffered with 5.10^{-3} M imidazole–HCl, pH 7.4. The whole homogenate was centrifuged at 550 *g* for 5 min to eliminate cell debris and nuclei. The supernatant was centrifuged at 10,000 *g* for 10 min; the resulting pellet corresponding to the mitochondrial fraction enriched in lamellate cytosomes was diluted to a final volume of 10 ml in the homogenization medium. Aliquots of this suspension (0.5 ml containing about 12 mg protein) were laid on top of a five layer discontinuous sucrose gradient (5 × 2.5 ml) of increasing molarity, 0.73 M, 1.01 M, 1.30 M, 1.58 M and 2.0 M respectively. After centrifugation for 210 min at 100,000 *g* in the SW 27.1 rotor of a Spinco model L2-65B ultracentrifuge, five fractions were yielded, designated M_1 to M_5, at each interface. The 10,000 *g* supernatant was centrifuged at 50,000 *g* for 60 min in a Spinco rotor 30. The resulting pellet containing the total microsomes was resuspended in the homogenization medium (17 mg/ml) and 2 ml of this suspension were layered over 3 ml of 1.3 M sucrose–5.10^{-3} M imidazole–HCl, pH 7.4. The purified smooth microsomal fraction was obtained at the interface of the 1.3 M sucrose layer after centrifugation in a SW 39 rotor for 210 min at 100,000 *g*.

Lipids were extracted from various subcellular fractions by 10 vol of chloroform–methanol (2/1; v/v) and the total lipid extract was washed according to the method of Folch *et al.* [8]. The cholesterol was determined by the method of Sperry and Webb [9]. Phosphorus was measured according to Fiske and Subbarow [10] and the phospholipids were calculated by multiplying the phosphorus value by 25.

The labelling of the membrane preparations was performed with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich) according to Shinitzky [6] in a Tris–HCl buffer, 0.1 M, pH 7.4. Measurements of fluorescence intensity and fluorescence polarization were performed with an Alscint Microviscosimeter, model MV-1a (Elscint LTD, Haifa, Israël). The excitation light was provided by a mercury lamp equipped with a cut-off filter (cut-off 365 nm) and a polarizer. The emitted light is detected in two independent cross-polarized channels after passing through a filter which cuts off wavelengths below 418 nm. From this measurements, polarization (P) was calculated according to the formula

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular, respectively, to the direction of the polarized excitation beam.

The $\bar{\eta}$ of the DPH-labelled systems is calculated as described [11, 12]. The method is based on a modified Perin equation (1).

$$\frac{r_o}{r} = 1 + v(r)\frac{T\tau}{\eta} \quad (1)$$

where r_o and r are the limiting (r_o = 0.362 [10]) and measured fluorescence anisotropies, T is the absolute temperature, τ is the life time of the excited state and $v(r)$ is a structural parameter. $v(r)$ is assumed to be constant [11]. r is obtained from P by the relationship

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I} = \frac{2P}{3 - P}.$$

Excited-state lifetimes (τ) were estimated from the total fluorescence intensity $F = I_{\parallel} + 2I_{\perp}$ and from the value of τ_o of 11.4 nsec [12]. Independent lifetime measurements were performed using an Ortec 9200 photon counting fluorescence lifetime instrument equipped with a deuterium spark-gas type flash lamp and appropriate filters. The decay of fluorescence was defined by 265 data points which were analyzed by computer [13]. The change of $\bar{\eta}$ with temperature can be evaluated from the expression (2)

$$\eta = Ae^{\Delta E/RT} \quad (2)$$

where A is a constant, ΔE is the flow activation energy, R is the gas constant and T is the absolute temperature. The sample compartment is temperature controlled to within 0.5°.

Administration of AC-3579 induces a considerable hypertrophy of SER and formation of lamellate cytosomes in rat hepatocytes as illustrated in Fig. 1a. Smooth membranes and lamellate cytosomes (Fig. 1b) were isolated after homogenization of treated liver. The concentration of phospholipids, cholesterol and AC-3579 were determined on these purified membrane fractions and compared to the values measured in smooth microsomes of the control; the results are collected in Table 1. In treated animals, the cholesterol/phospholipid ratio was decreased by about 8 fold in the smooth microsomes and 6.5 fold in lamellate

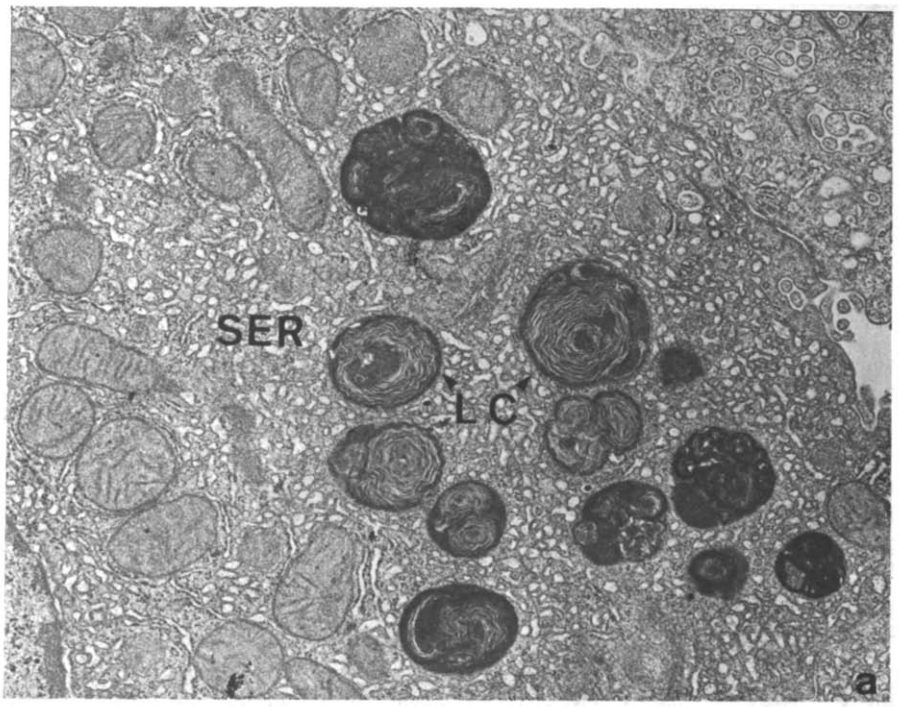

Fig. 1a. Hepatocyte after a 4 day administration of AC-3579. Lysosomes overloaded with membranous material (LC) and increased SER vesicular profiles (SER) are observed (× 12.000).

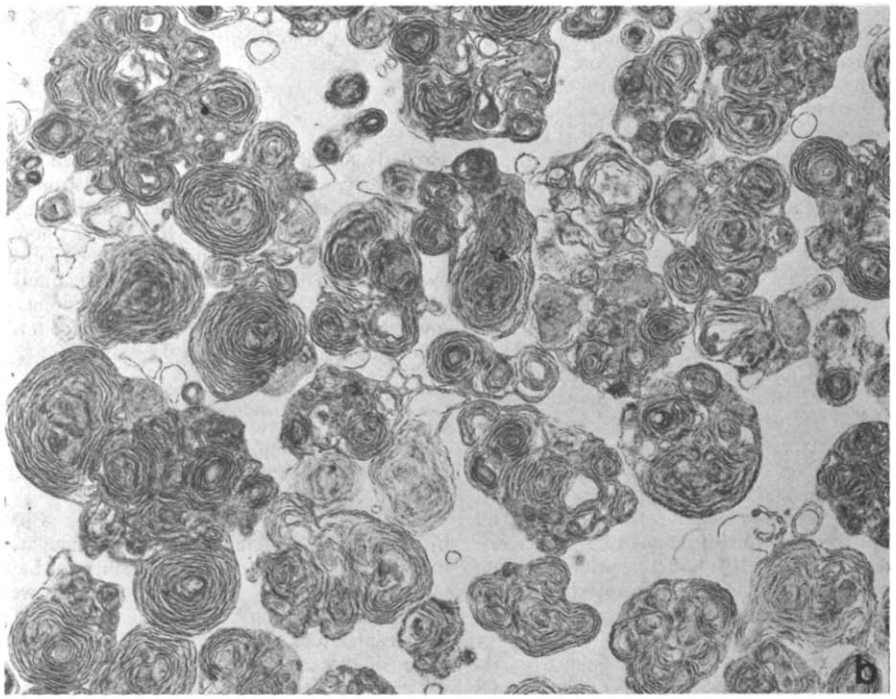

Fig. 1b. Fraction M_1 containing well preserved lamellate cytosomes with concentric membranous material (× 12.000).

Table 1. Cholesterol and AC-3579 level in untreated and treated SER membranes and lamellate cytosomes

Membranes		C/PL* (μg/mg)	AC-3579/PL* (μg/mg)	AC-3579/C* (μg/mg)
Control	SER	314 ± 14.8	—	—
AC-3579	SER	37.8 ± 3.9	0.72 ± 0.12	19.04 ± 1.13
treated	LC M_1	47.7 ± 7.9	5.37 ± 1.30	112.6 ± 7.4
rats	LC M_2	48.8 ± 5.4	5.68 ± 0.57	116.4 ± 1.1

* Mean values and standard deviations calculated for three experiments.

cytosomes as compared to the SER membranes of untreated rats. After 4 days administration, the drug content expressed in μg AC-3579/mg PL was 9 fold higher in lamellate cytosome fractions ($M1$ and $M2$) than in smooth microsomes. This elevated drug concentration enhanced considerably the AC-3579/cholesterol ratio in the two fractions containing pure lamellate cytosomes ($M1$ and $M2$). Labelling of the lamellate cytosomes and of the treated and untreated SER membranes was performed by mixing one volume of an aqueous dispersion of DPH (final concentration 10^{-6} M) to one volume of membrane suspension (2 mg PL/ml). Incorporation of DPH was followed by the increase of the total fluorescence intensity (F) which is constant after an incubation time of 45 min for microsomes and 90 min for the lamellate cytosomes at 37°. This intensity is more than 75-fold greater than those obtained with unlabelled membranes. Sequential dilution after washing the membrane suspensions revealed a constant value of P, thus excluding depolarization due to light scattering.

The excited-state fluorescence lifetime (τ) of DPH in the different membrane suspensions evaluated from the total fluorescence intensity (F) at each temperature was found to be constant and corresponds to a value of 9.9 ± 0.5 nsec. This indicates that AC-3579 does not modify the DPH spectral properties. Moreover, total fluorescence intensity (F) measurements and direct fluorescence lifetime (τ) measurements made on dimiristoyl-DL-α-phosphatidylcholine vesicles give an identical value of τ (9.9 ± 0.4 nsec) at 20° in the absence and in the presence of AC-3579 up to a concentration of 2 per cent of AC-3579 in the bilayer. This concentration is at least 10^3 times the drug concentration found in the membranous fractions studied (Table 1). In addition, the same measurements made on dimiristoyl-DL-α-phosphatidylcholine vesicles in absence of DPH show that AC-3579 by itself does not give any signal which could interfere with the measurement of P.

Figure 2 shows the temperature profiles of the $\bar{\eta}$ in the membranes of SER of treated and untreated animals and in the lamellate cytosomes. For the three membrane preparations, the decrease of $\bar{\eta}$ from low to high temperature is continuous. The absence of a breaking point indicates the absence of transition in the lipid matrix. The results obtained from Fig. 2 are summarized in Table 2. SER membranes of control and treated rats exhibit nearly the same characteristics. For the SER membranes of untreated rats, the values of the $\bar{\eta}$ and of ΔE are in good agreement with the results of Shinitzky [6], according to their cholesterol/PL ratio (Table 2).

The data obtained for SER membranes of treated rats do not fit with these results. Their $\bar{\eta}$ is higher than it would be expected from their cholesterol/PL ratio.

Lamellate cytosomes show a totally different behaviour. Firstly, their $\bar{\eta}$ is higher than that of the SER membranes although their cholesterol/PL ratio is of the same order of magnitude than that of the SER membranes of treated rats (Table 1). Secondly, the ΔE value is very low and fails out of the range of 6.5–8.5 kCal/mole as defined by Shinitzky [6] for natural membranes.

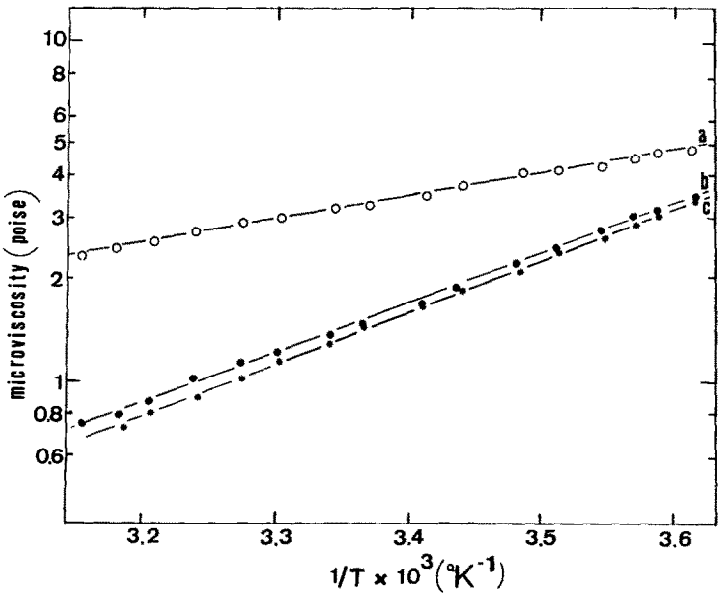

Fig. 2. Temperature dependence of the microviscosity ($\bar{\eta}$) of DPH in lamellate cytosomes (curve a) and smooth endoplasmic reticulum membranes of untreated (curve b) and treated (curve c) rats.

Table 2. Microviscosity and flow activation energy (ΔE) of untreated and treated SER membranes and lamellate cytosomes

Membranes		Microviscosity (P) 4°	25°	37°	ΔE kCal/mole
Control	SER	3.40	1.46	0.91	6.6
AC-3579	SER	3.25	1.36	0.84	6.6
treated	LC M_1	5.02	3.45	2.78	3
rats	LC M_2	5	3.44	2.79	3

Lamellate cytosomes result from an abnormal accumulation of lipid material produced by chronic treatment with certain amphiphilic drugs [1, 5]. The microviscosity of purified lamellate cytosomes induced by AC-3579 was studied by fluorescence polarization. The lamellar structures show a particular behaviour in comparison with smooth membranes of treated and untreated rats; indeed they exhibit an increase of the $\bar{\eta}$ and an important decrease of ΔE.

It was recently demonstrated that the cholesterol-PL ratio monitors the fluidity of natural membranes (6 and ref. therein) and model systems [15–18]; an increase of the cholesterol–PL ratio increase the $\bar{\eta}$. In lamellate cytosomes, however, the cholesterol–PL ratio is low. The abnormal increase of $\bar{\eta}$ is consequently attributed to the presence of high level of AC-3579. The drug effect is thus cholesterol-like but is amplified as compared to the cholesterol effect since AC-3579 content of the lamellate cytosomes only reaches 20 per cent of the cholesterol content.

Shinitzky and Inbar [6] showed that the values of ΔE of natural membranes are in the range of 6.5–8.5 kCal/mole. They attributed this averaging effect mainly to the membrane proteins movement. The ΔE of LC fails out this range. Shinitzky and Inbar also showed that, for model systems, the ΔE values can provide an index of the degree of order since the ΔE values decrease when the cholesterol/PL ratio increases. Since cholesterol–PL ratio of the LC is low, we attribute the decrease of ΔE to the presence of AC-3579. This effect is again cholesterol like. It may be attributed partially to the important hydrophobic group of the drug which consists of four hydrocarbon conjugated rings.

Other factors including the amount of sphingomyelin, the degree of saturation and the length of the phospholipid acyl chains and the presence of neutral lipids can modify the microviscosity of the lipid core but they are minor in comparison with the cholesterol–PL ratio [6]. In any case, they would not influence the dynamics of lipids in the different membrane fractions since the lipid pattern does not change in lamellate cytosomes after AC-3579 administration [19].

The mechanism of action of drugs inducing lamellate cytosomes, proposed by Lüllman *et al.* [5], postulates strong hydrophobic and hydrophylic interactions between the amphiphilic drugs and the phospholipids.

These interactions have already been described for various drugs [20, 21]. The present study shows that AC-3579 acts in the same way. In addition, it shows that AC-3579 is able to modify strongly the dynamic of the lipid matrix of natural membranes.

It has been shown in biological membranes [4] and in a model system [7] that the presence of AC-3579 in the lipid matrix was able to modify the activity of some exogeneous phospholipases A_2. These phospholipases are sensitive to the molecular packing of the substrate [22, 23]. Thus the non-degradation of the AC-3579 treated membranes by the intralysosomial phospholipases and their accumulation in the form of lamellate cytosomes in liver cells could be explained by the increase of $\bar{\eta}$ induced by the drug.

According to Shinitzky [6], the $\bar{\eta}$ of the SER membranes of treated rats is too high with regard to their cholesterol–PL ratio. This increase could be explained by the presence of small amounts of AC-3579 in SER membranes. Indeed, as it was observed in lamellate cytosomes, the cholesterol like effect of AC-3579 on the membranous viscosity appears to be very marked.

Acknowledgements—We would like to thank Miss M. Doriaux for carrying out the electron micrographs, Professor F. De Schryver (Scheikunde Departement, Katholieke Universiteit Leuven) for permission to use the fluorescence lifetime equipment and Dr. M. Van Derauwefaer for performing the fluorescence lifetime measurements.

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