decreased the serum concentration of triglycerides both in rats made hypertriglyceridaemic with fructose [15] and in normal rats deprived of food 12 hr before sacrifice (own unpublished data). Thus it is probable that the clofibrate-induced increase in carnitine compounds is not connected to the hypotriglyceridaemic effect of the drug. This is further supported by a similar lack of correlation between hepatic activities of carnitine acyltransferases and serum triglyceride level in clofibrate-treated rats [15].

The results demonstrate increased concentrations of carnitine and short chain length acylcarnitines in livers and plasma of clofibrate-treated rats. The changes in liver were intimately associated with the activity of carnitine acetyltransferase. The increased hepatic content of carnitine cannot be explained as a redistribution in the body because the total body carnitine was also increased. The dose-response relations do not support the view that hepatic carnitine has a role in the hypotriglyceridaemic effect of clofibrate.

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Effect of suloctidil* on Na^+/K^+ ATPase activity and on membrane fluidity in rat brain synaptosomes

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It has been clearly demonstrated that Na^+/K^+ ATPase is involved in the active transport of Na^+ and K^+ across plasma membranes [1]. At the level of the central nervous system, the Na^+/K^+ ATPase is concentrated in synaptic membranes [2]. Due to this localization, the enzyme is sensitive to modifications of the membrane structure produced by various exogenous or endogenous factors [3, 4]. As far as drugs are concerned, general and local anaesthetics and phenotiazines have been shown to inhibit Na^+/K^+ ATPase activity [5] and to fluidify the lipid matrix of the synaptosomes [6, 7]. However, with our present knowledge no clearcut relationships have yet been established between these two parameters [7, 8]. It has nevertheless been suggested that variations in the activity of the Na^+/K^+ ATPase would markedly alter information of transmission between nerve cells [9].

In this paper, the effects of suloctidil (and related compounds), chlorpromazine (CPZ) and ouabain on Na^+/K^+ ATPase activity and on membrane fluidity have been studied in rat brain synaptosomes. Suloctidil is an amino-alcohol endowed namely with vasoactive properties [10, 11] that could be related to a membrane effect.

Male Sprague–Dawley rats were used. Chemicals and their suppliers were as follows: suloctidil (erythro-*p*-(isopropyl-thio)-α[1-octylamino-ethyl] benzyl alcohol), CP 1136 S (erythro-*p*-(isopropylthio)-α-(1-aminoethyl) benzyl alcohol) and CP 894 S (erythro-*p*-(isopropylthio)-α-[1-(octylthio)-ethyl] benzyl alcohol) from Continental Pharma S.A., Brussels, Belgium, chlorpromazine from Rhone Poulenc S.A. and ouabain (g-Strophantin) from Boehringer, Mannheim GmbH (W. Germany).

Synaptosomes were prepared from rat brains using the procedure of Gray and Whittaker [12].

ATPase (Na^+ and K^+ activated, ouabain sensitive adenosine-5′-triphosphatase, EC 3.6.1.3) activity has been assayed by measurements of the free inorganic phosphate according to the colorimetric method of Fiske and Subbarow [13]. The synaptosome suspension was diluted 80 times in a medium containing 115 mM NaCl, 20 mM KCl, 2 mM MgCl, 1 mM EDTA and 40 mM Tris–maleate. The pH was adjusted to 7.4. 1.5 ml of the reaction mixture was incubated with 25 μl of DMSO either containing or not containing the drug to be investigated. The ATPase activity was initiated by the addition of 250 μl of a solution of ATP

7 mM. After 12 min at 37°, the enzymatic reaction was stopped by the addition of 250 μl of a solution of sodium dodecylsulphate (3.5% w/v). The mixture was chilled in ice-cold water. The precipitate which appeared in these conditions was removed by centrifugation at 1000 g for 10 min. One millilitre of the supernatant was used for the determination of inorganic phosphate.

The labelling of the synaptosome suspensions was performed with 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich) according to Shinitzky [14]. DPH stock solution (2.10^{-3} M in tetrahydrofuran) was diluted 1000 times in a Tris–maleate buffer (40 mM, pH 7.4) with vigorous stirring just before use. One volume of the diluted DPH dispersion was added to 1 volume of the synaptosome suspension. The mixture was incubated at 35° for 45 min. Incorporation of DPH was followed by an increase of the fluorescence signal which is constant after an incubation time of 40 min. Experiments were carried out with an Elscint Microviscosimeter Model MV-1a (Elscint Ltd, Haifa, Israel). This apparatus is designed to give directly the degree of fluorescence polarization (P) following equation (1):

$$P = \frac{I_{\parallel} - I_{\perp}}{I + I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallely and perpendicularly to the direction of polarization of the excitation beam respectively. This apparatus gives P with an accuracy better than 5 per cent. The temperature of the samples was controlled to within 0.5°. Correction for light scattering was made with reference samples without DPH [15]. Fresh stock solutions of the drug used were made daily in methanol. An appropriate amount of these stock solutions were added to the reaction mixture to give the desired final concentration. The methanol concentration in the reaction mixture never exceeded 0.5%. The ineffectiveness of methanol up to 1 per cent on P measurement was verified.

The evolution of the fluidity of the rat brain synaptosomes as inferred by the evolution of fluorescence depolarization (P) of DPH buried in the hydrocarbon core of the lipid bilayer as a function of the temperature as depicted in Fig. 1. With the absence of drugs, at low temperatures, the P values are high corresponding to a rigid lipid matrix. The increase of the temperature produces a concomitant diminution in the P values, which means an increase of the fluidity of the membrane. However, the evolution is nonlinear; a break-point is indeed observed at 20 ± 1°. It separates two domains where the evolution of the fluidity is directly proportional to the variation of the temperature. With the addition of suloctidil or CPZ to the aqueous phase, there is a reduction of P which is constant after an incubation of 10 min at 25°. It must be realised that the corresponding increase in fluidity is constant over the full range of temperatures studied for both compounds. Furthermore, the break-point is not displaced. At the same aqueous concentration, the effect of suloctidil on the membranous fluidity is, however, more marked as compared to CPZ. Ouabain, tested in the same conditions, has no measurable effect on synaptosomal fluidity. Figure 2 shows the evolution of the decrease in P (ΔP) as a function of drug concentration at 25° for suloctidil, CPZ and ouabain. ΔP is defined as the difference of the fluorescence polarization of DPH in the absence and in the presence of the drug, respectively, and corresponds in our experiments to an increase in the fluidity of the membrane. The fluorescence depolarization in the presence of the drug is recorded at the equilibrium. Qualitatively, the effect of suloctidil and CPZ are similar. However, quantitatively, the effect of suloctidil is greater since a variation of P is detected from 10^{-6} M for suloctidil and from 10^{-5} M for CPZ. Ouabain has no effect on the synaptosome membrane fluidity when tested in the same range of concentrations.

The results of concurrent measurements of the synaptosomal Na^+/K^+ ATPase activity are given in Table 1. Suloctidil appears as a potent inhibitor of the Na^+/K^+ ATPase since at 3.10^{-5} M it inhibits the activity of the enzyme by 50 per cent. At this concentration CPZ and ouabain inhibit the hydrolysis of ATP by 20 and 55 per cent respectively, values corresponding to those reported in the literature [16, 17].

The variation of fluidity (Fig. 2) and the enzymatic inhibition (Table 1) are related with a correlation coefficient (r) of 0.996 P ($P < 0.002$) for suloctidil and 0.965 ($P < 0.05$)

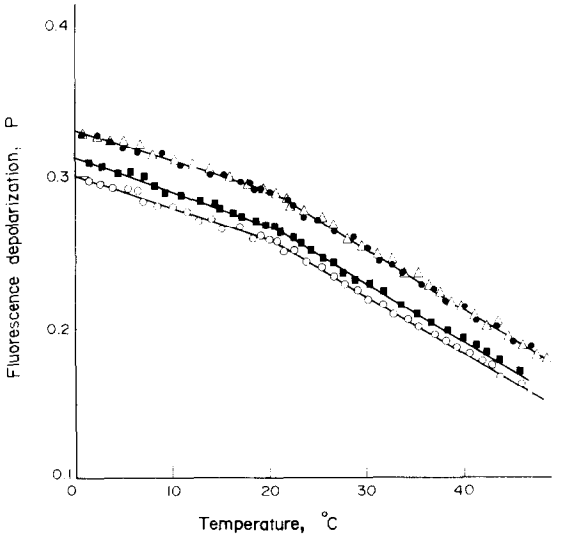

Fig. 1. Effect of suloctidil (○), CPZ (■) and ouabain (△) on the synaptosomial membrane fluidity (●) as a function of the temperature. Drug concentration: 10^{-4} M.

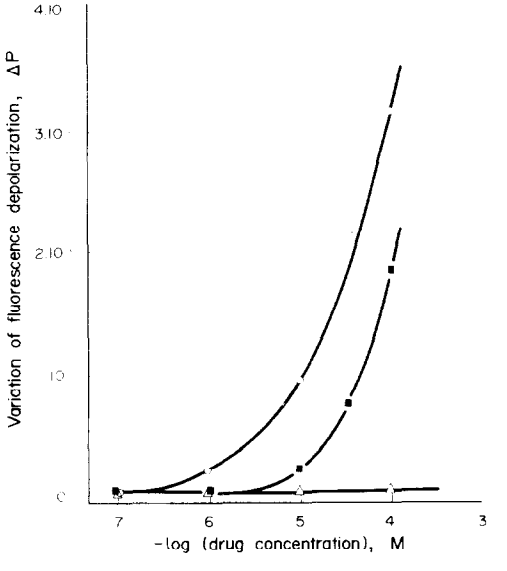

Fig. 2. Evolution of the increase in synaptosomial membrane fluidity (ΔP) as a function of drug aqueous concentration suloctidil (○), CPZ (■) and ouabain (△). $\Delta P = P_0 - P_d$ where P_0 and P_d are the degree of fluorescence polarization of DPH in absence and in presence of drug respectively. Temperature 25°.

Table 1. Na^+/K^+ ATPase activity expressed in per cent of the control

Compound	Concentration (M)* 10^{-6}	10^{-5}	10^{-4}
Suloctidil	96 ± 2 (10)	86 ± 3 (10)	26 ± 2 (10)
CPZ	95 ± 3 (6)	94 ± 4 (6)	66 ± 4 (6)
Ouabain		52 ± 2 (6)	36 ± 2 (7)
CP 894 S	100 ± 1 (2)	99 ± 2 (2)	97 ± 2 (2)
CP 1136 S	98 ± 2 (3)	97 ± 1 (3)	94 ± 2 (4)

* Each value represents the mean ± S.E.M. Number of experiments in parentheses.

for CPZ. Equations of the linear regressions are $y = 8.10^{-4} + 4.10^{-4}x$ for suloctidil and $y = -24.10^{-4} + 5.10^{-4}x$ for CPZ.

Two compounds, CP 1136 S and CP 894 S, related to suloctidil, were also tested on rat brain synaptosomes with respect to Na^+/K^+ ATPase activity and membrane fluidity. It must be recognized that, compared to suloctidil, CP 1136 S has no alkyl chain and that CP 894 S has a thio group instead of the amino function. At the concentration of 10^{-4} M, CP 1136 S produced a ΔP equal to 0.3 10^{-2} and inhibits the Na^+/K^+ ATPase activity by 6 per cent (Table 1) and CP 894 S produced a variation of polarization fluorescence ΔP of 3.8 10^{-2} M which is more significant than suloctidil but has practically no effect on the ATPase activity (Table 1).

In the present paper, it is reported that the addition at constant temperature of suloctidil and CPZ to the synaptosome suspension, produces:

(a) a fluidification of the lipid component of the synaptosomes, and
(b) an inhibition of the Na^+/K^+ ATPase activity.

The shift in membrane fluidity does not entail an increase of the Na^+/K^+ ATPase activity as expected from the temperature-activity observed between Na^+/K^+ ATPase activity and membrane fluidity [4]. On the contrary, a decrease of the enzymic activity is observed. This could imply either that the fluidity/ATPase activity relationship is not valid or is insufficient probably because the interactions between lipids and proteins are not fully understood or that suloctidil and CPZ have a direct effect on the enzyme.

With the latter possibility, a direct inhibition of the Na^+/K^+ ATPase activity has never been demonstrated. On the contrary, various papers suggest a strong non-specific interaction between CPZ and the lipid component of the membrane [5, 18–20]. It can tentatively be concluded, due to the similar behaviour of CPZ and suloctidil (Figs. 1 and 2, Table 1), that the latter compound has no direct action on the enzyme activity, but rather has an influence on the Na^+/K^+ ATPase through its solubilization in the lipid matrix.

This conclusion is further supported by the behaviour of the derivative 1136 S. Indeed, this compound which is structurally very close to suloctidil (the n-octyl chain is missing) affects neither the membrane fluidity nor the Na^+/K^+ ATPase activity. This seems to exclude a direct inhibition of the ATPase due to an interaction between the enzyme and the drug dissolved in the aqueous phase. Finally, from a comparison of the effect of suloctidil, CPZ and ouabain on the Na^+/K^+ ATPase activity and on synaptosome membrane fluidity; it appears that suloctidil and CPZ on one hand and ouabain on the other, could inhibit the Na^+/K^+ ATPase activity in different ways since the two former compounds modify the fluidity of the synaptosome while the latter one does not. (Figs. 1 and 2).

Moreover, the observation made that ouabain does not modify the fluidity of the synaptosome membrane indicates that ouabain has little or no interaction with the lipid component of the synaptosome. This is in agreement with the recognized mode of action of ouabain, which suggests the binding of the glycoside to the enzyme [16].

The analysis of the first possibility, namely the lipid fluidity/ATPase activity relationship, has already been developed in the literature [7, 8, 21]. Lenaz *et al.* [21] have proposed that general anaesthetics, through perturbation of membrane fluidity, may induce conformational changes in membrane proteins, which are on the basis of the changes in the catalytic power of membrane enzymes or carriers. A similar model has been postulated by Lee [8] for the action of local anaesthetics at the level of the Na^+ channel in nerve membranes: an increase in fluidity of the surrounding lipids allows the channels to close. The behaviour of suloctidil and CPZ fits with this hypothesis, unlike that of CP 894 S which is not positively charged. This stresses the importance of a possible electrostatic interaction between the positively charged drugs and some negatively charged phospholipids which have been shown to be necessary for the Na^+/K^+ ATPase activity [4, 20].

In conclusion, the results obtained on the effects of suloctidil on rat brain synaptosomes strongly suggest that the fluidifying effect and the corresponding Na^+/K^+ ATPase inhibition can be mainly ascribed to an electrostatic interaction with acidic phospholipids in the lipid matrix. This is supported by

(a) the analogy between the behaviour of suloctidil and CPZ,
(b) the behaviour of two analogues of suloctidil in which the ionic content (CP 894 S) or the hydrophobic-hydrophilic balance (CP 1136 S) has been modified and,
(c) by the difference of behaviour of ouabain, a potent inhibitor of the Na^+/K^+ ATPase, which has no effect on the synaptosome membrane fluidity.

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Escape from metaphase arrest by Ehrlich ascites cells grown *in vitro* during treatment with 5-chloropyrimidin-2-one

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Previous work has shown that 5-fluoro- and 5-chloropyrimidin-2-one exhibit metaphase arrest on human cells grown *in vitro* [1, 2], similar to that of colcemid and vincristine although at a higher concentration.

To study this group of inhibitors further, the effect of 5-chloropyrimidin-2-one on another mammalian cell line has been investigated. The cell line used originates from an Ehrlich ascites tumor cell line in mice and was grown in suspension culture. The inhibitor was found to exhibit an unexpected effect in this cell line. The cells overcame the metaphase arrest and returned to interphase without the appearance of telo- or anaphases or any change in cell number. Experiments describing this effect are reported.

5-Chloropyrimidin-2-one was synthesized by the method of Crosley and Berthold [3]. Colcemid and puromycin were commerical products.

The mouse cell line was obtained from Professor H. Klenow, University of Copenhagen, and was established as an *in vitro* culture by J. Allunans at the Public Health Laboratory in Oslo. The mean chromosomal number was 42. This cell line was grown with a doubling time of 15 hr at 37° in Eagle's Minimal Essential Spinner Medium (Gibco F 13) supplemented with 10% foetal calf serum, HEPES buffer (pH 7.3) at a final concentration of 15 mM, 1 ml of Flow 100× non-essential amino acids to 100 ml medium benzylpenicillin 100 u/ml and streptomycin 100 μg/ml.

The percentage of cells in prophase, metaphase, anaphase, telophase and interphase was determined by counting 1000 cells in smears of cells after fixation in Carnoy fixative (acetic acid–ethanol, 1:3, v/v) and staining with a 2% aceto-orcein solution.

The cell number was counted in a Bürker haemacytometer. The number of dead cells was determined after staining with 0.4% Trypan blue.

DNA histograms showing the distribution of cells among various phases of the cell cycle were obtained after staining with mithramycin [4] using a laboratory-built flow cytometer as described previously [5, 6]. The relative number of cells in G1, S and G2+M determined from the DNA histograms [7, 8] of untreated exponentially growing cultures were about 15 per cent in G1, 55 per cent in S and 30 per cent in G2+M. The mean phase durations calculated from six control histograms assuming cell cycle duration of 15 hr, were G1 = 1.6 ± 0.2 hr; S = 7.9 ± 0.6 hr and G2+M = 5.5 ± 0.7 hr.

The concentration of puromycin at which cell protein synthesis was inhibited was determined by studying the incoroporation of L-[^{14}C]leucine (0.007 μC/ml cell suspension sp.act. 588 Ci/mol) into the protein fraction after incubation for 30 min at 37° in the presence of puromycin. The cell pellet was treated with ice-cold 5% trichloroacetic acid for 30 min, washed with ethanol–ether (3:1 v/v), dissolved in soluene and counted in toluene containing 5 g/l PPO, 0.05 g POPOP and 30% Triton X-100.

The effect of 5-chloro-pyridin-2-one on the accumulation of cells in metaphase in exponentially growing cells is shown in Fig. 1. The concentration used was the lowest which would prevent the appearance of any ana- and telophase. The percentage of cells in metaphase increased for about 8 hr after drug administration, then declined and approached the control at the end of the experiment. The maximum value for the percentage of cells in metaphase and the time elapsed before reaching this value varied somewhat from experiment to experiment. Cells in prophase remained at approximately 1 per cent for 11 hr and subsequently no prophases were seen. The percentage of dead cells (judged from the uptake of Trypan blue) remained at 1 per cent for 21 hr which was the same as in the control. After 33 hr the figure rose to 3 per cent. When the percentage of cells in metaphase declined, the metaphase arrested cells presumably returned to interphase without dividing, since no ana- or telophases were seen during the whole experiment and the cell number remained constant (see lower part of Fig. 1).