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INHIBITION OF URIDINE INCORPORATION INTO RNA IN MAMMALIAN CELLS BY AGENTS AFFECTING THE PROPERTIES OF THE CELL MEMBRANE: CHLOROPROMAZINE, DIPYRIDAMOLE AND PHENYLETHANOL

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Abstract—The effects of chloropromazine, dipyridamole and 2-phenylethanol on the permeability of the cell membrane and on the metabolism of exogenous uridine by Ehrlich ascites cells were compared, by following the rate of fluorescein efflux from the cells, the incorporation of the labelled nucleoside into RNA and by determining the distribution of the label between the various metabolites. The main effect of chloropromazine was on the passive permeability of the cell membrane, decreasing it at low concentration and increasing it at higher ones; the latter effect was enhanced by irradiation with visible light. Dipyridamole did not modify the passive permeability, but blocked the entry of the nucleoside into the cell. Phenylethanol inhibited the entry of the nucleoside and increased the cell permeability.

PREVIOUS studies in this laboratory have shown that the polyenic antibiotic lucensomycin, which selectively binds to cholesterol moieties of the cell membrane[1–3] increasing the membrane permeability, causes a severe inhibition of the incorporation of externally added nucleosides into the nucleic acids of Ehrlich ascites cells.[4–6] The inhibition could be directly related to the increase of passive permeability, and was attributed to the inability of the nucleoside to reach an intracellular concentration sufficiently high for subsequent conversion to nucleotides and nucleic acids.[6] In the present study we have examined in Ehrlich ascites cells the effect of three different drugs—chloropromazine, dipyridamole and 2-phenylethanol—all of which inhibit the incorporation of exogenous uridine into RNA and have tried to relate the effects observed to the action of these drugs at the cell membrane level.

MATERIALS AND METHODS

Ehrlich ascites cells, Lipschütz IV tetraploid strain, obtained from Arsal, Pomezia, Italy, were grown in male Swiss mice and harvested 6–8 days after i.p. transplantation of 4–7 million cells. Cell viability was checked by the dye exclusion method using trypan blue, 1 mg/ml, in 0·9% NaCl solution, according to Tennant.[7] Only non-hemorrhagic cell samples containing more than 90 per cent viable cells were used.

Chemicals were obtained mainly from E. Merck, Darmstadt, Germany or from British Drug Houses, Poole, England. 2-phenylethanol was purchased from J. T. Baker

Chemicals N.V., Deventer, Holland. Lucensomycin* and chloropromazine were a gift of Farmitalia, Milan, Italy; dipyridamole† was a gift from C. H. Boehringer u. Sohn, Ingelheim, Germany. Uridine 5-^{3}H (sp. act. 5 Ci/m-mole), and 2-^{14}C (sp. act. 58 mCi/m-mole) were obtained from the Radiochemical Centre, Amersham, England. Radioactive samples were dissolved in 1 ml Soluene (purchased from Packard Instruments Co., Downers Grove, Ill., U.S.A.) and counted in a standard toluene-PPO-POPOP solution on a Packard Tri-Carb model 3380 liquid scintillator counter.

RNA biosynthesis in intact cells was evaluated by adding ^{3}H- or ^{14}C-labelled uridine to a suspension, equilibrated at 30°, of 2–6 $\times$ 10^{6} cells/ml, and by following, through repetitive sampling, the increase in time of the label in the acid-insoluble material. In some cases the nature of the labelled material was checked by alkaline hydrolysis, and it was confirmed that the label was in uridylic acid moieties of RNA. Under the conditions used, incorporation was linear from 5 to 30 min.[6]

For evaluation of the relative amounts of uracil, uridine and uridine nucleotides in the acid soluble material, the cells were taken at various times after addition of the label and lysed, without separating them from the incubation medium, with Triton X-100 (1%, v/v, final concentration); after addition of 0·4 M $HClO_4$, the supernatant was subjected to descending chromatography on Whatman no. 1 paper, using water-saturated *n*-butanol + 15 N ammonia (100:1, v/v) as developing solvent.[8] The chromatograms were analysed with a radiochromatogram scanner Packard model 7201, and the various components estimated from the area of their peaks. Under normal conditions, the labelled acid-soluble material was distributed in three spots, corresponding to uracil ($R_f = 0{\cdot}33$), uridine ($R_f = 0{\cdot}10$) and uridine nucleotides ($R_f = 0{\cdot}02$).

Cell permeability was evaluated by following, through repetitive sampling, the efflux of fluorescein from cells which had been loaded with the dye by previous incubation with fluorescein diacetate, as described by Celada and Rotman.[9] Since assays were performed with the final cell concentrations around 5–10 $\times$ 10^{6} cells/ml, back diffusion was negligible, due to dilution of fluorescein in the large extracellular volume. It has been shown[10] that fluorescein efflux follows an exponential law; if we indicate by C_t^0 the concentration of fluorescein in the extracellular fluid at a given time t and by C_∞^0 its concentration after at least 60 min at 42° (i.e. after an "infinite" time), a plot of ln $(C_\infty^0 - C_t^0)$ vs time yields a straight line, the slope of which, $-k$, is the efflux rate constant, proportional to the diffusion constant of fluorescein through the cell membrane.[10] This parameter was assumed to be a good index of membrane passive permeability to water-soluble low-molecular weight solutes in general.

Irradiation was performed by exposing the samples to the white light of two Osram 250 W lamps at 15–20 cm distance, keeping the temperature of the samples between 20° and 30°.

RESULTS

Effect of chloropromazine. Addition of chloropromazine was found to modify the rate of fluorescein efflux from Ehrlich ascites cells. As shown in Fig. 1, this effect was diphasic: at low concentrations the rate constant—which, as mentioned before, is proportional to the diffusion constant—was decreased (curve b) relative to the control

* Trade name Etruscomycin.
† Trade name Persantin.

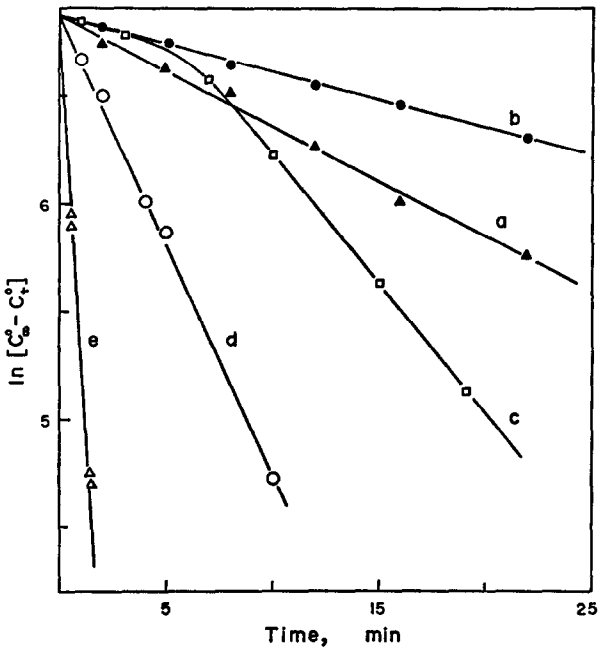

FIG. 1. Effect of chloropromazine on the rate of fluorescein efflux from Ehrlich ascites cells. The cells were loaded with fluorescein as described by Celada and Rotman[9], and then washed and resuspended. The concentration of fluorescein in the supernatant, C_t^0, was measured by its extinction at 490 nm after centrifugation at different times of incubation at 30°. The "infinite" value, C_∞^0, was obtained by incubating for 60 min at 42°. Curve (a) is the control without drug, curves (b), (c), (d) and (e) were obtained in the presence of 20, 100, 250 and 400 μM chloropromazine, respectively.

(curve a), while it increased at higher concentrations (curves d and e). It was sometimes observed (curve c) that, during incubation itself, the rate of efflux increased, being at first slower than the control, and becoming subsequently faster.

The drug exhibited a diphasic effect also on the hypotonic lysis of human erythrocytes (Table 1). Variations of the cell number did not affect the "protective" action—either as decrease of fluorescein diffusion rate or as protection against hypotonic lysis—of the drug at low ($\sim$ 25 μM) concentrations, while, if more cells are present, higher concentrations of the drug were needed to exert their "lytic" effect. Exposure to intense light increased the lytic effect of the drug at the high (e.g. 400 μM) concentrations.

The consequences of these modifications of membrane permeability upon the metabolism by the cells of added uridine are shown in Fig. 2. Incorporation of the labelled nucleoside into RNA was progressively inhibited by increasing concentrations of the drug, while the hydrolytic pathway leading to uracil was enhanced by the drug in the "protective" range of concentrations and only slightly—or not at all—inhibited by higher amounts. Synthesis of nucleotides exhibited an intermediate pattern. The polyenic antibiotic lucensomycin, which specifically increases the passive permeability of plasma membranes by interacting with cholesterol moieties, would have severely inhibited the incorporation into nucleotides and RNA, without any effect on the hydrolysis to uracil.[6, 10]

TABLE 1. EFFECT OF CHLOROPROMAZINE ON OSMOTIC LYSIS OF HUMAN ERYTHROCYTES

Chloropromazine (μM)	Osmolarity causing 50% lysis: Erythrocytes/ml (24×10^6)	(240×10^6)
0	0·154	0·145
0 + light	0·146	—
25	0·131	0·133
100	0·147	0·155
250	0·182	0·185
400	0·227	—
400 + light	> 0·3	—
500	> 0·3	0·204
1000	> 0·3	> 0·3

Erythrocytes, from a 10-fold concentrated suspension in isotonic NaCl, were added to solutions of NaCl of various osmolarity and containing different concentrations of the drug. pH was constantly 7·0. After 2 hr in the dark, the suspension was centrifuged and the extent of lysis evaluated from the amount of hemoglobin in the supernatant. In two experiments erythrocytes were added to the chloropromazine solutions (of varying osmolarity) kept under an intense white light.

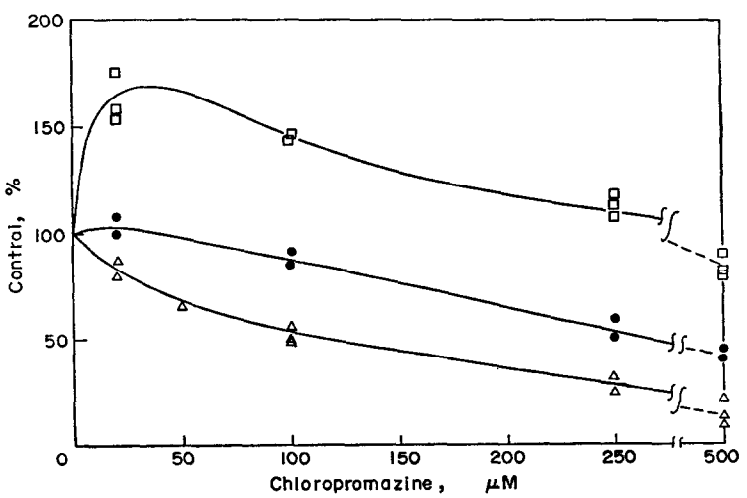

FIG. 2. Effect of chloropromazine on the transformation, by Ehrlich ascites cells, of labelled uridine to uracil, nucleotides and RNA. ^{14}C-Uridine, 1 μCi/ml, final concentration, was added to a cell suspension, 4×10^6 cells/ml, kept at 30°, at time 0. The distribution of the label between the various components of the acid soluble material was obtained by chromatography. The rate of incorporation into RNA was estimated from the appearance of the label in the precipitate. (△): Rate of incorporation into RNA; (●) and (□): amounts of labelled nucleotides and of uracil, respectively, in the acid-soluble fraction.

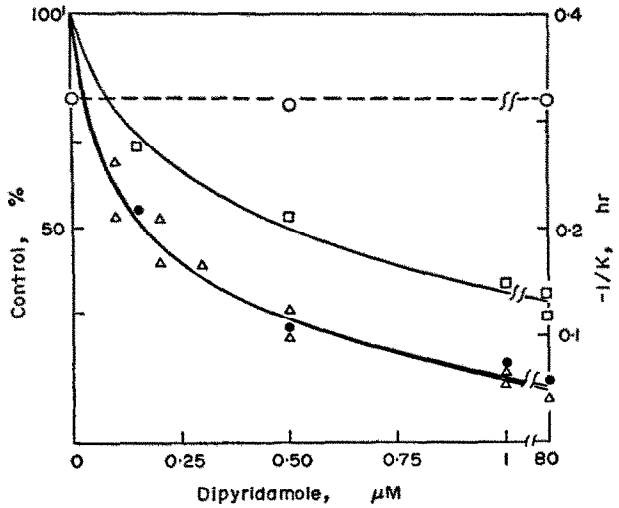

FIG. 3. Effect of dipyridamole on the rate of fluorescein efflux and on the transformation of labelled uridine to uracil, nucleotides and RNA. Same conditions and symbols as in Fig. 2. The broken line (O---O) shows that the rate of fluorescein efflux (indicated by its reciprocal on the ordinates at the right) is not affected by the drug.

TABLE 2. EFFECT OF DIPYRIDAMOLE AND LUCENSOMYCIN ON THE TRANSFORMATION OF LABELLED URIDINE TO NUCLEOTIDES AND URACIL, AND ON ITS INCORPORATION INTO RNA

	Time of incubation (min)	Per cent of total radioactivity in the acid-soluble material			Incorporation into RNA (p moles/min/10^6 cells)
		U(n)P	Uridine (residual)	Uracil	
Control	5	8·1	82·0	9·9	13·15
	15	16·6	59·7	23·7	
	25	20·4	49·4	30·2	
Dipyridamole (6×10^{-5} M)	5	0·9	95·3	3·8	1·12
	15	1·8	90·0	8·2	
	25	2·2	87·5	10·3	
Lucensomycin (2×10^{-5} M)	5	0	87·4	12·6	0·34
	15	0	77·8	22·2	
	25	0	70·7	29·3	
Dipyridamole (6×10^{-5} M) + lucensomycin (2×10^{-5} M)	5	0	75·9	24·1	0·18
	15	0	76·3	23·7	
	25	0	68·6	31·4	

^{14}C-Uridine 1 μCi/ml was added to the cell suspension, 4×10^6 cells/ml, kept at 30°, at time 0. At various times, cells were lysed and perchloric acid added. The distribution of the label between the various components of the acid-soluble material was obtained by chromatography. The rate of incorporation into RNA was estimated by the appearance of the label in the precipitate.

Effect of dipyridamole. Dipyridamole, up to 10^{-4} M, was without any effect on the rate of fluorescein efflux from Ehrlich cells, or on the extent of hypotonic lysis of erythrocytes. It had instead a dramatic inhibitory effect upon the entry of uridine into the cells, since both hydrolysis of the nucleoside to the free base and its phosphorylation to the corresponding nucleotides were prevented (Fig. 3). Incorporation of uridine into RNA was subsequently inhibited. Addition of agents, such as lucensomycin (or chloropromazine at high concentrations), which increase passive permeability, allowed the nucleoside to permeate the cell and to be hydrolysed to uracil, without restoring the ability to phosphorylate uridine to nucleotides (Table 2).

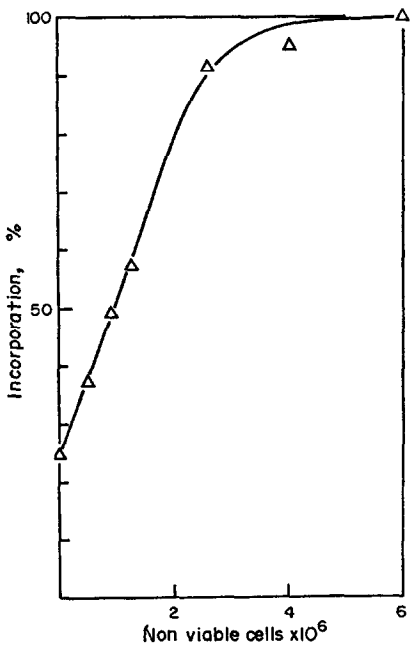

Fig. 4. Reversal by formalinized non-viable Ehrlich cells of the inhibition by dipyridamole of uridine incorporation in RNA of viable ones. The rate of uridine incorporation was estimated by repetitive sampling after addition of ^{3}H-uridine, 2·5 μCi/ml, to a suspension of 2·3 $\times$ 10^6 viable Ehrlich cells/ml (plus the indicated numbers of formalinized ones) kept at 30° and containing 0·6 μM dipyridamole. The incorporation is expressed as a percent of control incubations performed in the absence of dipyridamole.

The extent of inhibition by dipyridamole alone did not, within a wide range, depend upon the number of cells. In fact binding experiments with Ehrlich ascites cells or with erythrocytes, either intact or after formalinization, showed that only a small amount of the drug was bound to the cell membrane. Nevertheless, addition of large amount of formalinized, non-viable Ehrlich cells to a suspension of viable ones could displace the drug from the viable cells and relieve the inhibition of uridine incorporation into RNA (Fig. 4).

Effect of 2-phenylethanol. The effects of phenylethanol were somewhat intermediate between those of dipyridamole and those of permeabilizing agents. As shown in Table 3, the syntheses of nucleotides and RNA were inhibited much more severely

than the hydrolytic pathway. At the same time, passive permeability increased appreciably, though even at high concentrations of phenylethanol it did not reach the high rates obtained with chloropromazine or lucensomycin.

TABLE 3. EFFECT OF 2-PHENYLETHANOL ON PASSIVE PERMEABILITY AND ON URIDINE METABOLISM IN EHRLICH ASCITES CELLS

2-Phenylethanol (mM)	$-1/k$ (hr)	Uracil	Uridine nucleotides	RNA
0	0·312	100	100	100
8·36	0·23	—	36	34
20·9	0·13	50	1	2

The reciprocal, $-1/k$, of the rate of efflux of fluorescein was obtained by experiments similar to those indicated in Fig. 1. The rate of uridine incorporation into RNA was measured by following the appearance, at various times, of the label in the acid-insoluble material, upon incubation at 30°. The amounts of uracil and of uridine nucleotides were obtained by chromatography of the acid-soluble material. The values of uracil, uridine nucleotides and RNA are normalized to those obtained in the absence of phenylethanol, taken as 100.

DISCUSSION

In the present paper, three different drugs are compared which impair the ability of mammalian cells to incorporate exogenous uridine into RNA by acting at the cell membrane level, though with different mechanisms of action.

Dipyridamole appears to be the most specific one, being devoid of any effect on passive permeability, and inhibiting selectively the entry of nucleoside. These results confirmed those obtained by other authors.[11–15] Contrary to the results reported by Scholtissek,[12] phosphorylation was apparently not correlated to the permeation process since the hydrolytic and phosphorylating pathways were inhibited to a similar extent. Breakdown, e.g. by addition of lucensomycin, of the permeability barrier allowed the nucleoside to enter the cell, though, in our experimental conditions, the transformation to nucleotides, which required high intracellular uridine concentration, was not restored.

Experiments with isolated membranes—from beef erythrocytes or from Ehrlich ascites cells—aimed at estimating by fluorescence polarization the extent of binding of dipyridamole to the membranes were without success. The fraction of the drug bound, under the conditions used, was small and remained approximately constant if the concentration of dipyridamole was varied. This may be taken as indicating the presence of an indefinite number of sites with low affinity—or of a partitioning of the drug between the lipid phase of the membranes and the aqueous solvent. Working with whole cells, it was found that formalinization did not decrease the fraction of bound dipyridamole, but rather increased it, possibly because the breakdown of the permeability barriers allowed access of the drug to intracellular membrane systems. These results, though still preliminary afford an explanation of why the extent of inhibition of uridine permeation in the cells was, within a wide range, independent

from the number of cells, and why addition of formalinized cells could displace the drug from the viable, incorporating ones.

The mechanism of action of chloropromazine is more complex. The passive permeability of the membrane was modified in opposite directions according to the concentration of the drug. At low concentrations, the diffusion of water, fluorescein and probably also of other substances through the membrane was decreased. These effects are comparable to those described in similar and other membrane systems by other authors.[16–20] At high concentrations, a breakdown of the permeability barrier occurred giving, especially in anucleated cells, a "lytic" effect. Ehrlich ascites cells became fully permeable (i.e. $-k \to +\infty$), and were not able to exclude dyes such as trypan blue, eosin, nigrosin. This "lytic" effect, at variance from the "protective" one, had the following characteristics:

(1) the concentration of the drug required for a given increase of permeability was dependent upon the number of cells;
(2) the effect had a relatively slow onset (Fig. 1, curve c);
(3) it was enhanced by exposure to intense white light.

Since it is known that illumination induces the formation of a chloropromazine free radical,[21] the lytic effect might be tentatively attributed to the presence, in the solution, of such a radical, at equilibrium with the drug itself; the concentration of this highly reactive species would be increased by exposure to light or even, maybe enzymatically, by the sole presence of the cells.

These effects of chloropromazine on membrane permeability had different consequences upon the various metabolic paths of exogenous uridine. Incorporation into RNA was affected only by "lytic" concentrations of the drug, being progressively inhibited while permeability increased. The transformation to uracil was instead primarily affected by the "protective" concentrations of the drug, increasing up to 1·7 times the values found on untreated cells. At high concentrations of the drug, instead, the amounts of uracil formed were not significantly different from those obtained with untreated cells. Nucleotides had a behaviour somewhat intermediate between those of RNA and of uracil.

The reasons for such a pattern are not completely understood. The effect caused by "lytic" concentrations of chloropromazine is probably similar to that caused by addition to the cells of another permeabilizing drug, the polyenic macrolide antibiotic lucensomycin. Under such conditions, it had been shown that the hydrolytic pathway is practically unaffected. "Protective" concentrations of chloropromazine, on the other hand, can be expected, through a decrease of passive efflux, to keep within the cells higher concentrations of the uridine which had entered by an active or facilitated transport. This increase of intracellular nucleoside concentration should, however, have been reflected more on nucleotide and RNA synthesis than on hydrolysis to uracil, since the former path, and not the latter, is adversely affected by an increased leakage. It would seem necessary, to explain the experimental results, to admit the presence in the cells of a second nucleosidase or of alternative enzymes such as nucleoside phosphorylase (EC. 2.4.2.3) or even uracil phosphoribosyltranferase (EC. 2.4.2.9), which would start acting only at these higher uridine concentrations.

The action of phenylethanol was intermediate between those of dipyridamole and of chloropromazine at high doses: an inhibition of the active permeation of uridine,

as described by Plagemann,[14,15] together with a non-specific increase of membrane permeability. The phosphorylating pathway was therefore completely inhibited, while the efficiency of the hydrolytic one was partially reduced.

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