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Effect of Polyene Antibiotics on the Lectin-Induced Agglutination of Transformed and Untransformed Cell Lines†

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ABSTRACT: Treatment of transformed Py3T3, SV101-3T3, and L1210 cells, as well as mitotic and Pronase-treated untransformed 3T3 cells, with the polyene antibiotics filipin, nystatin, and amphotericin B inhibited agglutination by wheat germ agglutinin. The effect of polyene antibiotic treatment was lectin and cell specific. Concanavalin A induced agglutination was not inhibited, wheat germ agglutination induced agglutination of untransformed 3T3 interphase cells was not influenced, and other aggregation phenomena, including those of erythrocytes with blood group specific antibodies or divalent cations, were unaffected by polyene treatments. This suggests that the formation of polyene–cholesterol complexes in transformed and erythrocyte cell membranes may specifically affect wheat germ agglutinin receptors and/or secondary events necessary for wheat germ agglutinin induced agglutination. Fluorescence studies of membrane filipin–cholesterol complexes showed that pretreating the cells with wheat germ agglutinin, but not concanavalin A, perturbed the fluoresence properties of filipin. Electron spin resonance studies with spin-labeled fatty acids revealed at best only a slight decrease in fatty acyl chain flexibility following filipin treatment. These studies indicate that there are not only quantitative differences between the agglutinability of transformed and untransformed cells with wheat germ agglutinin but that qualitative differences exist as well.

In addition to alterations in growth control, the transformation of most animal cell lines leads, under most conditions, to alterations in the cell surface (Rapin & Burger, 1974). Among these changes is an increased susceptibility to agglutination with plant lectins (Burger & Goldberg, 1967; Inbar & Sachs, 1969; Benjamin & Burger, 1970; Eckhart et al., 1971). We have previously shown that the growth and temperature dependence of both concanavalin A and wheat germ agglutinin induced cell agglutination vary with the fatty acid composition of the membrane phosphatides (Horwitz et al., 1974). Here we consider the role of the other principal lipid in animal cell membranes, cholesterol, in lectin-induced agglutination.

Polyene antibiotics bind specifically and stoichiometrically to membrane cholesterol, rearranging it into complexes and disturbing membrane structure (Kinsky et al., 1969; Kinsky, 1970; Norman et al., 1972a,b, 1976; Hamilton-Miller, 1973; de Kruiff et al., 1974). The fluorescence properties of the membrane polyene–cholesterol complex (Drabikowski et al., 1973; Bittman et al., 1974) present an opportunity both to probe the environment of the polyene and to evaluate the effect of lectin binding on the lipid region of the membrane.

The following studies show that lectin binding perturbs the fluorescence properties of the membrane polyene–cholesterol complex in some cell types. Our results also suggest that the lipid environment of the receptors for Con A and wheat germ agglutinin differ and that the receptor(s) involved in wheat germ agglutinin induced agglutination may not be identical in transformed and untransformed 3T3 cells.

Materials and Methods

Concanavalin A (Con A)[1] was purchased from Miles, and the wheat germ agglutinin was prepared by the method of Bloch & Burger (1974). Filipin complex (lot no. U-5956/8393, 86% pure) was the generous gift of Dr. Fritz Reusser of Upjohn, and nystatin and amphotericin B were provided by Ms. Barbara Stearns of Squibb. The spin-labeled fatty acids, 5-doxylstearate and 10-doxylstearate, were generously supplied by Dr. J. Seelig, Department of Biophysical Chemistry, Biocenter of the University of Basel, Switzerland. Spin-labeled sterol (17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxyl) was purchased from SYVA (Palo Alto, CA).

Tissue Culture Cell Lines. Untransformed 3T3 and transformed SV101-3T3 and Py3T3 cells were grown as described previously (Noonan & Burger, 1973; Horwitz et al., 1974) on 100-mm Falcon tissue culture dishes containing 10

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[1] Abbreviation used: Con A, concanavalin A.

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mL of Dulbecco's modified Eagle's medium supplemented with 10% calf serum (GIBCO). The procedure used for the removal of the cells from the culture substratum for agglutination assays has been described (Burger, 1974). Ehrlich ascites cells and L1210 leukemic cells were maintained by serial passage in the peritoneal cavity of the mouse ($C57B1_6$ and Swiss, respectively). These cells were harvested and washed three times in saline (600 rpm, 5 min, 20 °C) for use in the agglutination assay. Prior to the agglutination assay cells were stored for short periods (up to 3 h) on ice (Ehrlich ascites and L1210 cells) or at room temperature (other cells). The cells remained viable, as judged by trypan blue exclusion as well as replating, for at least 3 h under these conditions.

Preparation of Mitotic Cells. Serum (50% in Dulbecco's modified Eagle's medium) was added 24 h after untransformed 3T3 cells had reached confluence. The 3T3 cells entered mitosis, as judged by the mitotic index, approximately 20 h after the addition of excess serum. Impulsecytophotometric analysis of the DNA content of the cells confirmed this result. Since mitotic 3T3 cells adhered less firmly to the culture substratum, they could be removed by shaking. After two saline washes (600 rpm, 5 min, 20 °C), the cells were used immediately for agglutination assays (Burger, 1974).

Agglutinations. Agglutinations of 3T3, SV101-3T3, and Py3T3 cells by the lectins Con A and wheat germ agglutinin were performed with cells grown to approximately 80% confluency. Ehrlich ascites and L1210 cells were prepared for these assays by the method described above, and erythrocytes were prepared by washing the cells three times in saline (1000 rpm, 10 min, 20 °C). Outdated human erythrocytes were supplied by the Swiss Red Cross.

Stock solutions of the polyene antibiotics were prepared by first dissolving the polyene (1.0 mg) in dimethylformamide (50 μL) and then diluting that solution to 20 mL in phosphate-buffered saline with vigorous vortexing. Filipin (76 μM), nystatin (54 μM), and amphotericin B (54 μM) stock solutions were stored at 0–4 °C in the dark. Cells were treated at 37 °C in the dark with filipin, nystatin, or amphotericin B at concentrations ranging from 0.5 to 50 μg/mL for time intervals ranging from 1 to 60 min. Unbound polyene was removed by low-speed centrifugation (600 rpm, 5 min, 20 °C), and the cells were subsequently washed three times with phosphate-buffered saline (600 rpm, 5 min, 20 °C). This procedure removed more than 95% of the unbound polyene. Further washing was avoided in order to minimize damage to the cells during centrifugation. Cells were used immediately for agglutination assays.

The amount of filipin bound to 3T3 cells probably in the form of a cholesterol complex was assayed spectroscopically at 356 nm and spectrofluoroscopically (excitation peak II and IV; see Figure 4 and Table II). 3T3 cells (1×10^6 cells/mL) were treated with 7.6 μM filipin for 1 h at 37 °C. After centrifugation for 5 min at 600 rpm and 4 °C and three subsequent washings with phosphate-buffered saline for 5 min at 600 rpm and 4 °C, the amount of filipin bound to the cells was measured at 356 nm (the ratio of the fluorescence emission peaks II to IV was identical with the one in Figure 4B and corresponded to the one found in Table II). For a pellet of 10^6 3T3 cells this value was 2.2 μg of filipin bound. Spectroscopically and spectrofluoroscopically no filipin or filipin–cholesterol complex was found in the supernatant of the third wash.

We found that both 3T3 cells as well as SV101-3T3 cells contained approximately 5 μg of cholesterol per 10^6 cells (Hatten, 1975) when the cells were harvested at approximately 80% confluency. Similar cholesterol values were found for Ehrlich ascites (4.97; Brennemann et al., 1974) and L5178 (3.4; Rothblatt & Kritchevsky, 1968) cells. The molar ratio of bound filipin to total cellular cholesterol was therefore 0.26 for 3T3 cells. The cholesterol content of these cells did not appreciably decrease in the cell pellet after washing and the supernatants of the first and second wash did not contain more than 5% and 1%, respectively, of the total cholesterol in the pellet, an amount approximately corresponding to the cells lost due to lysis rather than the extraction by filipin from intact cells.

The growth of 3T3 and SV101-3T3 cells in Dulbecco's modified Eagle's medium supplemented with calf serum 10% was found to be largely unaffected by the addition of filipin at a concentration less than 1.5 μM. Phase contrast and fluorescence microscopic examination, as well as trypan blue exclusion, were used routinely to evaluate the extent of cell damage or lysis following polyene treatment. Both preincubation with the polyene and the agglutination assays were performed over a temperature range between zero and 40 °C.

The details of the lectin agglutination assay have been described for cultured cells (Burger, 1974). Human erythrocytes, however, were agglutinated at 20 °C in a porcelain spot plate. For agglutination with blood group specific antisera (Sigma), the cells were resuspended at 2.0×10^6 cells/mL in a 1:100 dilution of the antiserum. After a 5-min incubation (20 °C) with gentle rocking, agglutination was scored. For agglutinations of erythrocytes with Ba^{2+} or Ca^{2+}, concentrations ranging from 1 to 50 mM were tested. After three saline washings (1000 rpm, 10 min, 20 °C), the cells were suspended at 2.0×10^6 cells per mL in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. To 100 μL of a suspension containing 2×10^5 cells in a porcelain spot well, $BaCl_2$ or $CaCl_2$ was added at the indicated concentration. The cells were incubated at 20 °C with occasional gentle shaking for 5 min. Agglutinated cells appeared as a single mass in the center of the well (100%), and lesser degrees of agglutination were easily scored by eye (75%, 50%, 0%). In some experiments, the cells were preincubated with filipin (0.76 μM, 30 min, 20 °C) in the dark. Unbound filipin was removed prior to the agglutination assay by washing the cells twice with phosphate-buffered saline (600 rpm, 5 min, 20 °C).

Lecithin Liposomes. Liposomes were prepared by a modification of the method of Kinsky et al. (1968). Chloroform solutions of egg lecithin (containing 50–90 μmol) and cholesterol at the desired molar concentration (0–90 μmol) were vortexed under N_2 until a thin lipid film resulted. Vesicles were prepared by adding 10 mL of phosphate-buffered saline followed by ultrasonic irradiation for 20 min under N_2 with a 20-kHz Branson sonifer (Model B-12) fitted with a solid tap horn (power level 3–5). Egg lecithin and cholesterol were purchased from Sigma, and their purity was confirmed by thin-layer chromatography on silica gel H–Na_2CO_3 plates developed with chloroform–methanol–water–acetic acid (25:15:3:1.5 by volume) for lecithin and chloroform–methanol–acetic acid–water (150:28:8:4 by volume) for cholesterol analysis.

Fluorescence measurements of filipin incorporated into cellular membranes or into liposomes were made using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A. Cells were pretreated with filipin (7.6 μM) for 1 h at 50 °C in the dark, and the spectra were recorded at 20 °C immediately after the excess filipin was removed by washing the cells in phosphate-buffered saline (600 rpm, 5 min, 20 °C). The emission and excitation fluorescence of the cell

Table I: The Effect of Polyene Antibiotics on the Half-Maximal Agglutination of Transformed and Untransformed Cells[a]

cells	polyene	agglutinin necessary for half-max agglutination: dose (μM)	WGA (μg/mL)	Con A (μg/mL)	polyene required for lysis (μM)
3T3	none		280	850	
	filipin	7.6	310	1000	no lysis at 76
	nystatin	5.4	280	850	no lysis at 54
	amphotericin	5.4	280	850	no lysis at 54
mitotic 3T3	none		75	300	
	filipin	7.6	400	350	29
	nystatin	5.4	200	350	30
	amphotericin	5.4	100	350	43
pronase-treated 3T3	none		50	400	
	filipin	7.6	420	400	
Py3T3	none		50	150	
	filipin	7.6	450	150	29
	nystatin	5.4	200	175	30
	amphotericin	5.4	90	150	43
SV101-3T3	none		50	120	
	filipin	7.6	500	150	29
	nystatin	5.4	200	120	30
	amphotericin	5.4	150	120	43
L1210	none		50	ND	
	filipin	5.0	500	ND	29
	nystatin	5.4	300	ND	27
	amphotericin	5.4	250	ND	45

[a] The cells were treated with the indicated polyene at the indicated dose as described in Materials and Methods. A cell sample of 2×10^6 cells/mL (both 3T3 and SV101-3T3 cells have a total cellular cholesterol content of approximately 10 μg per 2×10^6 cells; Hatten, 1975) was used for each assay. Half-maximal agglutination for wheat germ agglutinin and Con A was determined as described (Burger, 1974). Lysis in the presence of polyenes was determined both by trypan blue uptake and by phase contrast microscopy. WGA, wheat germ agglutinin.

sample (1×10^6 cells/mL) was scanned over the 280–650-nm range with excitation and emission at 320 nm and 480 nm, respectively. In some experiments, cells were preincubated with Pronase or trypsin (10–100 μg/mL, 15 min, 37 °C) or glutaraldehyde (0.05%, 15 min, 37 °C) before filipin treatment. In other cases, the cells were preincubated for 30 min at 37 °C with either wheat germ agglutinin (1–10 μg/mL) or Con A (1–40 μg/mL) before the addition of filipin. Following polyene treatment and washing, the cells were resuspended in phosphate-buffered saline (1×10^6 cells/mL), and emission or excitation spectra were recorded at 20 °C.

Spin Labeling of Cells and Electron Spin Resonance Measurements. 3T3, SV101-3T3, and L1210 cells were labeled with 5-doxylstearate and 10-doxylstearate as described elsewhere (Hatten et al., 1978). In some experiments, cells were pretreated with wheat germ agglutinin or Con A prior to labeling. In other experiments, cells were labeled with the sterol spin label 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxyl using the method described for fatty acid spin labels (Hatten et al., 1978). Spectra were recorded at 20 °C.

Results

Compared with other agglutination inhibitors, treatment with relatively low concentrations of polyene antibiotics had a dramatic effect on the wheat germ agglutinin induced agglutination of transformed cells. The wheat germ agglutinin

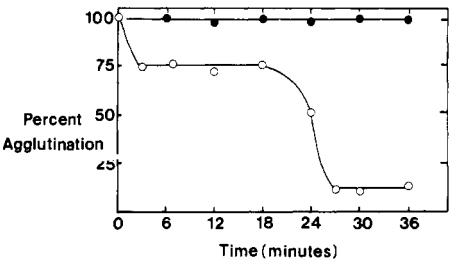

FIGURE 1: Time course of filipin-induced reduction in the wheat germ agglutinin-induced agglutination of L1210 leukemic cells. Cells were preincubated with filipin (input concentration, 7.6 μM, 37 °C) for the indicated time period prior to the removal of excess filipin, and agglutination was assayed at 20 °C as described in Materials and Methods. Further details are given in Table I.

concentration for the half-maximal agglutination of L1210, Py3T3, and the SV101-3T3 cells was greatly increased by low doses of polyenes (Table I). The agglutination of both untreated and treated cells (at higher concentrations of wheat germ agglutinin) was reversed by the addition of *N*-acetylglucosamine (0.8 mM).

Preincubation of the cells with cholesterol (30 μM in 0.25% ether) prevented the drop in wheat germ agglutinin induced agglutinability caused by polyene treatment. The addition of cholesterol in a tenfold molar excess to filipin reversed the inhibition of agglutination, if that addition was made within the first 3 min following the introduction of the polyene. After 3 min, however, the decreased agglutination caused by polyene treatment could not be reversed, even by larger excesses of cholesterol. Other sterols tested (cholestanol, epicholesterol, coprostanol, and cholesterol acetate) did not block the effect of polyene treatment on agglutination.

The decrease in wheat germ agglutinin induced agglutination occurred with a biphasic time dependence. After a 3-min incubation with filipin, agglutination was decreased by 25%, but a further reduction in agglutination did not occur until after a 20-min incubation with filipin (Figure 1). The second drop in agglutinability could be prevented by preincubation of the cells with NaN_3 (3.8×10^{-2} M), but azide did not prevent the initial decrease. In addition, the increased agglutinability of mitotic 3T3 and Pronase-treated 3T3 cells was abolished by incubation of the cells with filipin (Table I). In contrast, the agglutination of interphase 3T3 cells was not affected by polyene treatments applied over a similar concentration range (Table I).

When transformed cells were preincubated with filipin at 20 °C in the dark, wheat germ agglutinin induced agglutination occurred over the temperature range from 0 to 40 °C with the same time constant, but with a lowered half-maximal value. However, when the cells were pretreated with filipin at 0–5 °C in the dark and kept at that temperature for the agglutination assay, no inhibition of agglutination was observed. These results suggest that the removal of membrane cholesterol into complexes with filipin did not alter the temperature dependence of wheat germ agglutinin mediated agglutination. They also suggest, however, that the filipin–cholesterol complex was not formed at low temperatures.

In contrast to the effects of polyene treatment on the wheat germ agglutinin induced agglutination of these transformed cell lines, agglutination with the lectin Con A was only slightly inhibited by polyene treatment. No greater than a 20% decrease in agglutinability was observed for L1210, Py3T3, or SV101-3T3 cells, and no effect was observed with 3T3 cells (Table I). In all cases where polyene action induced an inhibition of agglutination, preincubation with cholesterol (500 μM) blocked the reduction.

The question was then raised as to whether filipin altered the cell surface to the extent that other aggregation phenomena were affected. This was not the case (Hatten, 1975). First, as mentioned above, although the wheat germ agglutinin induced agglutination of several transformed cell types was inhibited, the Con A induced agglutination of these cells was not affected by filipin treatment. This result was also observed with erythrocytes following filipin treatment (0.76 μM, 30 min, 30 °C, pH 7.2). Second, the agglutination of erythrocytes with blood group specific antibodies was not affected. Third, the agglutination of erythrocytes with divalent cations was also not affected by filipin treatment.

Three differential effects of polyene antibiotics were observed between transformed and untransformed cells. First, Py3T3, SV101-3T3, and L1210 cells were more susceptible than were interphase 3T3 cells to lysis in the presence of low doses of polyenes (Table I). Second, the agglutination of transformed cells, mitotic 3T3 cells, and Pronase-treated 3T3 cells by wheat germ agglutinin was inhibited following filipin treatment of the cells, whereas that of 3T3 cells was, to a large extent, unaffected (Table I). Third, transformed cells displayed more prominent morphological alterations as judged by phase contrast and fluorescence microscopy.

When treated with a low dose of filipin (7.6 μM, 10 min, 37 °C), transformed Py3T3, SV101-3T3, Ehrlich ascites cells, and L1210 cells became markedly swollen (2–3 times their normal diameter) and developed large fluorescent patches on their surfaces as judged by phase contrast and fluorescent microscopy. Some of the earliest morphological effects could be detected at 5–7 μM filipin. Untransformed 3T3 cells, on the other hand, did not develop fluorescent patches on their surfaces, even at doses up to 76 μM, although minor swelling (1.5 times their normal diameter) was sometimes observed after prolonged incubation with filipin (>30 min, 76 μM, 37 °C). At filipin doses greater than 58 μM, transformed cells failed to exclude the dye trypan blue and were fully lysed within 10 min (Table I). No lysis of 3T3 cells was observed, even for incubation times up to 1 h (37 °C) and doses up to 79 μM. In accordance with the results reported for model systems (Kinsky, 1970), the different polyene antibiotics were also shown here to have different efficacies for lytic action on transformed cells. Filipin and nystatin lysed these transformed cells at slightly lower doses than did amphotericin B (Table I).

The effects of cholesterol on the fluorescence properties of filipin are shown in Figure 2. In agreement with other results (Schroeder et al., 1972; Bittman & Fischkoff, 1972; Bittman et al., 1974; Drabikowski et al., 1973), the addition of cholesterol to an aqueous solution of filipin resulted in quenching of the fluorescence emission of filipin (Figure 2), and large changes in the peak ratios of the fluorescence excitation of filipin. A similar quenching and change in the peak ratios of the fluorescence properties of filipin were observed with lecithin liposomes containing cholesterol (lecithin to sterol ratio of 7:3) as well as 3T3, SV101-3T3, L1210, Ehrlich ascites cells, and human erythrocytes (Figures 2, 3, and 4). No differences in the quenching or peak ratios of the filipin emission and excitation spectra were observed between untransformed 3T3 and transformed SV101-3T3 cells (Figure 2C,D).

Since wheat germ agglutinin induced agglutination of transformed cells was shown to be inhibited by treating the cells with polyene antibiotics, the effects of pretreating the cells with lectins on the fluorescence properties of filipin (Figure 3) were tested. Pretreatment of 3T3 and SV101-3T3 cells, as well as erythrocyte ghosts with wheat germ agglutinin (1–10

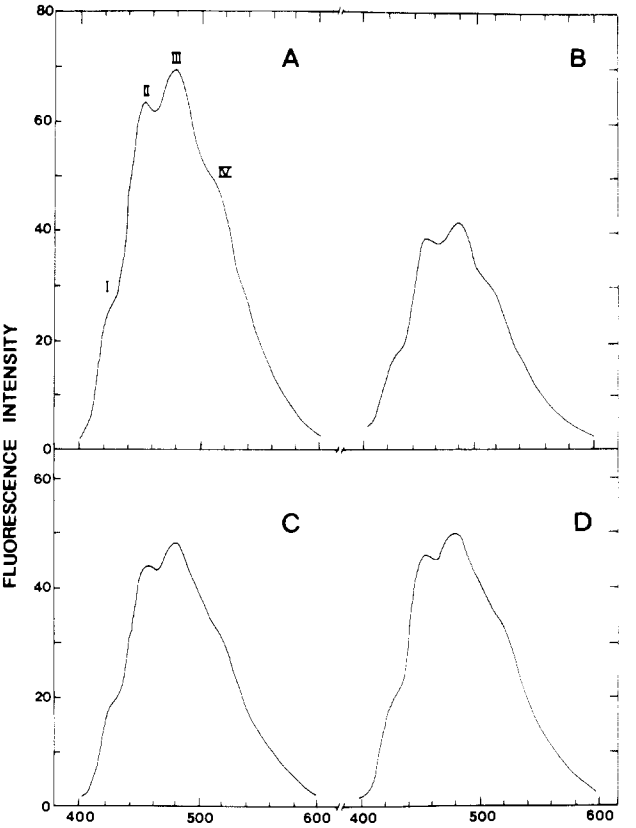

FIGURE 2: Fluorescence emission spectra of filipin and of filipin in the presence of cholesterol and 3T3 and SV101-3T3 cell membranes. The wavelength of excitation was 320 nm, and all spectra were recorded at 20 °C. Input concentrations were as follows: (A) filipin (7.6 μM) in PBS buffer; (B) filipin (7.6 μM) in PBS buffer containing 30 μM of cholesterol in 0.25% ether (final concentration). To remove the ether the solution was incubated for an hour at 37 °C. (C) Filipin (7.6 μM) in the presence of 3T3 cells (1×10^6 cells/mL) and (D) filipin (7.6 μM) in the presence of SV101-3T3 cells (1×10^6 cells/mL). The cholesterol concentration of 1×10^6 3T3 or SV101-3T3 cells is approximately 5 μg. Details are given in Materials and Methods.

μg/mL, 30 min, 37 °C), altered the ratio of peak III to peak II of the emission spectrum (wheat germ agglutinin concentration dependence shown in Figure 3C,D). In contrast, wheat germ agglutinin added to liposomes at a phospholipid to cholesterol molar ratio similar to that of fibroblasts (7:3) had no effect on the fluorescence emission of filipin (data not shown). Furthermore, preincubation of whole cells or erythrocyte ghosts with Con A (Figure 3A,B), blood group specific antibodies or divalent cations (in agglutinating doses) also did not affect the fluorescence emission of filipin incorporated into these cells. Pretreatment of the cells with Pronase or trypsin (10 μg/mL, 15 min, 37 °C) or glutaraldehyde (0.5%, 15 min, 37 °C) did not change these results.

The fluorescence excitation of filipin incorporated into either different whole cells (Table II) or erythrocyte ghost membranes (Figure 4) revealed some differences in the ratio of peak II to peak IV between the cell types tested (Table II). This ratio was altered by pretreating the cells with wheat germ agglutinin, in the case of erythrocyte ghost preparations (Figure 4), but not in the cases of 3T3, SV101-3T3, or L1210 cells. Con A, blood group specific antibodies, and divalent cations did not produce this result, even at agglutinating doses (Table II).

The order parameter obtained from spectra of fatty acid spin labels (5-doxylstearate and 10-doxylstearate) incorporated into transformed and untransformed cells pretreated with filipin revealed at best a very small change (average error

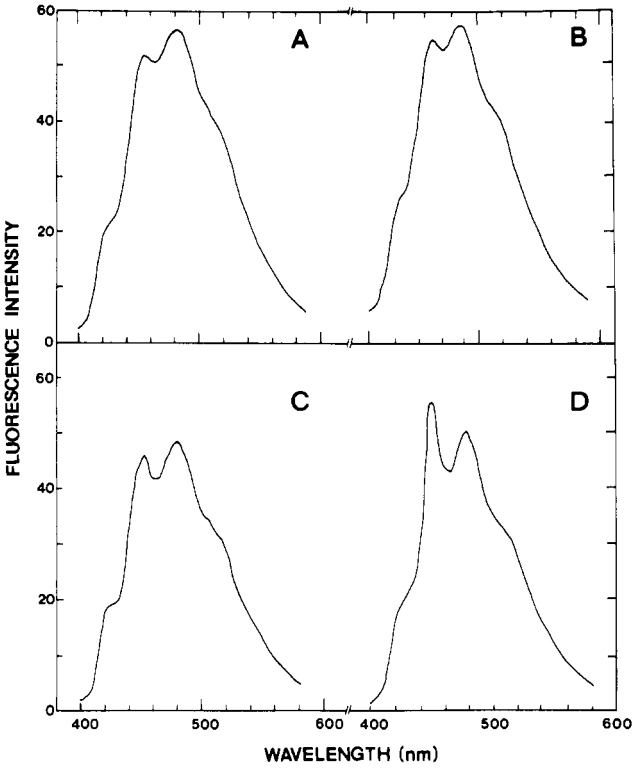

FIGURE 3: The effect of lectins on the fluorescence emission of filipin in the presence of human erythrocyte ghost membranes prepared from 1-mL packed erythrocytes. (A) Filipin (0.76 μM) in the presence of erythrocyte ghosts (erythrocyte cholesterol concentration, 60 μM). (B) Filipin (0.76 μM) in the presence of erythrocyte ghosts pretreated with 40 μg/mL of Con A, (C) 1.0 μg/mL wheat germ agglutinin, and (D) 10 μg/mL of wheat germ agglutinin. The wavelength of excitation was 320 nm and all spectra were recorded at 20 °C.

Table II: The Ratio of Peak II (325 nm) to Peak IV (357 nm) of the Fluorescence Excitation Spectrum of Filipin (Figure 4) at the Indicated Concentration in the Presence of the Indicated Cell Type (1×10^6 Cells/mL)[a]

solvent	filipin concn (μM)	ratio peak II: peak IV
(1) phosphate-buffered saline	7.6	0.63 (± 0.01)
(2) phosphate-buffered saline containing 25 μM cholesterol	7.6	0.85 (± 0.03)
(3) 3T3 cells	7.6	1.06 (± 0.02)
(4) SV101-3T3 cells	7.6	1.06 (± 0.02)
(5) L1210 cells	7.6	0.75 (± 0.04)
(6) Ehrlich ascites cells	7.6	0.80 (± 0.04)
(7) human erythrocytes	7.6	1.03 (± 0.01)
(8) human erythrocytes preincubated with:		
0.05 μg/mL wheat germ agglutinin	0.76	1.05 (± 0.03)
2.0 μg/mL wheat germ agglutinin	0.76	1.14 (± 0.03)
10.0 μg/mL wheat germ agglutinin	0.76	1.22 (± 0.03)
0.05 μg/mL Con A	0.76	1.03 (± 0.01)
40.0 μg/mL Con A	0.76	1.03 (± 0.01)

[a] The wavelength of emission was 480 nm, and measurements of the fluorescence intensity of each peak were made from spectra recorded at 20 °C. Details are given in Materials and Methods and Figures 3 and 4.

±0.005 in the s value as compared with that for untreated cells (Hatten, 1975). For 3T3 and SV101-3T3 cells, this value was 0.69 in the absence of filipin and in the presence of the antibiotic, 0.70. For L1210 cells the respective values were 0.70 and 0.72. Preincubation of the cells with either Con A or wheat germ agglutinin did not alter this result. In addition, no changes in the electron spin resonance spectrum of spin-labeled sterol derivatives were observed either between 3T3 and SV101-3T3 cells, or those cell types treated with filipin, or those cell types treated with both filipin and wheat germ agglutinin or Con A (Hatten, 1975).

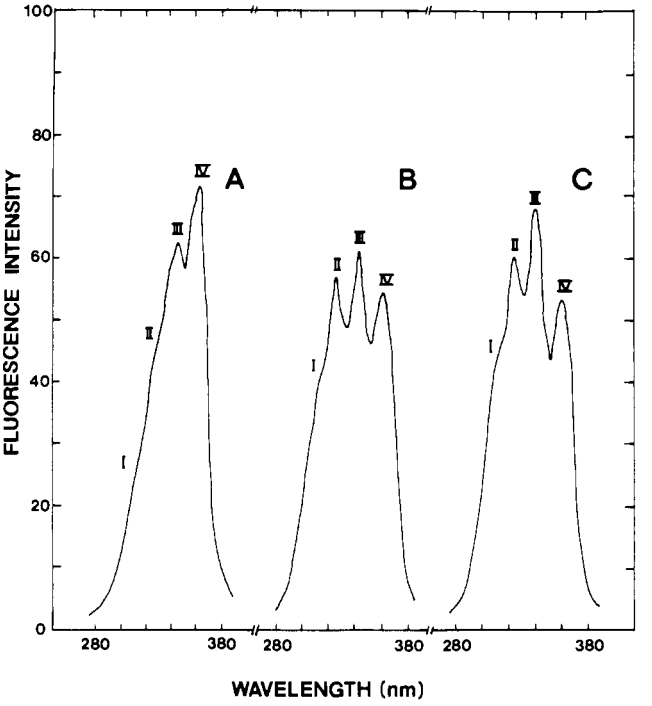

FIGURE 4: Fluorescence excitation spectra of filipin in PBS buffer (A), filipin in the presence of human erythrocyte ghosts (B), and filipin in the presence of human erythrocyte ghosts pretreated with wheat germ agglutinin (10 μg/mL) (C). The input concentration of filipin in each case was 0.76 μM, the wavelength of emission was 480 nm, and spectra were recorded at 20 °C. Details are given in Materials and Methods and Figure 3.

Discussion

The biphasic inhibition of wheat germ agglutinin induced cell agglutination caused by pretreating the cells with low doses of filipin is most simply explained by (1) a binding step that was reversed by the presence of excess cholesterol and (2) a membrane rearrangement step that was energy dependent and could not be reversed by simple competition for the polyene molecule. The specificity of the reaction of these polyenes with membrane cholesterol, previously demonstrated for lipid bilayer model membranes as well as some biological membranes (Kinsky et al., 1969; Dennis et al., 1970; Kinsky, 1970; Norman et al., 1972a,b; Bittman et al., 1974), was also supported here by the fact that cholesterol was the only sterol tested that inhibited the polyene-induced inhibition of agglutination.

The formation of polyene–filipin complexes in biological membranes has been shown to be temperature dependent (de Kruiff et al., 1974). This is consistent with our observation that treatment of transformed cells with filipin at 0 °C did not result in the inhibition of subsequent agglutination of the cells with wheat germ agglutinin at 0 °C. If, however, the cells were treated with filipin at 20 °C and wheat germ agglutinin induced agglutination was subsequently assayed at 0 °C, the same degree of inhibition observed at 20 °C was seen. These results suggest that the cholesterol domain required for the insertion of filipin into the membrane was not available at low temperatures. However, our results did not show that filipin treatment altered the temperature dependence of wheat germ agglutinin induced agglutination (Horwitz et al., 1974; Hatten, 1975).

The rearrangement of membrane cholesterol caused by filipin was relevant to wheat germ agglutinin induced agglutination, but not to any other aggregation phenomena tested. Filipin treatment inhibited cell agglutination induced by wheat germ agglutinin but not that by Con A. Furthermore, it did not inhibit the agglutination of human erythrocytes by blood group specific antibodies and did not influence the agglutination of erythrocytes or 3T3 and SV101-3T3 cells by divalent cations. This differential effect of filipin is consistent with studies by Puchwein et al. (1975), showing that the treatment of pigeon erythrocytes with filipin caused the inhibition of catecholamine-activated adenylcyclase, but not ATPase or 5′-nucleotidase activities.

In addition, pretreatment of the cells with wheat germ agglutinin, but not Con A or blood group specific antibodies (erythrocytes), caused alterations in the fluorescence excitation and emission spectra of filipin bound to membrane cholesterol in L1210, Ehrlich ascites, 3T3, SV101-3T3 cells, and erythrocytes. The changes in peak ratios of the fluorescence excitation of filipin are consistent with an increase in the amount of cholesterol available for interaction with filipin (Bittman et al., 1974), although other interpretations are certainly not excluded by these studies.

The results of electron spin resonance studies with spin-labeled fatty acids are consistent with the interpretation that the complexing of membrane cholesterol with filipin caused only a slight change in the mean molecular motion of the membrane phosphatide fatty acyl chains. Perhaps studies of the influence of membrane fluidity (as probed by spin labels) on lectin-induced agglutination (Horwitz et al., 1974; Hatten et al., 1978) do not support the extrapolation that such a small change in the order parameter alone would be sufficient to selectively inhibit wheat germ agglutinin-induced agglutination.

The results of electron spin resonance studies with a spin-labeled sterol are consistent with the interpretation that the complexing of membrane cholesterol with filipin did not change the mean molecular motion of membrane sterol. This could be due to the lower sensitivity of spin-labeled sterols, as probes of the fluid properties of the membrane owing to the restricted flexibility of cholesterol and to the fact that the presence of cholesterol retards fatty acid chain flexibility and increases the order (Hubbell & McConnell, 1971).

The question remains as to why polyene treatment inhibited the wheat germ agglutinin induced agglutination of transformed but not untransformed 3T3 cells. One previous study supports this finding of a higher sensitivity of transformed cells to polyene antibiotics (Mondovi et al., 1971); filipin treatment induces large changes in the membrane permeability of Novikoff hepatoma cells but not in that of Ehrlich ascites cells. It is possible that membrane permeability changes induced in transformed cells by polyene treatment either directly or indirectly interfere with the sequence of events required for the wheat germ agglutinin induced agglutination of these cells.

Another explanation for the exclusive effect of polyenes on the wheat germ agglutinin induced agglutination of transformed cells is the following. If there are subsets of wheat germ agglutinin receptors (Rapin & Burger, 1974), then the agglutination of transformed cells could be caused by the interaction of wheat germ agglutinin with a receptor subset that differs from the receptor subset responsible for the wheat germ agglutinin induced agglutination of untransformed cells. In this interpretation, the particular subset(s) of wheat germ agglutinin receptors required for the agglutination of transformed cells could be influenced either directly or indirectly by the formation of filipin–cholesterol complexes, whereas another subset(s) of wheat germ agglutinin receptors not required for agglutination could be unaffected. One of the latter subsets would be necessary for the agglutination of 3T3 cells with wheat germ agglutinin. This argument would also apply to secondary events such as the involvement of submembraneous elements in cell agglutination (Ash et al., 1977).

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References

Ash, J. F., Louvard, D., & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5584.

Benjamin, T. L., & Burger, M. M. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 929.

Bittman, R., & Fischkoff, S. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3795.

Bittman, R., Chen, W. C., & Anderson, O. R. (1974) *Biochemistry 13*, 1364.

Bloch, R., & Burger, M. M. (1974) *Biochem. Biophys. Res. Commun. 58*, 13.

Brenneman, D. E., McGee, R., & Spector, A. A. (1974) *Cancer Res., 34*, 2605.

Burger, M. M. (1974) *Methods Enzymol. 32*, 615.

Burger, M. M., & Goldberg, A. R. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 359.

de Kruiff, B., Gerritsen, W. J., Oerlemans, A., Van Dijck, P. W. M., Demel, R. A., & Van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 339*, 44.

Dennis, V. W., Stead, N. W., & Andreoli, T. E. (1970) *J. Gen. Physiol. 55*, 375.

Drabikowski, W., Lagwinska, E., & Sarzala, M. G. (1973) *Biochim. Biophys. Acta 291*, 61.

Eckhart, W., Dulbecco, R., & Burger, M. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1442.

Hamilton-Miller, J. M. T. (1973) *Bacteriol. Rev. 37*, 166.

Hatten, M. E. (1975) Ph.D. Thesis, Princeton University.

Hatten, M. E., Scandella, C. J., Horwitz, A. F., & Burger, M. M. (1978) *J. Biol. Chem. 253*, 1972.

Horwitz, A. F., Hatten, M. E., & Burger, M. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3115.

Howard, B. V., & Howard, W. J. (1975) *Prog. Biochem. Pharmacol. 10*, 135.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314.

Inbar, M., & Sachs, L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1418.

Kinsky, S. C. (1970) *Annu. Rev. Pharmacol. 10*, 119.

Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A., & Van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 152*, 174.

Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alving, C. R., & Kinsky, C. R. (1969) *Biochemistry 8*, 4149.

Mondovi, B., Strom, R., Agro, A. F., Caiata, P., Sole, P. D., Bozzi, A., Rotilio, G., & Fanelli, A. R. (1971) *Cancer Res. 31*, 505.

Noonan, K. D., & Burger, M. M. (1973) *Exp. Cell Res. 80*, 405.

Norman, A. W., Demel, R. A., DeKruijff, B., & Van Deenen, L. L. M. (1972a) *J. Biol. Chem. 247*, 1918.

Norman, W. W., Demel, R. A., DeKruijff, B., Geurts van Kessel, W. S. M., & Van Deenen, L. L. M. (1972b)

Biochim. Biophys. Acta 290, 1.
Norman, A. W., Spielvogel, A. M., & Wong, R. C. (1976) *Adv. Lipid Res. 14*, 127.
Puchwein, G., Pfeuffer, T., & Helmreich, E. J. M. (1975) *J. Biol. Chem. 249*, 3232.
Rapin, A., & Burger, M. M. (1974) *Adv. Cancer Res. 20*, 1.
Rothblatt, G. H., & Kritchevsky, D. (1968) *Exp. Mol. Pathol. 8*, 314.
Schroeder, F., Holland, J. F., & Bieber, L. L. (1972) *Biochemistry 11*, 3105.

Poly(riboadenylate)-Containing Messenger Ribonucleoprotein Particles of Chick Embryonic Muscles†

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ABSTRACT: Poly(A)-containing cytoplasmic messenger ribonucleoprotein (mRNP) particles were isolated from subcellular fractions of 12–14-day-old chick embryonic muscles by chromatography on oligo(dT)-cellulose and elution of the bound mRNP with 50% formamide. Two types of mRNP, free or nonpolysomal and polysome-derived, were obtained from the postpolysomal supernatant fraction and EDTA-dissociated polysomes, respectively. The mRNP were characterized by the absence of ribosomal RNAs and typical ribosomal proteins, by the presence of a limited number of characteristic proteins, and by the polydisperse sedimentation of the particles and their RNA moieties in sucrose gradients. Although the two types of particles show many similarities, their buoyant densities and $NaDodSO_4$–gel electrophoretograms of their protein moieties indicate that they represent two distinct types of macromolecular complexes. The free mRNP are relatively protein rich and contain 10 distinct proteins in the 40 000–100 000 molecular weight range. The polysome-derived mRNP are comparatively protein deficient and show two major proteins of about 52 000 and 78 000 daltons, which appear to be common to both classes of mRNP. The gel patterns of the poly(A)-associated proteins in both classes of mRNP are indistinguishable, suggesting that the complex protein patterns of the free mRNP are due to the presence of a set of additional proteins which are associated with the nonpoly(A) regions of their mRNA moieties. The presence of a major protein of 78 000 molecular weight in the poly(A)-protein fragments of both classes of mRNP suggests that the association of this protein to the poly(A) tracts is not influenced by translation. The distribution of pulse-labeled total poly(A)$^+$-RNA and two muscle-specific mRNAs, myosin heavy-chain mRNA and actin mRNA, between free mRNP and polysome fractions was about 35:65, respectively, and did not change during embryonic development between 11 and 17 days, suggesting that the two types of mRNP may exist in equilibrium in embryonic muscle cells. The possible relevance of these findings to a mechanism of translational control involving mRNP particles is discussed.

In eukaryotic cells mRNAs are complexed with proteins to form messenger ribonucleoprotein (mRNP)[1] particles (Perry & Kelley, 1968; Infante & Nemer, 1968; Henshaw, 1968; Kafatos, 1968; Cartouzou et al., 1969; Spohr et al., 1970, 1972; Olsnes, 1970; Lebleu et al., 1971; Jacobs-Lorena & Baglioni, 1972; Blobel, 1972, 1973; Bryan & Hayashi, 1973; Barrieux et al., 1975; Bag & Sarkar, 1975, 1976; Liautard et al., 1976; Jeffery, 1977; for a review see Spirin, 1969; Williamson, 1973; Greenberg, 1975). Two types of mRNP particles, polysome-derived mRNP, which are released by the dissociation of polyribosomes, and nonpolysomal free mRNP (also referred to as informosomes), have been reported in the literature. The polysomal mRNP particles derived from a large number of species and tissues contain two major proteins of about 52 000 and 78 000 molecular weight and a large number of additional polypeptides ranging usually from two to thirteen in the 16 000–150 000-dalton range (Lebleu et al., 1971; Blobel, 1973; Bryan & Hayashi, 1973; Barrieux et al., 1975; Burns & Williamson, 1975; Chen et al., 1976; Gander et al., 1973; Gedamu et al., 1977; Irwin et al., 1975; Lindberg & Sundquist, 1974; Kumar & Pederson, 1975; Morel et al., 1973; for a review see Greenberg, 1975). Although the reported variations in the size and number of the protein components of eukaryotic polysomal mRNP particles remain a matter of considerable dispute (Williamson, 1973; Greenberg, 1975), the widespread occurrence of the two major proteins of molecular weights 52 000 and 78 000 strongly suggests that they may be specifically associated with nucleotide sequences which are common to most mRNA molecules. With regard to the interrelationship of the protein moieties of free and polysomal mRNP particles, the few published reports in the literature have led to highly conflicting conclusions. Some of these studies indicate that the protein components of the two classes of mRNP, as judged by sodium dodecyl sulfate

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[1] Abbreviations used: mRNP, messenger ribonucleoprotein; poly(A), poly(riboadenylate); A_{260} unit, the quantity of material contained in 1 mL of a solution which has an absorbance of 1 at 260 nm when measured in a cell of 1-cm path length; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; hnRNP, heterogeneous nuclear ribonucleoprotein; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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