many other cell membranes (*2*). On the other hand, following up a report that ferritin-conjugated antibodies directed to blood group A antigen produce endocytosis in neonatal human erythrocytes, but not in adult cells (*3*), Schekman and Singer (*4*) have shown that the lateral mobilities of several receptors and antigens in the membrane of intact neonatal cells are greater than those for adult cells. For example, by the use of ferritin conjugates of concanavalin A, they showed that adding concanavalin A to neonatal mature erythrocytes causes a clustering in the plane of the membrane of the concanavalin A bound to its membrane receptors. The clustering was followed by endocytosis of the bound receptors. With adult mature erythrocytes, however, no such clustering or endocytosis was observed.

One possible explanation for this remarkable difference in mobility of receptors in the two membranes is that the lipid viscosity might be considerably less in the membrane of the neonatal cell than in the adult cell. Accordingly, we have measured the apparent lipid viscosity in the isolated membranes of the two cell types by a fluorescence polarization technique using the fluorescent probe, perylene (*5*, *6*). No significant difference between the two membranes was observed.

Adult erythrocytes were obtained from outdated blood from the San Diego Blood Bank and neonatal cells from cord blood immediately after a normal delivery. Erythrocyte ghosts were prepared from well-washed cells from which the buffy coat layer had been removed by lysis in 17 m*M* NaCl, $10^{-4}M$ EDTA, 0.5 m*M* tris-HCl, *p*H 7.4 (buffer A). The ghosts were centrifuged three times in this buffer in the cold and were used within 2 days. To erythrocyte ghosts at a concentration of about 1.5×10^8 ghost/ml in buffer A we added a $4 \times 10^{-4}M$ solution of perylene in ethanol to a final concentration of $4 \times 10^{-7}M$. Under these conditions the mole ratio of lipid to perylene in the suspension was greater than 500, which, according to other measurements we have made, eliminates the complicating effects of depolarization by excitation energy transfer between perylene molecules. After about 30 minutes, the fluorescence increase accompanying the absorption of perylene into the membranes reached equilibrium. Measurements of the intensity of polarized fluorescence were then immediately made with a sensitive spectrofluorimeter constructed in this laboratory (*7*). The cell suspension was excited at 404 nm with light polarized in a vertical direction. The emitted light was detected by two photomultiplier tubes, one of which measured the vertical component I_{VV} of polarized emission intensity while the other measured the horizontal component I_{VH}. The recording system gave digital readings of the values I_{VV}, I_{VH}, and $R = I_{VH}/I_{VV}$. The fluorescence anisotropy function A, a measure of the average angle through which the perylene molecules rotate during their fluorescent lifetime τ, was calculated from the expression

$$A = \frac{(1 - R) - (I_{VV}^S/I_{VV})(1 - R^S)}{(1 + 2R) + (I_{VV}^S/I_{VV})(1 + R^S)}$$

which includes correction factors for scattered light (*5*). The quantities with the superscript S were measured using an unlabeled cell suspension as a scattering blank. Membrane microviscosity was calculated from the anisotropy and lifetime (τ) of perylene by means of a calibration graph obtained with oils of known viscosity by the procedure of Cogan *et al.* (*6*). Lifetimes were measured with an instrument described elsewhere (*8*).

There was no difference in the microviscosities of neonatal and adult erythrocytes as measured by perylene (Table 1). The measured anisotropies are similar to those reported by Rudy and Gitler for the erythrocyte membrane at 37°C (*9*).

In studies such as this, perylene presumably serves as a probe for the fluidity in the interior hydrocarbon region of the membrane bilayer (*5*). It is conceivable that there are differences in the fluidity of surface regions of the membranes of neonatal and adult erythrocyte that are not measured with perylene. Nevertheless, any change in the dynamic properties of a lipid bilayer large enough to markedly alter the lateral mobilities of receptors in the membrane would probably be sensed by perylene.

In view of the evidence in this report, and of other data, Schekman and Singer (*4*) have proposed that the difference in lateral mobilities of receptors in the two different membranes is attributable to a different state of the spectrin complex attached to the cytoplasmic surfaces of the two membranes.

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References and Notes

1. F. Loor, L. Forni, B. Pernis, *Eur. J. Immunol.* **2**, 203 (1972); S. J. Singer, *Adv. Immunol.* **19**, 1 (1974).
2. M. Edidin, *Ann. Rev. Biophys. Bioeng.* **3**, 179 (1974).
3. P. L. Blanton, J. Martin, S. Haberman, *J. Cell Biol.* **37**, 716 (1968).
4. R. Schekman and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4075 (1976).
5. M. Shinitzky, A. C. Dianoux, C. Gitler, G. Weber, *Biochemistry* **10**, 2106 (1971).
6. V. Cogan, M. Shinitzky, G. Weber, T. Nishida, *ibid.* **12**, 521 (1973).
7. J. Yguerabide, unpublished results.
8. _____ and L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1217 (1971).
9. B. Rudy and C. Gitler, *Biochim. Biophys. Acta* **307**, 491 (1972).
10. Supported by PHS grants AI-06659 and GM-15971 to S.J.S. and PHS grant EY-01177 to J.Y. We thank E. Yguerabide for assistance in this work.

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13-*cis*-Retinoic Acid: Inhibition of Bladder Carcinogenesis in the Rat

Abstract. *Transitional cell and squamous cell cancer of the bladder was induced in Wistar/Lewis female rats by direct instillation of* N-*methyl*-N-*nitrosourea into the bladder. Feeding of the synthetic retinoid, 13-*cis*-retinoic acid, inhibited the incidence and extent of bladder cancer in these rats, even when 13-*cis*-retinoic acid administration was begun after completion of the carcinogen treatment.*

Bladder cancer in man continues to be a problem of major importance since death rates from this disease have not decreased appreciably within the past 20 years (*1*). The development of several animal models for the human disease, which is primarily transitional cell carcinoma, now allows intensive studies of agents which might be of use in prevention of bladder cancer (*2*, *3*). Recent studies have shown that synthetic analogs of vitamin A (retinoids) (*4*) may be used to prevent epithelial cancer of the skin, respiratory tract, and mammary gland in experimental animals (*5*). We therefore wished to see if these studies could be extended to prevention of cancer in bladder epithelium, which is known to depend on retinoids for maintenance of normal cellular differentiation (*6*). It has been previously reported that dietary deficiency of retinoids greatly increases susceptibility of bladder epithelium to chemical carcinogenesis (*7*). However, in these same experiments, feeding of high doses of natural vitamin A ester (retinyl palmitate) did not afford any protection from carcinogenesis (*7*). Therefore, for the present study we chose a different type of retinoid, which has pharmacokinetic proper-

Table 1. Inhibition of bladder carcinogenesis by 13-*cis*-retinoic acid. Under light pentobarbital anesthesia and sterile conditions, female Wistar/Lewis rats (Microbiological Associates), ages 6 to 7 weeks, were given either three biweekly doses, each of 1.5 mg of NMU (Ash Stevens Inc., Detroit, Michigan) in 0.15 ml of saline (groups A to E), or three doses of saline alone (groups F to H). 13-*cis*-Retinoic acid (Hoffmann–La Roche) was obtained as a gelatinized beadlet preparation and blended into the diet, which was fed continuously, starting with the first instillation of NMU or saline (groups B, C, G, and H) or 1 day after the last NMU instillation (groups D and E). Groups A and F were fed diet containing the control gelatinized beadlet material without retinoid, at same level fed to groups C, E, and H. All animals were given free access to the food. Total bladder neoplasms included carcinomas and noninvasive transitional cell papillomas. The diagnoses of transitional cell or squamous carcinoma were based on the presence of invasion of underlying connective tissue or invasion of smooth muscle and of moderate to marked histologic or cytologic atypia. Logistic regression tests (*13*) were performed, comparing the incidences of the various neoplasms in groups A to E. Since a decreasing trend of incidence was expected with increasing dose of 13-*cis*-retinoic acid, one-sided statistical procedures were used.

Group	Rats (No.)	Daily dose of 13-*cis*-retinoic acid (mg/kg of diet)	Bladder neoplasms		Transitional or squamous cell carcinoma		Squamous bladder carcinoma	
			Rats (No.)*	Bladder areas (No.)†	Rats (No.)‡	Areas (No.)*	Rats (No.)‡	Areas (No.)§
A	23	None	13 (57%)	30/69 (43%)	10 (43%)	20/69 (29%)	4 (17%)	6/69 (9%)
B	21	120	11 (52%)	18/63 (29%)	7 (33%)	12/63 (19%)	4 (19%)	4/63 (6%)
C	23	300	8 (35%)	13/69 (19%)	6 (26%)	9/69 (13%)	2 (9%)	2/69 (3%)
D	23	120	8 (35%)	13/68 (19%)	8 (35%)	13/68 (19%)	1 (4%)	1/68 (1%)
E	20	300	8 (40%)	10/58 (17%)	6 (30%)	7/58 (12%)	1 (5%)	1/58 (2%)
F	7	None	0	0	0	0	0	0
G	7	120	0	0	0	0	0	0
H	7	300	0	0	0	0	0	0

*P = .07. †P = .02. ‡P > .10. §P = .08.

ties entirely different from those of retinyl esters. In contrast to retinyl esters, it is well established that a retinoid such as retinoic acid is not stored in the liver (*8*) and is not transported in the blood by retinol-binding protein (*9*). Retinoic acid thus has an entirely different pattern of tissue distribution than retinyl esters. The synthetic analog, 13-*cis*-retinoic acid, was chosen for the present studies since it had been established that it could be fed to animals for long periods of time in relatively high dose without evident toxicity, as would be caused by all-*trans*-retinoic acid (*5*, *10*).

In our present experiments (Table 1), bladder cancer was induced in rats by direct instillation of *N*-methyl-*N*-nitrosourea (NMU) into the urinary bladder (*3*). Three doses, each 1.5 mg, were given at biweekly intervals to five groups of 23 rats each (groups A to E). Group A was fed powdered commercial laboratory chow (Purina) for the duration of the experiment. Groups B and C were fed 13-*cis*-retinoic acid, blended in the above diet at levels of 120 mg and 300 mg per kilogram of diet, respectively, starting at the time of the first instillation of NMU. Groups D and E were likewise fed 13-*cis*-retinoic acid, 120 mg and 300 mg per kilogram of diet, respectively; but in these groups treatment with retinoid was not started until 1 day after the three treatments with carcinogen had been completed. All surviving rats were killed 9 months after the initial dose of carcinogen. Their bladders were inflated with 10 percent formalin, and a ligature was placed around the neck of the bladder. After fixation, each bladder was sliced with two transverse cuts to yield an anterior dome-shaped area, a median open-cylindrical area, and a posterior cup-shaped area. Serial transverse sections (5 μm) were cut from each of the three areas and stained with hematoxylin and eosin. Thus, each bladder yielded three separate standardized areas for histological evaluation, and the extent of neoplastic involvement of the bladder as a whole could be evaluated (Table 1). For pathological diagnosis, slides were renumbered from a table or random numbers, taking one slide consecutively from each of the five experimental groups.

All slides were coded and evaluated by one of us (R.A.S.) for neoplastic and preneoplastic changes, and the results are presented in Table 1, which shows the distribution of carcinomas and noninvasive transitional cell papillomas in the various groups. The data indicate that 13-*cis*-retinoic acid inhibited the development of neoplasia of bladder epithelium. The retinoid had a particularly marked effect on the extent of neoplastic development in the bladder, since the number of areas of the bladder that had papillomas or carcinomas was significantly diminished by 13-*cis*-retinoic acid. In groups D and E, this inhibitory effect of 13-*cis*-retinoic acid was independent of any effects on initiation of cancer since the retinoid was not started until after all free carcinogen had disappeared. The half-life of NMU in aqueous solution is only a few hours at neutral *p*H (*11*). Although 13-*cis*-retinoic acid influenced the development of the more predominant transitional cell papillomas and carcinomas caused by NMU, inhibitory effects on development of squamous cell carcinoma of bladder epithelium were also evident. In human bladder cancer, foci of squamous cell carcinoma are often found scattered throughout highly malignant transitional cell carcinomas (*12*). In addition to an effect on bladder neoplasms, 13-*cis*-retinoic acid diminished the presence and extent both of flat, proliferative lesions, which had varying degrees of hypercellularity and atypia, and of squamous metaplastic lesions. These lesions were graded on a scale of 1 to 5, and a statistical rank test (*13*) was made for decreasing the severity of lesions with increasing dose of 13-*cis*-retinoic acid. This evaluation showed that the retinoid had a highly significant ability ($P < .005$ for both types of lesion) to diminish the severity of these preneoplastic lesions.

The mechanism of the inhibition of bladder carcinogenesis by 13-*cis*-retinoic acid is not known, although the results were clearly not caused by some generalized toxic effect of 13-*cis*-retinoic acid. At the doses used, no manifestations of "hypervitaminosis A" (*14*) were seen, and the average weights of the rats fed 13-*cis*-retinoic acid were essentially identical to those fed chow diet alone. Moreover, bladder calculi were not seen in the inbred Wistar/Lewis female rats used in our experiments. In random-bred Wistar rats (*3*), NMU does cause stone formation and the presence of calculi has been a complicating factor in interpretation of bladder carcinogenesis studies (*15*). More positively, it is now known from organ culture studies that retinoids can arrest or reverse the progression of lesions

caused by chemical carcinogens in prostatic and tracheal epithelium (*16*). Whether retinoids can directly modify the development of preneoplastic lesions in bladder epithelium in vivo is not known; nor is it known whether 13-*cis*-retinoic acid has an effect on endocrine or immune mechanisms that might modulate bladder carcinogenesis. However, the recent development of an organ culture system in which both normal and carcinogen-treated bladder epithelium can be maintained in vitro (*17*) should further our understanding of the mechanism or mechanisms whereby 13-*cis*-retinoic acid inhibits bladder carcinogenesis.

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References and Notes

1. E. Silverberg and A. I. Holleb, *Ca 25*, 2 (1975).
2. E. Erturk, S. M. Cohen, J. M. Price, G. T. Bryan, *Cancer Res.* **29**, 2219 (1969); N. Ito, Y. Hiasa, A. Tamai, E. Okajima, H. Kitamura, *Gann* **60**, 401 (1969).
3. R. M. Hicks and J. S. Wakefield, *Chem.-Biol. Interact.* **5**, 139 (1972).
4. The word "retinoids" has been introduced to include the set of molecules comprised of natural forms of vitamin A and their synthetic analogs, in a manner analogous to the naming of carotenoids or steroids [M. B. Sporn, N. M. Dunlop, D. L. Newton, J. M. Smith, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1332 (1976)].
5. W. Bollag, *Eur. J. Cancer* **10**, 731 (1974); *Chemotherapy (Basel)* **21**, 236 (1975); C. D. Port, M. B. Sporn, D. G. Kaufman, *Proc. Am. Assoc. Cancer Res.* **16**, 21 (1975); C. J. Grubbs, R. C. Moon, M. B. Sporn, *ibid.* **17**, 68 (1976).
6. S. B. Wolbach and Pr. R. Howe, *J. Exp. Med.* **42**, 753 (1925); R. M. Hicks, *Biol. Rev.* **50**, 215 (1975).
7. S. M. Cohen, J. F. Wittenberg, G. T. Bryan, *Cancer Res.* **36**, 2334 (1976).
8. J. E. Dowling and G. Wald, *Proc. Natl. Acad. Sci. U.S.A.* **46**, 587 (1960).
9. J. E. Smith, P. O. Milch, Y. Muto, D. S. Goodman, *Biochem. J.* **132**, 821 (1973).
10. P. Nettesheim and M. L. Williams, *Int. J. Cancer* **17**, 351 (1976).
11. H. Druckrey, R. Preussmann, S. Ivankovic, D. Schmähl, *Z. Krebsforsch.* **69**, 103 (1967).
12. G. R. Prout, Jr., in *Cancer Medicine*, J. F. Holland and E. Frei, Eds. (Lea & Febiger, Philadelphia, 1973), pp. 1670–1680.
13. See D. R. Cox, *Analysis of Binary Data* (Methuen, London, 1970); E. L. Lehmann, *Nonparametrics: Statistical Methods Based on Ranks* (Holden-Day, San Francisco, 1975).
14. T. Moore, in *The Vitamins*, W. H. Sebrell and R. S. Harris, Eds. (Academic Press, New York, ed. 2, 1967), vol. 1, pp. 280–294.
15. D. B. Clayson and E. H. Cooper, *Adv. Cancer Res.* **13**, 271 (1970).
16. I. Lasnitzki, *Br. J. Cancer* **9**, 434 (1955); _____ and D. S. Goodman, *Cancer Res.* **34**, 1564 (1974); D. P. Chopra and L. J. Wilkoff, *J. Natl. Cancer Inst.* **56**, 583 (1976); B. P. Lane and S. L. Miller, *Proc. Am. Assoc. Cancer Res.* **17**, 211 (1976).
17. D. H. Reese, R. D. Friedman, J. M. Smith, M. B. Sporn, *Cancer Res.* **36**, 2525 (1976).
18. We thank Hoffmann–La Roche Inc. for 13-*cis*-retinoic acid, D. Feeser for assistance with instillation of NMU, and D. Overman for help with data recording.

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Liposome-Cell Interaction: Transfer and Intracellular Release of a Trapped Fluorescent Marker

Abstract. *When small, unilamellar lipid vesicles containing a high concentration of the fluorescent dye 6-carboxyfluorescein are incubated with either frog retinas or human lymphocytes, fluorescence distributes widely throughout each cell. Since "self-quenching" largely prevents the dye from fluorescing as long as it remains sequestered in vesicles, it is clear that a considerable amount of dye is released from the vesicles and diluted into the much larger volume of the cell.*

In recent years liposomes have been considered as vehicles for introducing membrane-impermeant substances into cells, either by membrane fusion or by phagocytosis. The list of substances whose injection has been attempted includes antitumor drugs (*1*, *2*), chelating agents (*3*), enzymes (*4*), hormones (*5*), cyclic nucleotides (*6*), and polynucleotides (*7*). Some of these studies have been done with cell suspensions or with tissue cultures, others with isolated tissues or whole animals, including human subjects (*2*). However, basic uncertainties remain about the mechanisms of interaction between liposomes and cells, in particular about the intracellular fate of incorporated material (*8*, *9*). In one study cyclic adenosine monophosphate trapped in small, unilamellar liposomes (vesicles) was reported to slow the growth of tissue culture cells (*6*). Some nucleotide was evidently released into the cytoplasmic space, but its distribution there was not determined directly, and the amount remaining sequestered in cell-associated vesicles could not be ascertained. Clearly a rapid and simple method is needed to determine the distribution in cells of substances whose injection from vesicles is being attempted. We now report results of a sensitive fluorescence technique that distinguishes between material still remaining in vesicles and that released intracellularly. The intracellular distribution of released material is determined by conventional fluorescence microscopy.

Small vesicles containing the water-soluble fluorophore 6-carboxyfluorescein (6-CF) were prepared either by sonication of a phospholipid suspension (*10*) or by injection of an ethanolic solution of lipid into an aqueous solution (*11*). The two approaches gave qualitatively similar results in studies of incorporation by cells. In the most frequently used protocol 25 mg of either dioleoyl (DOL) or dipalmitoyl (DPL) lecithin (Applied Science) was hydrated with 4 ml of a 200 m*M* aqueous solution (*p*H 7.4) of recrystallized 6-CF (Eastman). The suspension was mixed by vortexing for several minutes, then sonicated for approximately ten times the period required for optical clarification (*12*). Free 6-CF was removed by passing the sonicate through a short column (9 by 200 mm) of Sephadex G-25 at 5°C, with 135 m*M* NaCl (buffered to *p*H 7.4 with 10 m*M* Hepes) as eluant. Vesicles emerged in the void volume, whereas free dye was retarded by the gel. Lipid purity was assessed by thin-layer chromatography and gas-liquid chromatography (*13*). Quasi-elastic laser light scattering on DOL vesicles (*14*) showed more than 95 percent of the lipid in the vesicle suspension to be in the form of particles 250 to 350 Å in diameter (presumably unilamellar vesicles), and calculations based on the ratio of phospholipid to 6-CF gave an average value of about 250 Å for the vesicle diameter (*15*), assuming that the vesicles did indeed contain dye at the original 200 m*M* concentration. 6-Carboxyfluorescein closely resembles fluorescein itself in spectral properties (excitation maximum, 490 nm; emission maximum, 520 nm) but was chosen for these studies because it is more polar than fluorescein and leaks out of vesicles more slowly (*16*).

A vital phenomenon for our purposes is the "self-quenching" commonly seen with fluorescent systems. In dilute solutions of 6-CF the fluorescence is proportional to the number of dye molecules present, but as concentrations are raised above about 10 m*M* the yield per molecule drops off rapidly because of interaction between fluorophore molecules. Thus, a suspension of vesicles containing 200 m*M* 6-CF fluoresces only slightly, but the fluorescence increases more than 30-fold when dye is released (for example, by detergent) and diluted into the entire solution volume. Self-quenching thus allows material remaining in vesicles to be distinguished from that released and diluted into the much larger volume of a cell. To appreciate the magnitude of the dilution factor, consider that the internal volume of a vesicle 300 Å in diameter is about 4×10^{-18} cm^3 and that of a lymphocyte is about 2×10^{-10} cm^3 (*17*). Release into a lymphocyte of the contents of one ves-