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IONIC TRANSPORT THROUGH MODEL MEMBRANES

II. FUNCTION OF CHOLESTEROL AND STEROID HORMONES

A. WESLEY HORTON, ALLAN R. SCHUFF and DAVID W. McCLURE

Department of Biochemistry and Division of Environmental Medicine, University of Oregon Medical School, Portland, Oreg. 97201 (U.S.A.)

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SUMMARY

1. The kinetics of transfer of a water-soluble azo dye, Orange IV, from an aqueous into phospholipid micelles in an adjacent organic phase have been investigated as a method of evaluating the possible effects of various hydrocarbons on the structure of the lipid micelles and interfacial films. The transport of this dyestuff probe apparently requires the presence of unsaturated alkyl chains in the lipid, provided by egg phosphatidylcholine or by synthetic lecithin (1-oleyl-2-hexadecyl ether).

2. The kinetics had previously been shown to be retarded by those *n*-alkanes that are promoters of carcinogenesis. On the basis of this correlation it had been predicted *n*-$C_{18}H_{38}$ and *n*-$C_{20}H_{42}$ should be the most active biologically of the *n*-alkanes. This has been confirmed in detail by biological tests for promoting activity.

3. Inclusion of cholesterol and various steroid metabolites, including androgens and progestogens, had profound effects on the rate of dye transport by egg phosphatidylcholine (very little transfer to the bulk organic phase was achieved by the steroids without the phospholipid). Cholesterol had the maximum retarding effect, but increased somewhat the ultimate equilibrium transfer of dye to the micelles in the organic phase.

4. The C_{19} steroids, dihydrotestosterone, testosterone, androsterone, and epiandrosterone enhanced the rate of transport by lecithin–cholesterol micelles in different critical ranges of concentration. The four steriods were thus ranked in the same order of effective concentration in the model transport system as they are in assays of their biological activities as androgens. The metabolic intermediate, pregnenolone, affected the transport kinetics similarly to testosterone. In contrast, the C_{21} hormone, progesterone, and the estrogen analog, diethylstilbestrol, retarded ionic transport in this model.

INTRODUCTION

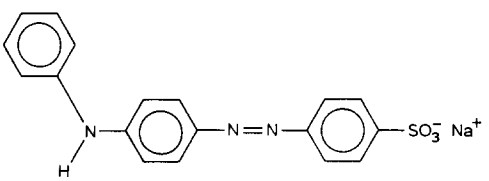
Cholesterol is perhaps the most ubiquitous organic molecular species in the lipoprotein membranes of mammalian cells[1]. However, its function has remained a matter of speculation. Perhaps the most acceptable hypothesis has been that of Chapman to the effect that the presence of cholesterol causes the hydrocarbon chains of

differing phospholipids to be in an "intermediate fluid" condition permitting some latitude in the length and degree of unsaturation of the alkyl chains[2].

We have introduced cholesterol into the carrier micelles of a model transport system, described earlier,[3] and found that it has a profound retarding effect on the kinetics of ionic transport in this physical model, when it is the only sterol present. This paper will focus on the physical–chemical characteristics of the model system and their dependence on the structure of the steroids introduced into the phospholipid micelles.

It will be shown that micelles formed of the combination of egg phosphatidylcholine and cholesterol provide one end of the interfacial permeability scale (the low end), but that it requires combinations with certain more polar steroids to achieve high rates of ionic transport across the model interface employed. Certain of the sex hormones and metabolic intermediates have served as useful models of the more polar steroids. There is growing indication that the properties they exhibit in this system may be relevant to some of their functions as hormones.

The model transport system remains essentially the same as that previously described. The model simulates transfer of organic ions across one cytoplasmic interface of the biological membrane. Briefly, this physical system involves the phospholipid-facilitated transfer of the water-soluble dyestuff, Orange IV



from a bulk aqueous phase across a liquid–liquid interface into lipid micelles in a bulk hydrocarbon phase, decahydronaphthalene (presence of micelles, mol. wt $2.2 \cdot 10^6$, shown by light scattering. (Horton, A. W. and Schuff, A. R., unpublished)).

The dyestuff acts in effect as a probe to investigate these lipid structures. The rate of its adsorption to the interface will be seen to be a function of the lipid composition. Further, the second step involving the transformation of the lipid aggregates in the interfacial state to those in the bulk organic phase will be shown to be a very sensitive function of the structure and concentration of the steroids added to the phosphatidylcholine solution.

Certain predictions of the first paper based on a correlation of the physical and biological properties of normal alkanes have been subjected to experimental test on mice. In general it had been found that the *n*-alkanes that are promoters of skin carcinogenesis retard anionic transport when introduced into the physical model. On the basis of quantitative differences in their effects in the physical system, it had been predicted that the peak of promoting activity *in vivo* would be for the C_{18} and C_{20} chainlengths. The biological tests (which will be reported elsewhere) have confirmed this prediction in detail and have shown that as little as 10% *n*-octadecane has significant promoting activity for skin carcinogenesis.

MATERIALS AND METHODS

Egg phosphatidylcholine and dipalmitoylphosphatidylcholine, General Biochemicals. Synthetic DL-lecithin (1-oleyl,2-hexadecyl ether), Calbiochem. Orange IV, sodium *p*-(*p*-anilinophenylazo)-benzene sulfonate, Matheson, Coleman and Bell, recrystallized twice from doubly distilled water and once from 95% ethanol. Aqueous phase, 134 mM KCl, 1 mM phosphate buffer [MCB BX1635-5, pH 7 concentrate, 3.2 ml diluted in 1 l doubly distilled (glass) water]. *Cis*- and *trans*-decalin, Matheson, Coleman and Bell, practical grade, chromatographed on activated silica gel to remove impurities, especially those responsible for ultraviolet absorbance at 260–280 nm (*e.g.* tetralin).

Cholesterol, Merck, USP, triply recrystallized from methanol. Dihydrocholesterol, Nutritional Biochemicals; testosterone, dihydrotestosterone, progesterone, pregnenolone (Δ^5-3-β-ol-20-one), epiandrosterone, and dehydroepiandrosterone, Schwarz-Mann; androsterone, K and K Laboratories and Schwarz-Mann.

The purity of all steroids was confirmed by thin-layer chromatography on silica gel using *n*-hexane–ethyl acetate (70:30, v/v) as the developing solvent.

Experimental procedure

The procedure employed to study the effects of the added materials on the rate of transport is essentially identical to that described in the previous paper[3]. The concentration of egg phosphatidylcholine in the organic phase is reduced to 0.3 mM to minimize the undesirable stabilization of emulsion sometimes resulting from higher concentrations. Mixed micelles of lecithin *plus* steroids are allowed to equilibrate in the decalin solution in the 50-ml round bottomed flask for 2 h before contact with the aqueous dye solution.

The azo dye (40 μM) is dissolved in the buffered KCl solution and then shaken with an equal volume of the equilibrated solution of lipids in decalin for specified periods of time at a temperature of 25 ± 3 °C. The emulsions are broken by centrifugation at $1700 \times g$ and the absorbance of the clarified phase determined in a Beckman DB spectrophotometer.

After centrifugation of the emulsion is completed the recovered volumes are determined in graduated cylinders and the volume of residual emulsion estimated (frequently but not always zero.) The actual recovery of dyestuff in the clarified aqueous and organic phases is calculated on the basis of their absorbance at the maximum in the region of 410–440 nm and the volume of each phase recovered. Conformance to Beer's Law in the range of concentrations employed has been demonstrated. The distribution of dyestuff is reported as the percent of the original quantity that has disappeared from the aqueous phase after specified periods of mixing, the percent found in the organic phase, and the percent remaining at the interfaces (by difference). Any material remaining in the unbroken emulsion is therefore included in the percent accounted for as remaining at an interface.

RESULTS

In Fig. 1 is presented a detail comparison of the kinetics of transport in the basic systems involving egg phosphatidylcholine micelles (0.3 mM) alone and in com-

bination with resrystallized cholesterol. From examination of the results in Fig. 1, Table I, and the lower curve of Fig. 3, two conclusions may be drawn immediately. Cholesterol retards both the adsorption of dyestuff to the interface and its transfer to the bulk organic phase. However, at concentrations of cholesterol up to about 21 mM (in the bulk organic phase) the equilibrium is shifted to practically complete

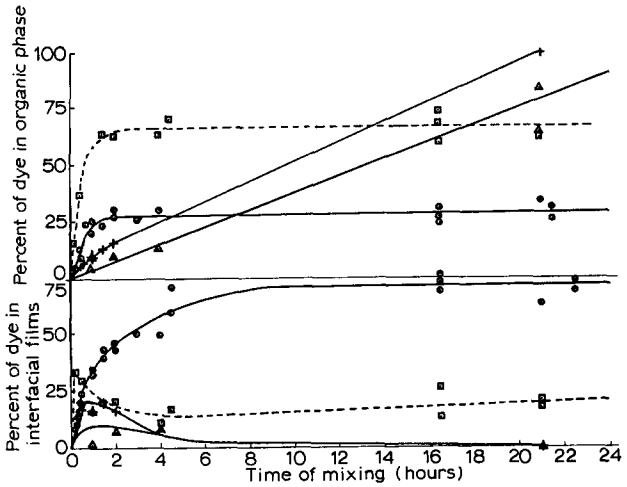


Fig. 1. Effect of cholesterol on rate of anionic transport by egg lecithin (0.3 mM). ⊡---⊡, transport with lecithin alone; △—△, transport with 6 mM cholesterol; +—+, transport with 15 mM cholesterol; ⊙—⊙, transport with 30 mM cholesterol.

TABLE I

EFFECT OF CHOLESTEROL ON IONIC TRANSPORT BY EGG LECITHIN

Lipid composition (mM) in organic phase		*Distribution of dyestuff after 21-h mixing*		
Egg lecithin	*Cholesterol*	*Organic phase* (%)	*Disappearance from aqueous phase* (%)	*Interfacial films* (%)
0.3	0	63	80	18
0.3	0	63	84	21
0.3	2	75	86	11
0.3	4	80	80	0
0.3	6	72	69	1
0.3	9	100	96	0
0.3	12	95	89	0
0.3	15	100	95	0
0.3	18	86	91	5
0.3	21	96	93	0
0.3	24	38	95	57
0.3	27	32	95	63
0.3	30	34	96	63

transfer of dye to the organic phase. At higher concentrations an abrupt decrease in the transformation of interfacially adsorbed dye to the bulk organic phase is seen.

With few exceptions, cholesterol itself among all the sterols studied has the greatest retarding effects on the kinetics in the model transport system. Saturation of the B ring of cholesterol did not alter its effects on the system.

It should be noted that cholesterol is by far the most soluble of the sterols studied in this system. Solutions containing lecithin and up to 200 mM cholesterol in decalin have been prepared and are stable at 25 °C. In Table II are shown the solubilities of various steroids in a solution of 0.3 mM egg lecithin in decalin. These solubilities are generally about 1 mM greater than in decalin in the absence of the lecithin micelles. Light scattering studies indicate that cholesterol forms comparatively small micelles in decalin, mol. wt approx. 2300 (Horton, A.W. and Schuff, A.R., unpublished).

TABLE II

STEROID SOLUBILITIES (mM) IN 0.3 mM EGG LECITHIN/DECALIN AT 22 °C

		Solubility in presence of equimolar cholesterol
Cholesterol	> 200	
Dihydrocholesterol	> 50	
Epoxycholesterol		10
7-Ketocholesterol		> 15
Pregnenolone	4	> 5
Progesterone	45	
Testosterone	10	12
Dihydrotestosterone		> 10
Androsterone	8	10
Epiandrosterone		13
Dehydroepiandrosterone	15	
Diethylstilbestrol	1	
Estrone	0.17	
Estradiol	0.3	

For ready reference the structures of the principal steroids used are shown in Fig. 2. All have the *trans* configuration of rings A/B and, exept for cholesterol and progesterone, contain keto and secondary hydroxyl groups situated essentially at opposite ends of the molecule.

Most of the comparisons of the effects of the various steroids that follow will be based upon the rate of transfer of the dye to the clarified bulk organic phase. Examination of the upper set of curves in Fig. 1 indicates that perhaps the most useful comparison of difference in transport rates associated with the different compositions can be made by comparing the percent of the dye transferred to the organic phase in one hour of mixing.

In Fig. 3 the effects of addition of some C_{19} and C_{21} steroids to the system are compared with that of cholesterol and lecithin. The response curve for the combination of cholesterol and the C_{19} androsterone is included in Fig. 3 to show the close

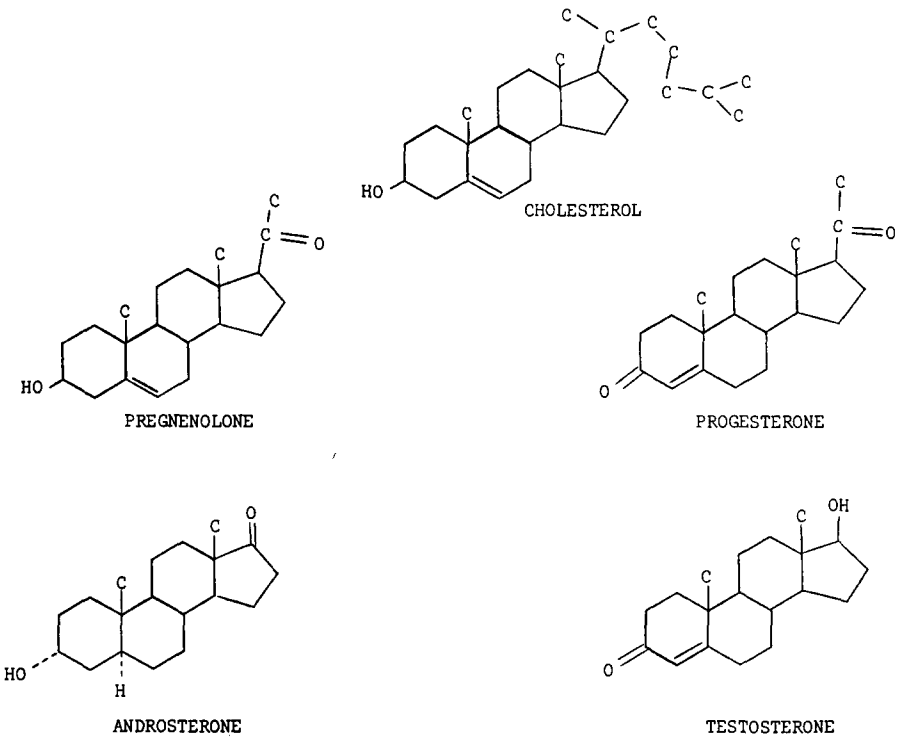


Fig. 2. Molecular structure of steroids.

parallel between the effects of the androgen and of progesterone at concentrations up to 6 mM and the wide divergence at higher concentrations. Similar parallels have been noted in the behaviour of the androgen and the progestagen (at 3–7 mM) in systems containing 20–30 mM cholesterol.

In Fig. 4 are shown the effects of the C_{19} androgens on the transport properties of the lecithin–cholesterol micelles. Again the pattern obtained with the hydroxyketonepregnenolone occurs but the concentration of polar steroid required to produce a maximum rate of transport varies with its structure. In general, the optimal concentrations of the various androgens rank in the same order as their activities in certain biological assay systems (see Discussion).

Similar differences between the effects of the androgens and progesterone may be shown without the inclusion of cholesterol in the system. However, the enhancement of transport rates by the androgens is generally increased by equimolar cholesterol. Progesterone still retards transport in the absence of cholesterol, but to a lesser extent.

Insufficient data are available at this time on the effects of estrogens on ionic transport by egg lecithin. The preliminary picture using the synthetic analog, diethylstilbestrol, is that equilibrium is reached very rapidly, but that interfacial adsorption of the dyestuff probe dominates. The transfer to the micelles in the bulk organic phase in 1 h parallels that for progesterone (Fig. 3), but the concentration of diethylstilbestrol required for a given percent transfer is only 1/10 that of progesterone.

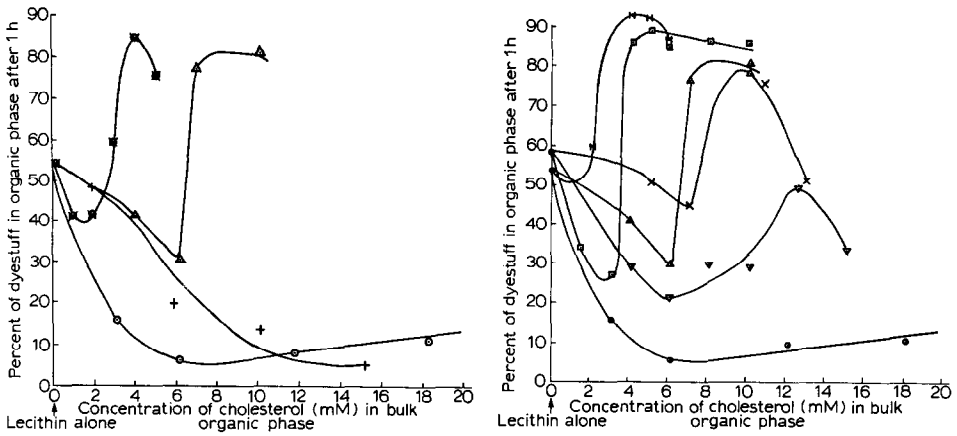


Fig. 3. Effect of equimolar cholesterol and steroids on rate of ionic transport by egg lecithin (0.3 mM). △—△, transport with equimolar androsterone and cholesterol; ⊗—⊗, transport with equimolar pregnenolone and cholesterol; +—+, transport with equimolar progesterone and cholesterol; ⊙—⊙, transport with lecithin and cholesterol.

Fig. 4. Effects of equimolar cholesterol *plus* androgens on rates of transport by egg lecithin (0.3 mM). ⊡—⊡, transport with testosterone and cholesterol; ▽—▽, transport with dehydroepiandrosterone and cholesterol; △—△, transport with androsterone and cholesterol; ⊙—⊙, transport with lecithin and cholesterol; ×—×, transport with epiandrosterone and cholesterol; |•|—|•|, transport with dihydrotestosterone and cholesterol.

TABLE III

IONIC TRANSPORT BY STEROIDS AND SYNTHETIC LECITHINS

Lipid composition (mM) in organic phase				*Time of mixing (min)*	*Distribution of dyestuff*		
Synthetic lecithin		*Steroids*			*Organic phase (%)*	*Disappearance from aqueous phase (%)*	*Interfacial films (%)*
Dipalmitoyl	*1-Oleyl, 2-hexadecyl diether*	*Testosterone*	*Cholesterol*				
0.1	0	0	0	20	0	6	6
0.1	0	0	0	60	0	6	6
0.1	0	0	0	180	0	9	9
0.1	0	6	0	20	0	11	11
0.1	0	6	0	60	0	11	11
0.1	0	6	0	180	0	16	16
0	0	6	9	10	0	31	31
0	0	6	9	30	0	31	31
0	0	6	9	90	0	31	31
0	0.3	0	0	60	19	56	37
0	0.3	0	6	60	12	27	15
0	0.3	6	0	60	53	87	34
0	0.3	6	6	60	73	96	23

That the unsaturated phospholipids are essential to the transport of dyestuff Orange IV is shown in Table III. The results of 3 series of experiments are presented involving, in the first series, the saturated synthetic dipalmitoylphosphatidylcholine at 0.1 mM (its solubility limit in decalin) alone and in combination with testosterone. In the second series, the effect of using a mixture of testosterone and cholesterol without phospholipid was investigated. In these two series of experiments some interaction with the interfacial lipid occurred but no transfer into the micelles in the bulk organic phase.

In contrast, the synthetic unsaturated lecithin did facilitate the transfer of the dyestuff to lipid micelles in the organic phase. However, the rate was much reduced as compared to that obtained using egg lecithin. The dye distribution after 60 min with the synthetic phospholipid was almost identical to that obtained in 10 min using the natural product. However, the responses to the addition of the two steroids to this synthetic lecithin were entirely parallel to those obtained with the mixture represented by egg lecithin.

DISCUSSION

What does this use of an anionic azo dye as a probe suggest about the normal function of cholesterol and its metabolites in cell or organelle membranes? Even though they are non-ionogenic, these steroids may play a critical role in determining the rate of phospholipid-facilitated diffusion of ionic substances between cellular "compartments". Cholesterol, the major membrane sterol, and dihydrocholesterol, a minor component, retard such ionic transport. An increase in the ordering of bilayers by cholesterol and a similar retarding effect on ionic and carbohydrate permeability of liposomes have been observed by other investigators of phospholipid model systems[4–8].

In contrast the hydroxyketonic steroids seem to increase the solubilization of the probe in the phospholipid mesophase, the extent depending upon the concentration and structure of the polar steroids. Thus the differing phospholipid–sterol compositions of the various intracellular membranes may exert their control of diffusion rates by determining the probability of a favorable orientation of the substrate in the micellar palisade.

Although the rate of adsorption of dye into the interfacial lipid film is reduced by cholesterol, the ultimate equilibrium transfer out of the aqueous phase is increased. Apparently cholesterol alters the structure of the interfacial film in such a way that the probability for a favorable orientation for interdigitation of the semi-planar dye molecule is decreased. This retards the adsorption and the effect reaches a maximum in this system at 6 mM in the bulk organic phase. However, it is apparent that once the dye molecule does find its way into the cholesterol–lecithin film, it is bound more strongly than in the absence of the sterol. Further, the transformation from the interface to micelles in the bulk organic phase is particularly efficient in the presence of cholesterol so long as its concentration in the organic phase is below 24 mM. The nature of the phase transition that apparently occurs at this sterol concentration has not been determined as yet.

The primary site of action and the chemical mechanisms by which androgens function at the cellular level have eluded discovery. Various considerations have led

to the hypothesis that one important aspect of the mechanisms by which the steroid hormones function may be by some permeability control mechanism (possibly influencing the rate or selectivity of transport of nuclear RNA to the cytoplasm)[9]. Our experimental observations on the effects of steroids on the rate of ionic transport in the physical model system lead us to suggest that, especially in the case of the androgens, a lipoprotein membrane may be one important locale of a rate-controlling step in their hormonal action.

According to Saunders[10] and Heftmann[11] the androgens may be ranked in order of decreasing growth promoting activity on target tissues or secondary sexual characteristics as follows: dihydrotestosterone, testosterone, androsterone, dehydroepiandrosterone, and epiandrosterone. Note in Fig. 4 that the concentrations of four of these required to produce a peak rate of ionic transport are in the same order. The fifth, dehydroepiandrosterone, is weakly androgenic but not very effective as a transport-enhancer in the model system. It is also the only one of the five that has the same structure in the A and B rings as cholesterol.

The C_{21} steroid, pregnenolone, occupies a key position in the biosynthesis of the androgens, but is devoid of androgenic activity, as measured by effects on growth of seminal vesicles or ventral prostate. However, pregnenolone is effective in maintaining testis weight in rats for 20 days following hypophysectomy[12].

In its effects on transport kinetics in the physical model, pregnenolone proved to be at least as active as the most potent androgen tested. Hence, we would suggest that those biological phenomena in which this C_{21} intermediate has properties like the androgens are the ones in which the importance of their direct effects on permeability control mechanisms is dominant.

Bangham *et al.*[8] found that 2 mM pregnanolone, progesterone, or androsterone all increased the rate of Na^+ release from liposomes composed of egg lecithin, cholesterol, and dicetyl phosphate. Testosterone showed a similar effect[13]. These results on liposomes may be compared qualitatively with those in Figs 3 and 4 on azo dye transport by lecithin and cholesterol. At 2 mM all of the added steroids retarded transport of the anionic dyestuff compared to the lecithin control. A detailed study of the dependence of the transport rates on the concentration of the added steroids demonstrated the dramatic differences in the effects of the progestagen and the androgens and the quantitative distinction between the effects of the different androgens.

The requirement of unsaturated chains in the phospholipid for ionic transport is worthy of further discussion in relation to the profound effects of these hydroxyketonic steriods on the rate of the process. The location of the double bonds of the most common ester chains, oleate, linoleate, and linolenate, found in phospholipids has been determined by biochemical evolution to preserve a chain of seven CH_2 groups between the carbonyl group and the first double bond (10.5 Å in the extended configuration between the carbonyl oxygen and the 9,10 unsaturation). It is noteworthy that the distance between the two oxygen atoms of the biologically important steroids, testosterone and estradiol, is also 10.5 Å.

More than a coincidence is suggested. An interaction in the lipid micelle might involve hydrogen bonding of a steroid hydroxyl to the ester carbonyl oxygen of the phospholipid or to the highly structured bound water about the lecithin head group. Specificity may be provided by the orientation required for a second interaction (permanent dipole–induced dipole) between the other oxygen function of the steroid

and the highly polarizable 9,10- (or 12,13-) double bond of the unsaturated phospholipid chain.

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