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Nuclear Magnetic Resonance Studies on Liposomes: Effects of Steroids on Lecithin Fatty Acyl Chain Mobility

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Summary. The effects of fourteen sterols on the NMR spectra of liposomes derived from egg yolk phosphatidylcholines were studied by continuous-wave and Fourier-transform measurements at 60 MHz. Sterols were compared for their ability to broaden the acyl methylene resonances of phosphatidylcholine, when incorporated into liposomes at 25% molar ratio. The ratio of the phosphatidylcholine peak heights (acyl methylene: choline N-methyl) was used as a criterion of the relative condensing activity for the different sterols. This ratio was inversely proportional to the molar volume of the incorporated sterol, as measured by the parachor of the compound. Small sterols had little condensing effect, and the larger sterols such as cholesterol and ergosterol had maximum condensing effects. The study confirmed the importance of the sterol side-chain at C-17 as a requirement for sterol-phospholipid interaction.

Key Words. Liposomes, NMR, sterols, phosphatidylcholine

Sterols are present in eucaryotic membrane often in an approximate 1:1 molar ratio with phospholipids. Most sterols are insoluble in water but are dispersed in smectic mesophases formed by phospholipids in natural membranes and in artificially-prepared liposomes. The observed differences in the interaction between various steroids and phospholipids indicate the importance of certain structural features of the steroids. It has been shown that for maximal steroid-phospholipid interaction the steroid must possess a planar sterol nucleus, a 3β-hydroxy group and an intact side chain at the 17-position of the steroid nucleus (De Kruyff, Demel & Van Deenen, 1972; DeKruyff *et al.*, 1973; Demel, Bruckdorfer & Van Deenen, 1972). The 17-side chain of cholesterol was recognized as necessary for steroid-lecithin interactions by Demel *et al.* (1972) who found that androstan-3β-ol, a cholesterol analog lacking a 17-side chain, had no effect on the permeability of egg yolk lecithin liposomes to small neutral molecules such as glucose and glycerol. Butler, Smith & Schneider (1970) showed that androst-5-en-3β-ol had a smaller

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condensing effect than cholesterol in oriented multi-lamellae of beef brain lipids. Taylor & Haydon (1965) studied the relative effects of progesterone and cholesterol on the pressure-area curves of a monolayer of lecithin films at the air-water interface. At the same pressure cholesterol produces a greater decrease of the area per molecule than progesterone which has a much shorter side chain. In an ESR study, Suckling & Boyd (1976) used cholesterol and its analogs possessing a variety of side chains at the 17-position of the steroid nucleus and found the ordering effects of the cholesterol analogs to be proportional to the length of the steroid side chain. Suckling & Boyd (1976) also mention that preliminary results in their laboratory suggest that the degree of condensation of an egg phosphatidylcholine (PC) monolayer by cholesterol analogs decreases as the side-chain length is reduced. The effects of cholesterol on natural and artificial membranes have been extensively studied by NMR (Chapman & Penkett, 1966; Darke *et al.*, 1972; Lee *et al.*, 1972; Phillips, Kamat & Chapman, 1970) and other techniques (Papahadjopoulos & Kimelberg, 1973). There are, however, few comparative NMR studies of the relative condensing effects of sterols other than cholesterol. We studied the effects of 14 sterols, among them estrogens, androgens, progestagens, corticoids and membrane sterols, by 60 MHz proton NMR in liposomes of purified egg yolk phosphatidylcholine. The NMR results were examined for correlations with a molar volume parameter, the parachor, since we have shown previously that many nonspecific actions of steroids can be correlated with their molar volume (Ahmad, Fyfe & Mellors, 1975; Ahmad & Mellors, 1976; Ahmad, 1977).

Materials and Methods

Steroids

The fourteen steroids used were: estradiol [1,3,5(10)-estra-triene-3,17β-diol], testosterone (17 β-hydroxy-4-androsten-3-one), progesterone (4-pregnene-3,20-dione), deoxycorticosterone (DOC) (21-hydroxy-4-pregnene-3,20-dione), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), cortisone (17,21-dihydroxy-4-pregnene-3,11,20-trione), cortisol (11β,17,21,-trihydroxy-4-pregnene-3,20-dione), triamcinolone (9α-fluoro-11β, 16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione), dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxyl-1,4-pregnadiene-3,20-dione), Schering-12600 (6-chloro-6-dehydro-16-methylene-17α-acetoxy-progesterone), flumethasone (6α,9α-difluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione), cholane-24-ol, cholesterol (3β-hydroxy-cholest-5-ene) and ergosterol (3β-hydroxy-24-methyl-cholest-5,7,22-triene). Schering-12600 was obtained as a gift from the Schering Corporation, U.S.A.; cholan-24-ol was purchased from ICN Pharmaceuticals Inc., Cleveland, Ohio, and all other steroids were purchased from Sigma Chemical Co., St. Louis, Missouri.

Stock solutions of the steroids were prepared in chloroform at a concentration of 30 mM. Those steroids which were insoluble in chloroform were sonicated (Biosonik III, Bronwill Scientific, Rochester, N.Y.) to form a fine suspension in chloroform prior to use in liposome preparations.

Lecithins

Egg yolk lecithin (phosphatidylcholine; 1,2-diacyl-3-sn-O-glyceryl-phosphorylcholine), a mixed lipid of heterogeneous acyl composition was extracted and purified from fresh eggs by the method of Bangham, Hill and Miller (1974). The purified egg phosphatidylcholine (PC) gave a single spot by thin layer chromatography using silica gel G plates, a chloroform/methanol/water (65:25:4) solvent system, detection with iodine vapour (R_f-value 0.33) and comparison with an authentic standard. The purified egg PC was stored as a chloroform stock solution at a concentration of 30 mM under N_2 at about -12 °C.

Preparation of Liposomes in Deuterium Oxide

Appropriate aliquots of stock chloroform solutions of PC and steroids were used. For the preparation of PC liposomes, 3.0 ml stock chloroform solution (90 μmoles PC) was evaporated to dryness in a 50-ml round-bottomed, ground-joint flask *in vacuo* on a Buchi rotatory evaporator to obtain a fine layer of lipid coated on the inner surface of the flask. Deuterium oxide (3.0 ml) containing 5 μmoles of the NMR reference standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was added to the flask, and before shaking and sonicating the flask was flushed with nitrogen to minimize oxidation of lipids. For PC-steroid liposomes, 3.0-ml stock chloroform solution of PC and 1.0 ml of each steroid stock solution were evaporated to dryness in a similar manner and 3.0 ml of the DSS-containing D_2O was added to the flask followed by flushing with nitrogen. Hence in all preparations, the PC concentration was kept constant at 30 μmoles of PC per ml of D_2O and the molar ratios of egg PC/steroids were 3:1. A set of 1:1 molar ratio preparations were also made for some steroids. The flasks were gently shaken for 3 min and then sonicated in a water bath-type sonicator (Sonogen-Z Ultrasonic Transducer-Automatic Cleaner, frequency 25 kHz) for 1 hr at about 40 °C water bath temperature, under an atmosphere of nitrogen. Thin layer chromatography of the lipids after sonication showed no presence of oxidation products. A translucent dispersion was obtained after sonication with PC-only liposomes and with several steroids. Such preparations appeared to be stable for a few hours. For certain other steroids, the liposome preparations were milky and were not stable for more than an hour. The preparations of liposomes in D_2O with added DSS were transferred to regular 5 mm NMR sample tubes. The NMR spectra of each liposome preparation was obtained in the continuous-wave mode on the Varian T-60 NMR spectrometer (Varian Associates, Palo Alto, Calif.) and also in the Fourier-transform pulse mode using a Bruker WP-60 FT-NMR spectrometer. In FT-NMR measurements the repeat time of the repetitive pulse sequence was kept constant for all the systems studied to eliminate spurious effects in signal intensities due to relaxation effects and was also kept long compared to the known T_1's in that system so that the results would be comparable to those obtained in the CW mode.

From the NMR spectra, the ratios of the peak heights for (i) $-(CH_2)_n-/{}^+N(Me)_3$, (ii) $-(CH_2)_n-$/DSS, and (iii) ${}^+N(ME)_3$/DSS were calculated. These ratios of the peak heights were plotted against the parachors of the steroids to check whether there is any correlation between the effect of steroids and their parachors.

Here we may mention the experimental problems encountered with the 1:1 (PC/steroid) liposomes. Some steroids with a relatively lower lipophilicity, e.g., cortisol, estradiol, etc., do not mix with phosphatidylcholine bilayers unless sonicated. Sonication of such steroids with PC produces milky or turbid dispersions, which are unstable colloidal suspensions and rapidly flocculate within half an hour after sonication, particularly at 1:1 molar ratio of PC and steroid. It is possible to obtain the NMR spectra of such liposome preparations in a short time by the continuous-wave technique, but it is difficult to obtain Fourier-transform NMR spectra of these preparations because they start to flocculate by the time required for 100 or 500 scans at 5 or 10-sec repeat times. The 3:1 (PC/steroid) molar ratio preparations are more stable and some of them remain visibly uniform even after several days. For this reason only five steroids were examined at a 1:1 molar ratio, and fourteen steroids were examined at a 3:1 molar ratio with phosphatidylcholine.

Computation of the Parachors

The parachors of organic compounds can be obtained in two ways: (i) by summation of the parachors of all the atoms, bonds, and other structural features occurring in the chemical structure of the compound, and (ii) by experimental determinations of surface tension and density. We used the parachor tables of Quayle (1953) for the calculation

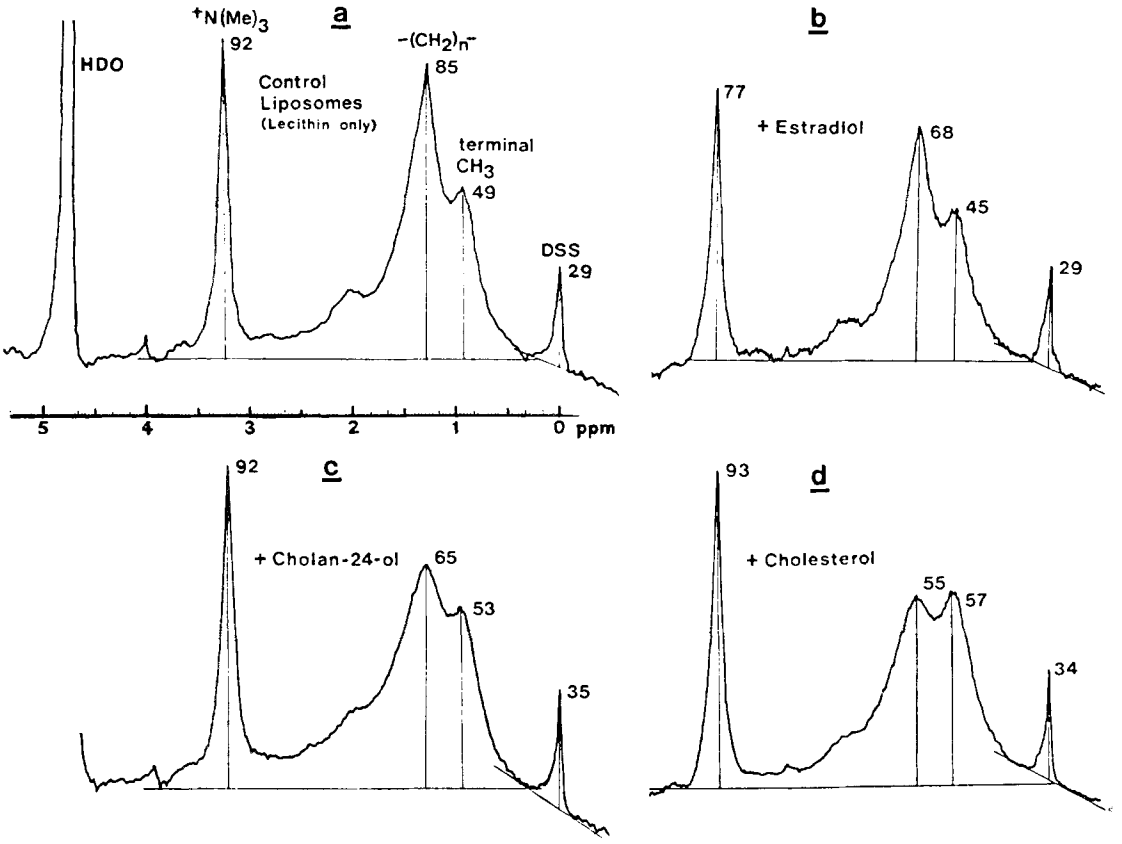

Fig. 1

of all parachors reported in this paper. With the help of the parachor tables, it is possible to calculate the molecular parachor of any compound if the chemical structure is known.

Results and Discussion

Some sample 60-MHz NMR spectra of liposomes from our study have been reproduced in Fig. 1: (a) control (egg PC only, no steroid) liposomes, (*b*) PC+estradiol (3:1), (*c*) PC+cholan-24-ol (3:1), and (*d*) PC+cholesterol (3:1). Table 1 gives the ratios of the peak heights of the 60 MHz continuous-wave NMR resonance signals (Varian T-60) and the parachors of the steroids. Cholesterol at 25% mole-fraction of the

Table 1. Data from the continuous-wave proton-NMR (Varian T-60) study of liposomes of egg-PC and steroids in 3:1 molar ratio, showing the ratios of the peak heights and the parachors of the steroids

Steroids	Parachor of steroids [*P*]	Ratio of Peak Heights			
		$\frac{-(CH_2)_n-}{{}^+N(Me)_3}$	$\frac{-(CH_2)_n-}{DSS}$	$\frac{{}^+N(Me)_3}{DSS}$	$\frac{t\text{-}CH_3}{DSS}$
Control liposomes	–	0.98	5.16	5.26	1.95
(egg PC only)	–	0.96	4.67	4.90	1.86
(1) Estradiol	623.7	0.95	5.40	5.67	2.27
(2) Testosterone	680.8	0.84	4.07	4.87	1.93
(3) Progesterone	751.3	0.80	3.65	4.55	1.95
(4) DOC	765.6	0.86	4.05	4.70	2.00
(5) Corticosterone	781.2	0.86	4.59	5.35	2.18
(6) Cortisone	787.3	0.83	3.25	3.95	1.55
(7) Cortisol	796.8	0.87	3.32	3.82	1.55
(8) Triamcinolone	809.6	0.75	2.74	3.65	1.57
(9) Dexamethasone	834.1	0.79	2.58	3.29	1.42
(10) Schering 12600	844.4	0.81	2.67	3.28	1.29
(11) Flumethasone	844.7	0.82	4.25	5.20	1.85
(12) Cholan-24-ol	878.7	0.71	3.19	4.48	2.00
(13) Cholesterol	994.7	0.54	2.32	4.27	1.50
(14) Ergosterol	1008.1	0.57	2.10	3.67	1.46

Regression equations ($n=14$)	r	s	F
$\frac{-(CH_2)_n-}{{}^+N(Me)_3} = -0.00099\ P + 1.58879$	0.90	0.05	53.0
$\frac{-(CH_2)_n-}{DSS} = -0.00734\ P + 9.42157$	0.80	0.59	21.9

where P is the parachors of the steroids; n is the number of data points, r is the correlation coefficient; s is the standard error of the estimate, and F is the variance ratio of Snedecor.

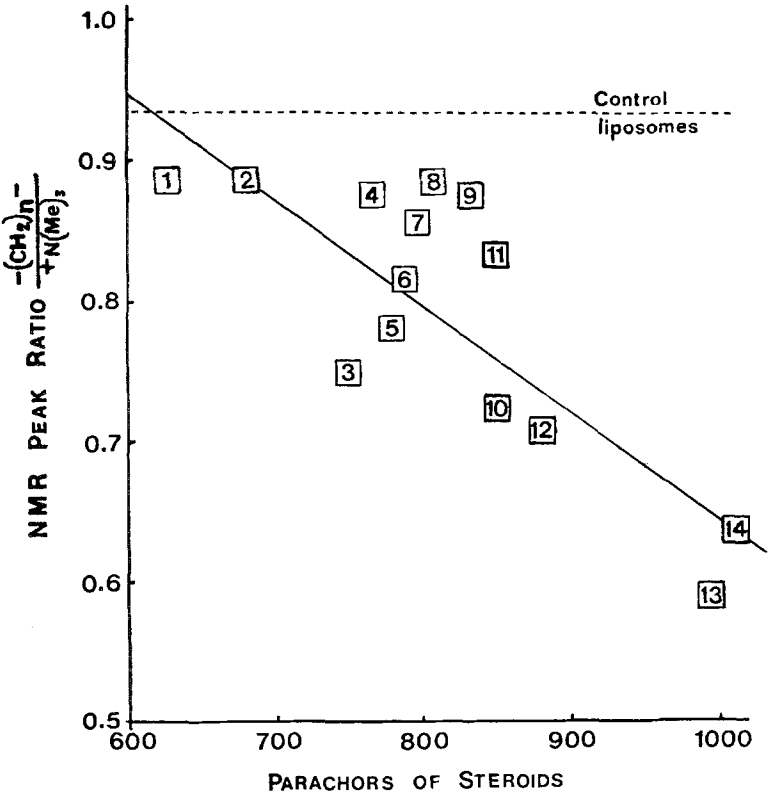

Fig. 2. 60-MHz Fourier-transform NMR (Bruker WP-60) study of the liposomes of egg-PC and steroids in 3:1 molar ratio at 23 °C, showing a correlation between the ratio of the peak heights of the $-(CH_2)_n-$ signals to the peak heights of the $^+N(Me)_3$ signals and the parachors of the steroids. The ratio of the peak heights (acyl methylenes/choline N-methyls) is a measure of the relative condensing activity of the steroids on the liposomes. The dashed line on the top portion of the figure is drawn from the value obtained with the control (PC only, no steroid) liposomes. The final concentrations of egg-PC and each steroid in the liposomal preparation are 30 μmoles and 10 μmoles per ml D_2O, respectively; DSS (the NMR reference standard: sodium 2,2-dimethyl-2-silapentane-5-sulfonate) is added at a final concentration of 5 μmoles per ml D_2O. The details of liposome preparation are given in *Materials and Methods*. In FT-NMR measurements the repeat time of the repetitive pulse sequence was kept constant for all systems studied to eliminate spurious effects on the signal intensities due to relaxation effects and was also kept long compared to the known T_1's in these systems so that the results would be comparable to those obtained in the continuous-wave mode. The steroids incorporated are: (1) estradiol, (2) testosterone, (3) progesterone, (4) DOC, (5) corticosterone, (6) cortisone, (7) cortisol, (8) triamcinolone, (9) dexamethasone, (10) Schering 12600, (11) flumethasone, (12) cholan-24-ol, (13) cholesterol and (14) ergosterol. The best-fit line is drawn by a regression analysis, using the regression equation ($n=14$):

	r	s	F
$\frac{-(CH_2)_n-}{^+N(Me)_3} = -0.00074\ P + 1.39017$	0.79	0.06	20.2

where P is the parachors of the steroids, and the statistical terms have their usual meaning

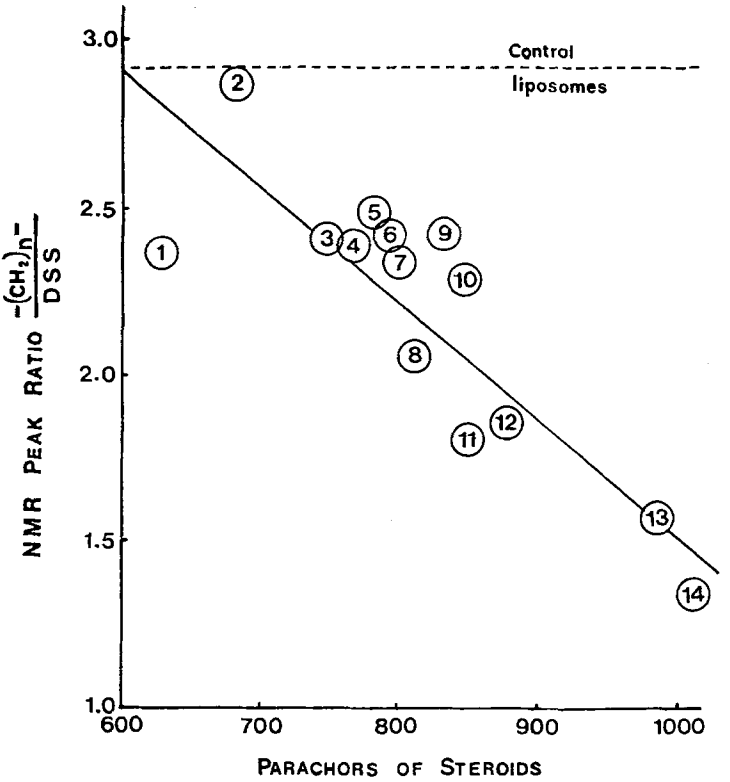

Fig. 3. 60-MHz Fourier-transform NMR study of the liposomes of egg-PC and steroids in 3:1 molar ratio at 23 °C, showing a correlation between the ratio of the peak heights of the $-(CH_2)_n-$ signals to the peak heights of the DSS signals and the parachors of the steroids. The ratio of the peak heights is a measure of the fluidity of liposomes and of the relative condensing activity of the steroids on the liposomes. The broken line on the top indicates the value obtained with the control (PC only, no steroid) liposomes. The experimental details are the same as described in the legend to Fig. 2. The steroids incorporated are: (1) estradiol, (2) testosterone, (3) progesterone, (4) DOC, (5) corticosterone, (6) cortisone, (7) cortisol, (8) triamcinolone, (9) dexamethasone, (10) Schering 12600, (11) flumethasone, (12) cholan-24-ol, (13) cholesterol and (14) ergosterol. The best-fit line is drawn by a regression program, using the regression equation ($n=14$):

	r	s	F
$\frac{-(CH_2)_n-}{DSS} = -0.00330\ P + 4.88123$	0.84	0.23	27.6

where P is the parachor of the steroids, and the statistical terms have their usual meaning

lipids (i.e., PC/sterol=3:1) affects the acyl methylene and the choline N-methyl proton-NMR signals differently, severely broadening and lowering the acyl methylene signal and leaving the choline N-methyl signal relatively unchanged. A measure of this action of cholesterol would

be the ratio of the peak heights (acyl methylenes/choline N-methyls), which is used in this study as a criterion of the relative condensing activity of 14 different steroids. A linear regression for the condensing activity of these steroids *vs.* their parachor indicates a good correlation. The condensing effect can also be seen in the ratio of the acyl methylenes to the reference standard DSS. However, ratios of the choline N-methyl/DSS and the terminal-methyl/DSS peak heights change relatively little; and show no correlation with the steroidal parachors.

Figures 2 and 3 show similar results obtained using a Bruker WP-60 Fourier-transform pulse-NMR spectrometer. Fig. 2 is a plot of the ratio of the peak heights of the $-(CH_2)_n-$ resonance signal to the peak heights of the $^+N(Me)_3$ signal *vs.* the steroidal parachors. In Fig. 3 the ratio of the peak heights of the acyl $-(CH_2)_n-$ signal to the peak heights of the reference standard DSS is plotted against the parachors of the steroids. Except for some minor differences in numerical values, the results of the two sets of experiments are quite similar.

The low-parachor steroids produce a lesser condensing effect than the high-parachor steroids, although all steroids have some condensing effect due to hydrophobic interactions between the acyl methylene chain of phosphatidylcholine and the steroid nucleus. As listed in the tables and figures, the first eleven steroids, namely estradiol, testosterone, progesterone, deoxycorticosterone (DOC), corticosterone, cortisone, cortisol, triamcinolone, dexamethasone, Schering 12600 and flumethasone produce minor condensing effects within 20% of the control value. More significant condensing effects are seen with the last three steroids namely, cholan-24-ol, cholesterol and ergosterol, having side-chains at the 17-position larger than two carbons.

We also made liposomes of egg-PC and five steroids in 1:1 molar ratio. The peak height ratios for these 1:1 molar ratio liposomes are shown in Figs. 4 and 5. In *Materials and Methods*, we have discussed the lack of stability for 1:1 PC/sterol liposomes. It should be pointed out here that the steroid-proton resonances are not detectable under the conditions of these experiments. Presumably the steroid-proton signals are broadened and buried under the narrow PC signals. This suggests that the steroids are in a relatively immobilized state, compared to the bulk of the phosphatidylcholine.

Although there is a definite trend, the abilities of C_{18}-steroids (estrogens), C_{19}-steroids (androgens) and C_{21}-steroids (pregnanes and corticoids) to restrict the motion of the acyl methylene groups are not significantly different from one another despite the structural variety of the

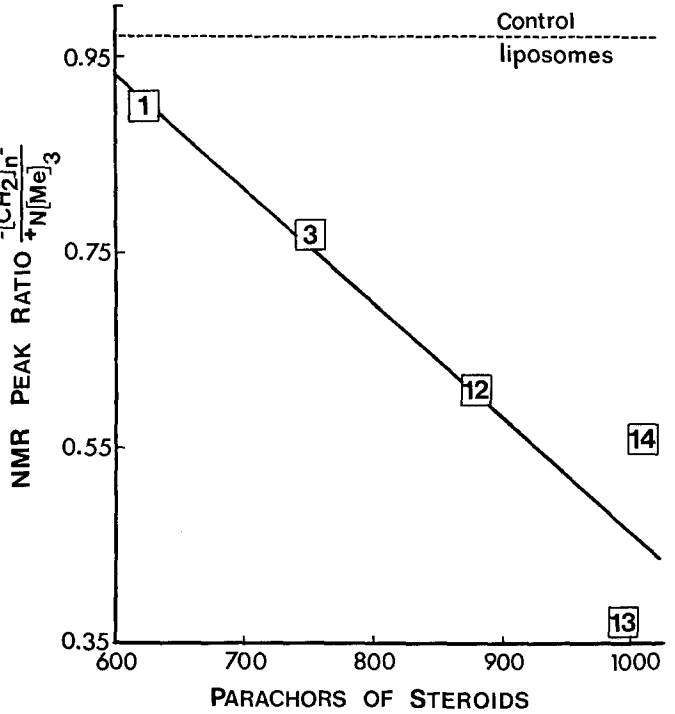

Fig. 4. 60-MHz NMR study of the liposomes of egg-PC and steroids in 1:1 molar ratio, at 23 °C, showing a correlation between the ratio of the peak heights of the $-(CH_2)_n-$ signals to the peak heights of the $^+N(Me)_3$ signals and the parachors of the steroids. The ratio of the peak heights [acyl methylenes/choline N-methyls] is a measure of the relative decrease in fluidity of the liposomes produced by the steroids. The broken line on the top portion of the figure indicates the mean of the values obtained with the control (PC only, no steroid) liposomes. The final concentrations of egg-PC and each steroid in the liposomal preparation are 30 μmoles and 30 μmoles per ml D_2O, respectively; the NMR reference standard DSS is added at a final concentration of 5 μmoles per ml D_2O. The details of liposome preparation are given in *Materials and Methods*. The steroids incorporated in these 1:1 liposomes are: (1) estradiol, (3) progesterone, (12) cholan-24-ol, (13) cholesterol and (14) ergosterol. The numbers used in the figure for these 5 steroids are the same as in Table 1 and Figs. 2 and 3. From a least-squares regression analysis, the best-fit line is drawn by using the regression equation ($n=5$):

	r	s	F
$\frac{-(CH_2)_n-}{^+N(Me)_3} = -0.00115\ P + 1.62418$	0.93	0.08	20.2

where P is the parachor of the steroids, and the statistical terms have their usual meaning

nucleus. A notable effect is observed when the side chain at the 17-position of the steroid nucleus is longer than two carbon atoms, *viz.* cholan-24-ol, cholesterol and ergosterol, whose action is evident in all figures and tables. C_{21}-steroids with a two-carbon side chain show inter-

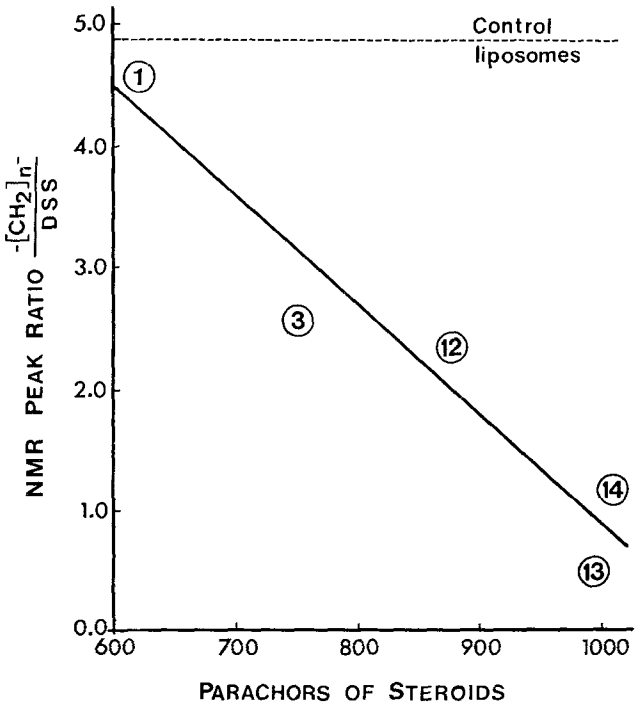

Fig. 5. 60-MHz NMR study of the liposomes of egg-PC and steroids in 1:1 molar ratio at 23 °C showing a correlation between the ratio of the peak heights of the $-(CH_2)_n-$ signals to the peak heights of the DSS signals and the parachors of the steroids. The ratio of the peak heights [acyl methylenes/DSS] is a measure of the relative decrease in fluidity of the liposomes produced by the steroids. The broken line on the top portion of the figure indicates the mean of the values obtained with the control (PC only, no steroid) liposomes. The experimental details are the same as described in the legend to Fig. 4. The steroids incorporated in these 1:1 liposomes are: (1) estradiol, (3) progesterone, (12) cholan-24-ol, (13) cholesterol and (14) ergosterol. The numbers used in this figure for the 5 steroids are the same as in Table 1 and Figs. 2, 3, and 4. From a least-squares regression program, the best-fit line is drawn by using the regression equation ($n=5$):

	r	s	F
$\frac{-(CH_2)_n-}{DSS} = -0.00897\,P + 9.87878$	0.95	0.54	29.5

where P is the parachor of the steroids, and the statistical terms have their usual meaning

mediate effects whereas C_{18}-steroids and C_{19}-steroids lacking the 17-side chain do not affect the liposomes significantly when compared with spectra of the control (PC only, no steroid) liposomes. This suggests that the length of the side chain at the 17-position of the steroid nucleus is an important factor in the ability of steroids to restrict the motion of the acyl methylenes of the phospholipids in the bilayers of biological membranes.

The decrease in the ratios of the peak heights of acyl methylenes/choline N-methyls are found to be in the order:

$$C_{27} \text{ and } C_{28} > C_{24} > C_{21} > C_{19} \text{ and } C_{18}\text{-steroids.}$$

The ratio of the height of the methylene peak divided by the height of the standard DSS peak gives the absolute height of the methylene peak in terms of a fixed reference standard. A comparison of the $-(CH_2)_n-$ $/^+N(Me)_3$ and $-(CH_2)_n-$/DSS ratios indicate that the steroid effect is solely on the methylene chain and not due to any effect on the choline N-methyls. It appears that these effects are governed by overall molar sizes of the steroids, as measured by the parachors of the steroids, with some exceptions. However, within each class of steroids, these effects are not strictly proportional to the small changes in the parachors, indicating specific group interactions. Despite the anomalous behavior of some individual steroid members, there is a trend for increasing condensing capacity with increasing molar volume of the steroids.

We have also studied the effect of several steroids on the permeability of these liposomes to a neutral marker, ^{14}C-glucose (Ahmad, 1977) and found that steroids with long side chains at the 17-position, such as cholesterol, are much more effective in reducing the permeability of liposomes to glucose. Similar observations have been reported by other workers (Demel *et al.*, 1972). Several actions of cholesterol, such as decreasing the mobility of fatty acyl chains of phospholipids as seen by NMR and ESR, reducing the permeability of liposomes to nonelectrolytes and ions, condensing lipid films (Papahadjopoulos & Kimelberg, 1973) may be rationalized by considering the packing of cholesterol molecules into the phosphatidylcholine bilayers. A good fit of the molecular models of PC and cholesterol in intercalating bilayers of 1:1 molar ratio has been demonstrated in a recent interpretation by Huang (1977*a*, 1977*b*) of the experimental results of Yeagle and coworkers (Yeagle *et al.*, 1975; Yeagle *et al.*, 1976; Yeagle & Martin, 1976). The uniqueness of cholesterol molecules for an interaction with membrane bilayers has been indicated by many other studies. It is possible that the smaller steroids pack into bilayers in a manner similar to cholesterol and it can be visualized that the smaller steroids would create "cavities" in the bilayer, thus allowing the terminal half of the fatty acyl chains (from C_{10}-methylene to terminal-CH_3) to undergo unrestricted motion. The importance of the 17-side chain of steroids and its length, for steroid-phospholipid interaction in liposomes has been demonstrated.

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