THE EFFECT OF SIDE CHAIN STRUCTURE ON THE INCORPORATION OF STEROIDS INTO LIPID BILAYERS (LIPOSOMES)

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1. Introduction

A number of investigations have now been made into the relationship between the structure of steroid molecules and their ability to enter phospholipid bilayers or cellular membranes [1–8]. However, most attention has been focussed on the steroid ring system and although it is known that modifications of the side chain at position 17 can greatly alter uptake of steroids by natural or artificial membranes, no systematic study has been carried out.

Here, we report an investigation into the incorporation of a series of testosterone esters into liposomes formed from egg lecithin. The relationship between the incorporation and the lipophilic character of the esters was also explored. It was found that for esters with saturated, straight side chains, incorporation increased with chain length up to a maximum at 8 C atoms and then declined. Insertion of double bonds or branching of the chain lowered incorporation. The pattern of incorporation did not correlate with the lipophilic character of the steroids.

2. Materials and methods

Testosterone and its acetate, propionate and benzoate were obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Other esters were prepared by standard procedures [9] from the appropriate acyl chloride or acid anhydride. They were all assayed by measuring the absorbance at 240 nm in 95% ethanol. This showed a linear relationship with concentration for all compounds studied and was not significantly altered by the presence of lecithin in the concentrations used in the experiments. Egg lecithin was prepared and determined as before [2].

2.1. *Preparation of liposomes*

Liposomes were prepared by dissolving 12.8 μmoles of lecithin and 20 μmoles of the steroid in a little chloroform in a 25 ml beaker and evaporating off the solvent to leave a thin film of lipid. 10 ml of water were added and the mixture, immersed in an ice bath, exposed to the maximal output of an MSE 100 W ultrasonic probe for five periods of 90 sec interrupted by 20 sec cooling periods. The resulting dispersion was centrifuged at 74,000 *g* for 20 min and the clear supernatant, containing the liposomes, removed. Samples were taken for lecithin determination and, after dilution with 19 vol of ethanol, for recording the absorption spectrum.

Control experiments were performed in which the steroid, without any lecithin, was subjected to the above procedure. Testosterone itself dissolves in water to a significant extent [10] and the apparent incorporation into liposomes had to be corrected for this. Corrections for testosterone esters were trivial.

2.2. *Assessment of lipophilic character*

Rather than measure the oil/water partition coefficient, we assessed the lipophilic character of the steroids by reversed phase TLC on silica gel G plates impregnated with silicone oil [11]. The mean of four R_f determinations was used to determine the R_M value from the formula

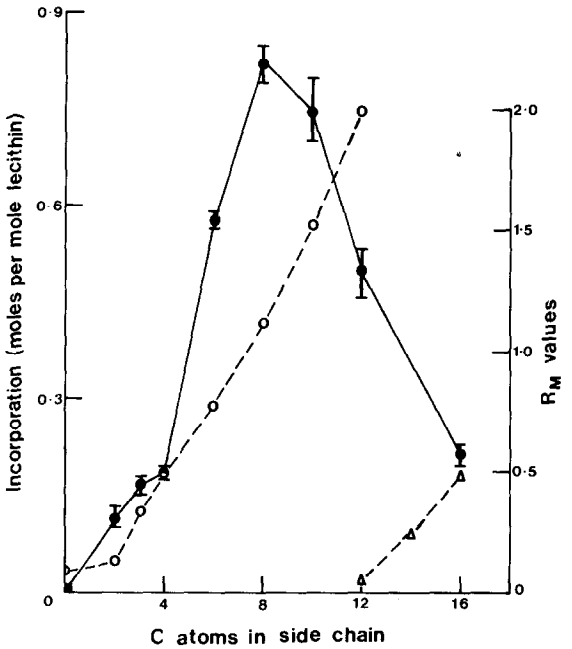

Fig. 1. Relationship between the incorporation into liposomes, the length of the side chain and the lipophilic character (R_M) of testosterone esters. (●—●—●): Incorporation into liposomes; (○—○—○): R_M value in methanol–water (65:35 v/v); (△—△—△): R_M value in methanol–water (85:15, v/v). Incorporation into liposomes is given as the mean of four determinations ± range.

$$R_M = \log(^1/R_f - 1)$$

R_M values were determined using four different methanol–water systems but methanol–water (65:35, v/v) was found most useful for the range of compounds used.

3. Results

The results obtained with saturated, straight chain esters containing 2 to 16 C atoms in the side chain are shown in fig. 1. As expected, the lipophilic character of the esters (R_M value) increases almost linearly with chain length. The R_M value for testosterone palmitate could not be determined in the standard solvent system because it did not leave the origin, but the results given for methanol–water (85:15 v/v) show that it is not an anomalous member of the series.

Table 1
Effect of unsaturation and branching of the side chain upon incorporation of testosterone esters into liposomes.

Testosterone ester	R_M value	Incorporation* (moles per mole lecithin ± range)
Crotonoate	0.30	0.14 ± 0.01
Benzoate	0.59	0.04 ± 0.01
2-Octenoate	0.98	0.56 ± 0.03
3-Octenoate	0.98	0.55 ± 0.04
Undecylenate	1.53	0.56 ± 0.08
2-Methylpropionate	0.45	0.11 ± 0.01
4-Methylpentanoate	0.79	0.25 ± 0.02

*Average of 4–5 determinations.

Incorporation of the steroids into liposomes is markedly dependent on the length of the side chain. Although others have reported some incorporation of testosterone [1, 12] we found that the amount dispersed under our conditions was the same in controls to which no lecithin was added as in the test experiments (about 60 μg per ml). Thus it seems that no testosterone had entered the liposome structure. Incorporation of its straight chain esters increased with the number of C atoms in the side chain up to a maximum at 8 C atoms and then declined. This maximum incorporation, obtained with testosterone octanoate, was 0.82 mole per mole of lecithin which approaches the 1:1 molar ratio obtained with cholesterol under these conditions.

Since the side chains of the natural membrane sterols are branched, the 2-methylpropionate and 4-methylpentanoate of testosterone were compared with their straight chain analogues (table 1). The R_M values of straight and branched isomers were almost identical but in each case, branching of the side chain reduces the incorporation by about half. Further comparison of the results given in table 1 with those in fig. 1 shows that the presence of a double bond in the side chain also lowers the extent to which a given ester is incorporated into liposomes. A terminal double bond appears to have a relatively small effect as shown by the results obtained with testosterone undecylenate. (Although the undecanoate was not investigated, it can be inferred from fig. 1 that about 0.62 mole would

be incorporated per mole of lecithin). On the other hand when a double bond is inserted in the 2 or 3 position of the octanoate side chain, incorporation decreases by about 30%. With testosterone benzoate, incorporation into liposomes is very small even though it has a relatively high R_M value.

4. Discussion

Steroid molecules do not need a side chain at position 17 for incorporation into liposomes [4, 5, 7] and it is probably the dipolar nature of the testosterone molecule which renders it difficult to incorporate, as Gale and Saunders [13] have pointed out in discussing solubilization of steroids by lysolecithin. If the polar 17β hydroxyl group is esterified, the molecule becomes more lipophilic, as shown by the increased R_M value, but at the same time, the balance of polarity is altered in favour of the A ring and the net dipole increases. These effects become more marked as the side chain becomes longer and incorporation into liposomes increases with the R_M value but only for saturated, unbranched chains of up to 8 C atoms. This suggests that the correlation seen between the R_M values of some testosterone esters and their penetration into cell membranes [11] should not be extended into a general rule.

It is noteworthy that maximum incorporation is obtained with testosterone octanoate, since the natural membrane sterol, cholesterol, also has a side chain of eight C atoms. The lowering of incorporation caused by branching or incorporating double bonds into the side chain [table 1] is also seen with cholesterol [2, 3, 7, 8]. However, the cholesterol side chain is shortened by being branched at two points while the testosterone octanoate side chain is extended by the ester link itself. Thus the testosterone ester side chain is appreciably longer than that of cholesterol. Further, it appears from the shape of the curve shown in fig. 1 that the nonanoate would be incorporated into liposomes even better than the octanoate. On the other hand, these unbranched side chains will have more conformational freedom than the cholesterol side chain. The importance of flexibility of the side chain and the restrictions imposed by branching and unsaturation have been inferred before [4, 8]. Obviously, this effect of a double bond should be small if it is in a terminal position and this appears to be so.

The cholesterol molecule is shorter than most of the fatty acids present in natural phospholipids, and as the hydroxyl group is anchored at the aqueous interface, the sterol side chain will not extend into the centre of the lipid bilayer of a liposome or cell membrane. In this central region, the ends of the fatty acid chains show a high degree of motion and there is a low electron density [14–16]. Rothman and Engelman [17] have shown that in membranes or liposomes containing cholesterol and phospholipids, the lower region of the phospholipid hydrocarbon chains will be in a 'liquid-like' state and the upper region more ordered, because the cross-sectional area of the cholesterol side chain is only about half that of the ring system. As incorporation of testosterone esters progressively decreases with side chain length beyond 8 or 9 C atoms, it appears that these chains do not extend across from one side of the bilayer to fill in this space below the steroid ring on the other side. In the liquid crystal state, the hydrocarbon chains have to have a certain degree of motion and this will be important at the junction between the two halves of the bilayer, especially if natural phospholipids, containing a mixture of fatty acids of different chain length, are involved. If packing of hydrocarbon in this region becomes too tight, the conformational adaptations needed for maximum interaction will be hindered and this can determine the solubility of the whole steroid molecule. Within this limitation, optimum incorporation (maximum solubility) will be obtained with the molecule giving maximum van der Waals interactions and so side chain structural requirements are very specific. The same considerations will not apply to monolayers and care should be taken in extrapolating results obtained with such preparations.

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