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THE SOLUBILITY OF CHOLESTEROL AND ITS EXCHANGE BETWEEN MEMBRANES

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It has been proposed that exchange between membrane cholesterol pools occurs by desorption of molecules into the aqueous environment rather than by formation of a transitory collision complex between the membranes. The rate of exchange is likely to be determined by the rate of dissociation of cholesterol from the membrane bilayer and by the concentration of cholesterol monomers or aggregates of cholesterol molecules in solution. The aim of this study was to measure the effects of agents known to increase cholesterol exchange rates on cholesterol solubility, critical micellar concentration and on the activation energy of exchange. A comparison was also made with regard to these parameters, of the exchange of cholesterol to that of 4-cholesten-3-one, another steroid which exchanges more rapidly than cholesterol. Acetone and dimethylsulphoxide increased cholesterol exchange between liposomes and erythrocytes, but only modestly increased the apparent solubility of cholesterol in saline and had no effect on the activation energy of the exchange process. However, acetone and dimethylsulphoxide increased the critical micellar concentration of the cholesterol 3-fold, although tetraethylammonium iodide, which had a smaller effect on exchange, did not. 4-Cholesten-3-one had a lower solubility and critical micellar concentration than that of cholesterol, but had the same activation energy for exchange. It is concluded that the apparent solubility of steroid aggregates are unlikely to determine the rate of exchange, but that agents which substantially increase exchange also increase the critical micellar concentration. The low critical micellar concentration of cholestenone suggests that the actual monomer concentration in an exchange system is low and that the rate of dissociation of the molecules from the liposomes must determine the exchange rate. This is not reflected in the activation energy measurements since these are a composite of all the elements of the exchange process.

Introduction

The mechanism of exchange of 5-cholesten-3β-ol (cholesterol) has been a matter of debate for a number of years. One of the theories proposed that a collision complex forms between the cholesterol carrying structures [1] which may involve the transitory fusion of the outer leaflet of the membrane bilayers [2]. However, the weight of evidence now favours the theory that exchange occurs by desorption of cholesterol molecules from a 'donor' membrane and transfers to an 'acceptor' in a water-soluble form since it is a first-order process [3,4]. Other workers have found an effect of acceptor cholesterol concentration on exchange [5,6], but it has been calculated that these findings can still be interpreted in terms of the dissolution of cholesterol molecules [7,8]. Haberland and Reynolds [9] have shown that cholesterol has an apparent solubility in water of 4.6 μM represent-

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ing aggregates of cholesterol monomers, although recent work suggests that only crystals not micelles are in suspension [10]; the apparent critical micellar concentration of cholesterol is much lower (25 nM).

A number of factors have been shown to alter the rate of cholesterol exchange between erythrocytes and lipoproteins including temperature [11], pH and the presence of dimethylsulphoxide, acetone or tetraethylammonium iodide [12]. Also 4-cholesten-3-one (cholestenone) when incorporated into erythrocytes can replace membrane cholesterol, but appears to exchange more rapidly than cholesterol itself [13,14]. Cholestenone is a metabolite in the conversion of cholesterol to cholestanol [15].

The aim of this study was to determine the extent to which the solubilities of critical micellar concentrations of the cholesterol were related to the exchange. Similarly, a comparison was made of the solubilities of cholesterol and cholestenone and related to their exchange rates.

Materials and Methods

[1α,2α(n)-^{3}H]Cholesterol, [4-^{14}C]cholesterol and cholesterol[1-^{14}C]oleate were obtained from Amersham International PLC, Bucks., U.K. 4-Cholesten-3-one was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. and recrystallized from ethanol before use. Cholesterol oxidase was supplied by Boehringer Corporation (London) Ltd. Lewes, Sussex, U.K.; egg phosphatidylcholine and cholesterol by Sigma U.K. Ltd., Poole, Dorset.

Exchange experiments

1. Preparation of liposomes. 5 mg cholesterol, 10 mg egg phosphatidylcholine, 2 μCi [1α,2α(n)-^{3}H]cholesterol (spec. act. 50 mCi/mmol) and 0.4 μCi cholesterol [1-^{4}C]oleate spec. act. 55 mCi/mmol were mixed in chloroform, dried and sonicated in 9 ml buffer (140 mM NaCl/12.5 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4)/45 mM sucrose/80 mg · l^{-1} penicillin/200 mg · l^{-1} streptomycin sulphate) using a 150 watt sonicator (M.S.E. Ltd., Crawley, U.K.) for 5 min in a flask surrounded by iced water. The sonicate was centrifuged for 30 min at 40000 × *g* in a M.S.E. Superspeed 65 centrifuge (10 × 10 ml rotor) (M.S.E. Ltd., Crawley, U.K.). Aliquots of the supernatant were extracted by the method of Folch et al. [16] and analysed for cholesterol [17], and also for phospholipid [18]. The cholesterol/phospholipid molar ratio was approximately 0.9 : 1 in these preparations. In some cases mixed liposomes of phospholipid, cholesterol and cholestenone were prepared and the molar ratio of the steroids determined by GLC using a Pye Unicam 104 gas chromatograph with flame ionization detectors and a 2 m glass column packed with 3% QFI on Gas Chrom Q (Applied Science Europe B.V., Holland) and a flow (120 ml · min^{-1}) of 95% argon/5% CO_2 at 205°C, detector temperature 230°C. Cholestane was added to lipid extracts as an internal standard.

2. Erythrocytes. Human blood was collected into citrate anticoagulant [19] and washed free of plasma by repeated centrifugation at 2000 × *g* for 15 min in isotonic saline. Aliquots of packed cells were extracted [20] and the cholesterol contents analysed.

Incubations

Mixtures of liposomes and erythrocytes were prepared in buffer pH 7.4 (see above) at 37°C to give an excess of erythrocyte cholesterol over liposome cholesterol by at least 5 : 1 to limit back exchange [21]. Dimethylsulphoxide, acetone and tetraethylammonium iodide were added to give the desired concentrations in some incubations. At 0, 1, 2, 3 and 4 h during the incubation, 2-ml aliquots of the incubation mixture were removed and centrifuged at 2000 × *g* for 5 min and samples were taken of the supernatant which contained the liposomes. Measurement of the initial exchange also reduces the effects of back exchange. 0.2-ml samples of supernatant in triplicate were counted for ^{3}H and ^{14}C in a liquid scintillant containing per litre toluene 16 g 2,5-diphenyloxazole/0.1 g 1,4-bis-2(5-phenyloxazolyl)benzene/300 ml Triton X-100 and using a Packard Tricarb Spectrometer Model 3385).

Exchange rates and activation energy

The exchange at each time interval was calculated assuming that cholesterol [^{14}C] oleate is a non-exchangeable marker and that exchange is

represented by a change in the $^3H/^{14}C$ ratio in the lipsomes. In this procedure the calculation of exchange is not influenced by adhesion or fusion of liposomes with erythrocytes. The fraction of [^{3}H]cholesterol remaining in the liposomes was calculated as described by Backer and Davidowicz [21].

$$\text{Fraction of [}^3\text{H]cholesterol remaining in the liposomes} = \frac{H_s}{C_s} \cdot \frac{C_o}{H_o}$$

where H_o and C_o are [^{3}H]cholesterol and cholesterol [^{14}C]oleate counts in the original liposomes. H_s and C_s are the [^{3}H]cholesterol and cholesterol [^{14}C]oleate counts in the supernatant liposomes after incubation for time s.

The absolute amount of cholesterol and cholestenone was measured in the original liposomes and in the supernatants by analysis of GLC. The fraction remaining of cholestenone was calculated by substituting the weight of the steroid in the lipsomes at time 0 and time s in the formulae for H_o and H_s.

The rate constant k was calculated from the equation:

$$-kt = \ln(\text{fraction of cholesterol remaining in the liposomes})$$

The fractions remaining at 0, 1, 2, 3 and 4 h were plotted and the slope determined by the least squares procedure to give the apparent rate constant. From k, the half-time of exchange ($t_{1/2}$) was determined

$$t_{1/2} = (\ln 2)/k$$

The values for k were determined over a range of temperatures to calculate the activation energy ΔH of the exchange process.

$$\Delta H = 2.303R \frac{\mathrm{d}\log k}{\mathrm{d}(1/T)}$$

where R = gas constant and T = temperature (K). $\log_{10} k$ was plotted against $1/T$ and the activation energy determined from the slope.

$$\Delta H = \text{slope} \cdot 2.303 \cdot 1.987 \cdot 4.186 \text{ kJ} \cdot \text{mol}^{-1}.$$

Preparation of [^{14}C]4-cholesten-3-one

2.5 mg of cholesterol including 12.5 μCi [4-^{14}C]cholesterol in 0.5 ml isopropanol was incubated with 3.0 ml phosphate buffer pH 7.6, 0.0005 ml Triton X-100 and 10 units (1 mg) cholesterol oxidase (EC 1.1.3.6) for 25 min at 37°C. The buffers were gassed with O_2 before and during the reaction. The reaction was stopped by addition of chloroform/methanol (2 : 1, v/v) and the lipids extracted [16]. The extract was applied to an aluminium-backed TLC plate made with Kieselgel 60 (E. Merck, Darmstadt, F.R.G.) and run in benzene/ethylacetate (9 : 1, v/v). The bands were identified with suitable standards, eluted and their identity confirmed on the GLC system described above and the presence of radio-activity in the cholestenone peak demonstrated using radio detection equipment Model F0112/ F3222/ RRG-121 (Panax Equipment Ltd., Mitcham, U.K.). The specific activity of the cholestenone was the same as that of the remaining and original cholesterol. Cholestenone of higher specific activity was prepared to determine the critical micellar concentration by reducing the amount of unlabelled cholesterol to 0.5 mg.

Steroid solubilities

The solubilities were determined using the methods of Haberland and Raynolds [9]. Solutions of ^{14}C-labelled steroids (cholesterol spec. act. 3.2 mCi · mmol^{-1} and cholestenone spec. act. 0.66 mCi · mmol^{-1}) were blown to dryness under N_2 in a glass tube (100 mm long × 10 mm diameter) fitted with a Teflon-lined cap and the tube was rotated to obtain an even film of the steroid on the glass surface. 5 ml solute which was saline (9 g/l) with or without other agents was added to each of the tubes which contained 21–104 μg steroid per ml solute. The tubes were rotated for 5 or 24 h at 37°C; 0.2 ml samples were taken for counting radio-activity in a liquid scintillant containing Triton X-100 (see above). The remaining solutions were centrifuged in a MSE 65 centrifuge at 37°C for 30 min at 20000 × g. 0.5-ml samples were removed from a point midway between the top and bottom of the liquid in the tube and counted. In all cases the sampling pipette was washed with scintillation fluid to minimize adsorption. The apparent solubility was calculated in the centrifuged and non-centrifuged solutions from the counts obtained and the known specific activities of the original steroids.

Determination of critical micellar concentration of steroids

Again the method of Haberland and Reynolds [9] was used to determine the apparent critical micellar concentration. Cholesterol solutions were prepared as described above and adjusted to a value of 375 nM; 5 ml was introduced into Visking dialysis tubing (8/32 inch) with a pore diameter of 4.8 nm which excludes spherical particles greater than 12000–14000 daltons. The dialysis sac was doubled over and placed in a plastic universal tube containing 15 ml of saline or saline/agent mixture which was stirred with a small magnet. The tubes were closed except during times of sampling and maintained at 37°C for up to 50 h. Samples were taken at intervals for counting and the cholesterol concentration determined uisng the specific radioactivity of the original sample. The apparent critical micellar concentration is indicated when the values for cholesterol concentration plateau after several hours. The approximate value can be determined from the tangents to the initial slope and the final slope which intersect at the critical micellar concentration. A similar procedure was employed to determine the critical micellar concentration of cholestenone.

Results

Effects of acetone, dimethylsulphoxide and tetraethylammonium iodide on cholesterol exchange

When liposomes containing [^{3}H]cholesterol and cholesterol [^{14}C]oleate were incubated with an excess of human erythrocytes in terms of cholesterol

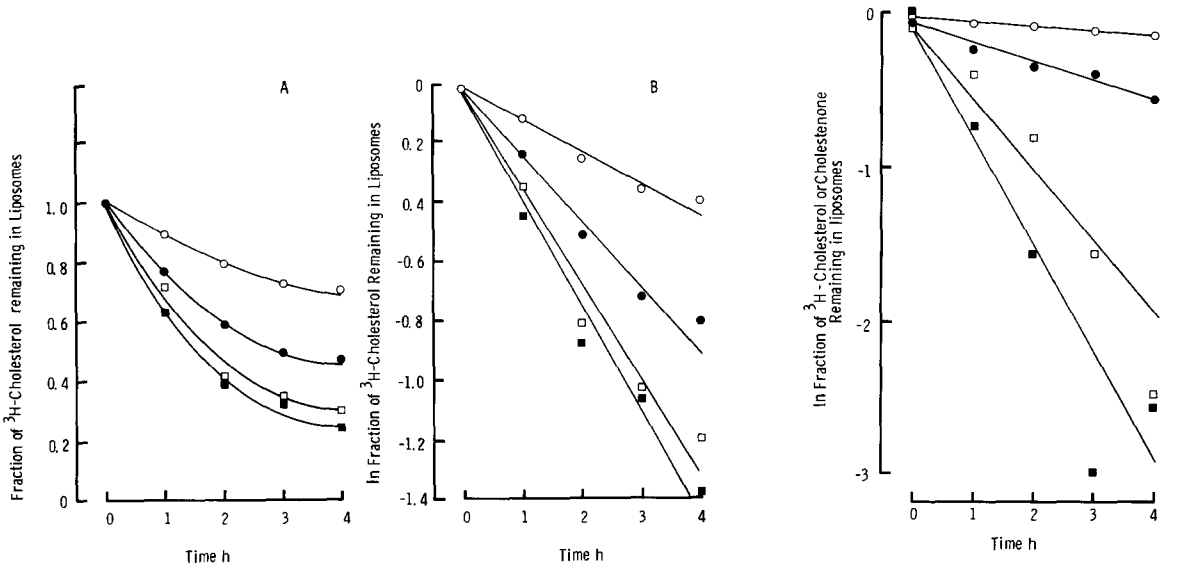

Fig. 1. (Left) The effect of tetraethylammonium iodide, acetone and dimethylsulphoxide on the rate of cholesterol exchange between liposomes and erythrocytes. Liposomes containing egg phosphatidylcholine, [^{3}H]cholesterol (0.111 mg) and cholesterol [^{14}C]oleate (^{3}H/^{14}C ratio 10:1) were incubated with erythrocytes containing 0.786 mg cholesterol at 37°C and the exchange of cholesterol determined as described under Materials and Methods. The ^{3}H/^{14}C ratio was used to calculate the fraction of cholesterol remaining in the liposomes (Materials and Methods) in the presence of: saline alone (○), 0.5 M tetraethylammonium iodide (●), 1.5 M dimethylsulphoxide (□) and 1.5 M acetone (■) (A). The rate constant k for the exchange was calculated from the slope of a plot of ln(fraction cholesterol remaining in the liposomes) against time (B). Each point is the mean of six incubations.

Fig. 2. (Right) The rate constant for cholesterol and cholestenone exchange between liposomes and erythrocytes. Liposomes containing egg phosphatidylcholine, [^{3}H]cholesterol (0.305 mg), cholestenone (0.28 mg) and cholesterol [^{14}C]oleate (^{3}H/^{14}C ratio 10:1) were incubated with human erythrocytes containing 1.685 mg cholesterol at 37°C and the exchange of cholesterol and cholestenone was measured (see Materials and Methods). The rate constants for the exchange and the effects of 1.5 M dimethylsulphoxide were calculated from the slope of the plot of ln(fraction of steroid remaining) against time ($n = 4$). ○——○, Cholesterol in saline; ●——●, cholesterol in dimethylsulphoxide; □——□, cholestenone in saline; ■——■, cholestenone in dimethylsulphoxide.

content, the labelled unesterified cholesterol is transferred to the erythrocytes by exchange with unlabelled cholesterol in the erythrocyte membrane (Fig. 1A). The transfer of labelled cholesterol from the liposomes was accelerated by the presence of 1.5 M acetone and 1.5 M dimethylsulphoxide and to a lesser extent by 0.5 M tetraethylammonium iodide. The rate constant k for the exchange process was determined from plots of ln(fraction of cholesterol remaining in the liposomes) against time (Fig. 1B). With no agents $k = 0.10\ h^{-1}$, with tetraethylammonium iodide $k = 0.18\ h^{-1}$, with dimethylsulphoxide $k = 0.31\ h^{-1}$ and acetone $k = 0.37\ h^{-1}$. The corresponding half-times were control 6.9 h, tetraethylammonium iodide 3.58 h, dimethylsulphoxide 2.24 h, and acetone 1.87 h. Acetone increased the rate of exchange by almost 4-fold. The plots in Fig. 1B suggest that the process may not be precisely monoexponential, but this may be due to the effects of back exchange after 4 h.

The exchange of cholestenone

In this experiment liposomes were prepared containing ^{3}H-labelled cholesterol, cholesterol [^{14}C]oleate marker and unlabelled cholestenone. They were incubated with erythrocytes and the changes in the amount of cholesterol and cholestenone in the liposomes determined in relation to the counts from the non-exchangeable marker. Cholestenone transferred rapidly from the liposomes and dimethylsulphoxide accelerated this process slightly (Fig. 2). The rate constant for cholestenone transfer was calculated to be 0.58

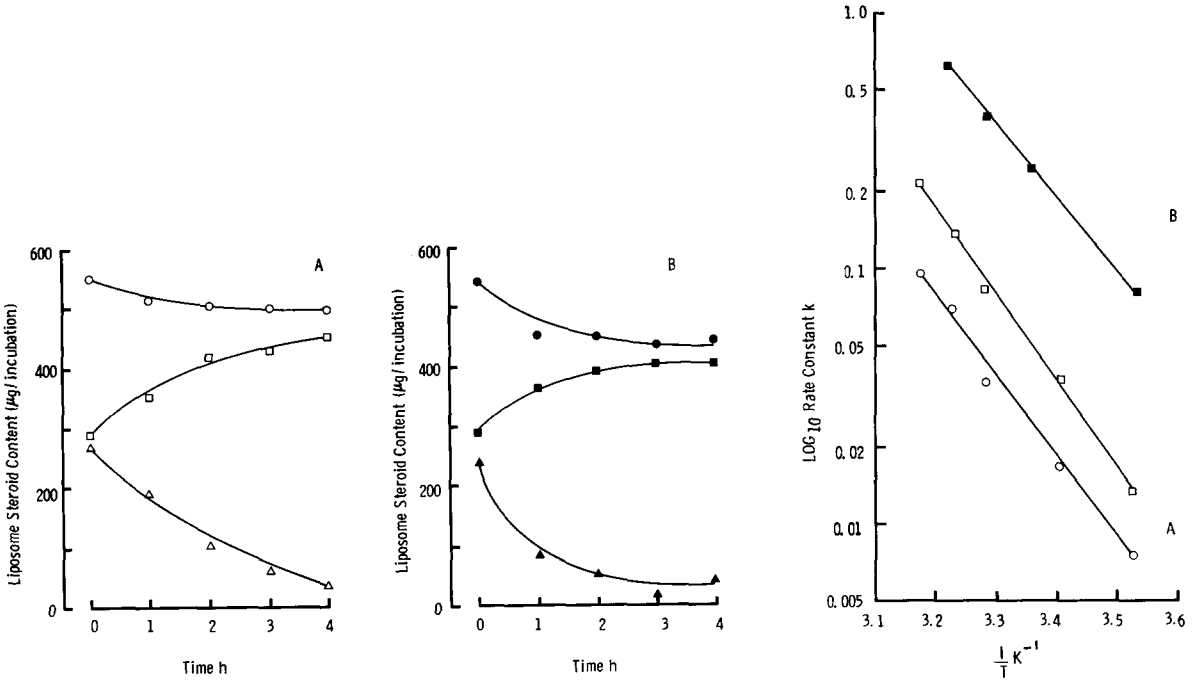

Fig. 3. (Left) The mass transfer of cholesterol and cholestenone exchange between liposomes and erythrocytes. For details of exchange experiment see legend to Fig. 2. The change in the mass of cholesterol and cholestenone in the liposomes normalized to the non-exchangeable cholesterol marker was determined by GLC at each time interval. (A) Control (saline only): ○———○, total steroid; □———□, cholesterol; △———△, cholestenone. (B) Dimethylsulphoxide: ●———●, total steroid; ■———■ cholesterol; ▲———▲, cholestenone.

Fig. 4. (Right) Activation energies for cholesterol exchange in the presence of dimethylsulphoxide and for cholestenone exchange. Exchange experiments (see legends of Figs. 1 and 2) were performed at various temperatures to determine the rate constants k in each case. The values for $\log_{10} k$ were plotted against $1/T$ where T is temperature K. The activation energy was determined from the slope. In experiment A the effect of 1.5 M dimethylsulphoxide on activation energy of cholesterol exchange was determined (○———○, control; □———□, dimethylsulphoxide). In experiment B the activation energy of cholestenone exchange was found (■———■).

h^{-1}, over 5-times greater than that of cholesterol in the absence of cholestenone. When dimethylsulphoxide was present in the incubation medium the rate constant was 0.65 h^{-1}. The rate constant for cholesterol exchange was lower ($k = 0.03$ h^{-1}; $t_{1/2} = 23$ h) compared to experiments where cholesterol is the only steroid. Nevertheless, dimethylsulphoxide increased the cholesterol rate constant to 0.13 h^{-1}, a similar percentage increase to that found in the previous experiment, but which was lower than the value found in the absence of cholestenone.

The changes in liposome total steroid content are shown in Fig. 3. In all cases there was a small decrease in the total steroid content compared with the original liposomes, which was more marked in the presence of acetone. As indicated above the amount of cholestenone was sharply decreased over 4 h and the cholesterol content increased, although not sufficiently to replace all the transferred cholestenone.

Activation energies of exchange

The exchange of cholesterol in the presence and absence of dimethylsulphoxide was measured between 10 and 42°C. The rate constants were determined in each case and plotted against the inverse of the absolute temperature (Fig. 4), to determine the activation energy of exchange. The value for cholesterol exchange was 49.8 kJ · mol^{-1}, but was not different from that found in the presence of dimethylsulphoxide (53.2 kJ · mol^{-1}). In another experiment (Fig. 4) the exchange of cholestenone over a range of temperatures showed an activation energy of 49.6 kJ · mol^{-1}, but that of the cholesterol present in the mixed liposome was difficult to measure because of the small amount of exchange which occurred at all temperatures.

The solubility of cholesterol and cholestenone

The solubility of cholesterol in saline at 37°C was similar to the values found previously at 22°C [9] (Table I). Centrifugation of cholesterol solutions at 20000 × *g* for 30 min reduced this apparent solubility to a value less than 25% of the original which indicates the large size of many of the aggregates of cholesterol molecules. The solubility of cholestenone in saline was approximately half that of cholesterol without centrifugation and of a similar value to that of cholesterol after centrifugation (Table I). Cholestenone, therefore, also forms aggregates, which may be smaller than cholesterol aggregates.

The presence of acetone, dimethylsulphoxide and tetraethylammonium iodide at concentrations known to increase exchange of cholesterol all increased its solubility by over 50% (Table II). Centrifugation of the particles reduced these values except in the case of tetraethylammonium iodide where the solubility was unchanged. This was shown to be an effect of the higher density of the solutions and not an effect on aggregate size (Bruckdorfer, K.R., unpublished observations). Only in the case of dimethylsulphoxide was there a genuine increase in solubility in the centrifuged solutions which indicated a smaller size of cholesterol aggregates.

TABLE I

THE SOLUBILITY OF CHOLESTEROL AND CHOLESTENONE IN ISOTONIC SALINE AT 37°C

[4-^{14}C]Cholesterol or [4-^{14}C]cholestenone were used to determine the solubility of the steroids (Materials and Methods). The solutions were centrifuged at 20000 × *g* for 30 min at 37°C and the apparent solubility in the supernatant calculated from the remaining radioactivity. The results are the means of eight experiments ± S.D.

μg solid steroid/ ml saline	Solubility (μg · ml^{-1})			
	Before centrifugation		After centrifugation at 20000 × *g*	
	Cholesterol	Cholestenone	Cholesterol	Cholestenone
21	1.82 ± 0.76	0.88 ± 0.08	0.45 ± 0.08	0.38 ± 0.03
63	1.69 ± 0.91	0.91 ± 0.03	0.40 ± 0.07	0.35 ± 0.05
104	1.95 ± 0.38	0.97 ± 0.10	0.38 ± 0.20	0.29 ± 0.09
Mean value	1.79 ± 0.17	0.92 ± 0.05	0.41 ± 0.04	0.34 ± 0.05

TABLE II

THE EFFECT OF AGENTS WHICH INCREASE THE RATE OF CHOLESTEROL EXCHANGE ON THE SOLUBILITY OF CHOLESTEROL IN WATER

[^{14}C]Cholesterol was used to determine the solubility of cholesterol in several solvents which enhance exchange. The solutions were centrifuged at 20000 × g and further samples taken to determine the concentration of cholesterol in the supernatant. The results are the means of twelve experiments ± S.D.

Solvent	Cholesterol solubility ($\mu g \cdot ml^{-1}$)	
	Uncentrifuged	Centrifuged at 20000 × g
Water	1.64 ± 0.43	0.31 ± 0.03
Saline (9 g/l)	1.83 ± 0.37	0.35 ± 0.06
0.5 M tetraethyl-ammonium iodide	2.92 ± 0.34	2.39 ± 0.35
1.5 M dimethyl-sulphoxide	2.82 ± 0.76	0.93 ± 0.10
1.5 M acetone	2.81 ± 0.35	0.42 ± 0.32

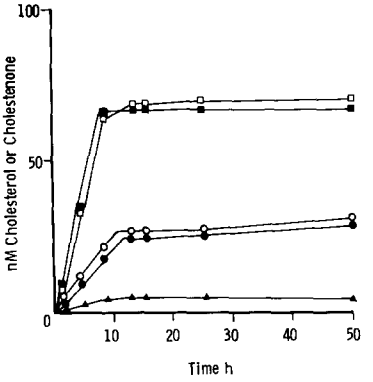

Fig. 5. The critical micellar concentrations of cholesterol and cholestenone. [^{14}C]Cholesterol (○——○) or [^{14}C]cholestenone (▲——▲) were dissolved in saline and diluted to 375 nM and dialysed against saline for up to 50 h and the critical micellar concentrations were determined (see Materials and Methods) for both steroids and for cholesterol in the presence of tetraethylammonium iodide (●——●), dimethylsulphoxide (□——□) and acetone (■——■). Each point is the mean of 3–8 determinations.

The critical micellar concentration of cholesterol and cholestenone

The solutions of the steroids were prepared and centrifuged as described above and adjusted to a concentration of 375 nM, approx. 15 times the critical micellar concentration reported earlier for cholesterol [9]. Dialysis of this solution against saline showed that cholesterol did pass through the membrane reaching a concentration of 26 nM at 37°C after 13 h (Fig. 5). Dialysis with either 1.5 M acetone or dimethylsulphoxide on both sides of the membrane showed a significant increase to

TABLE III

SUMMARY OF DATA FOR RATE CONSTANTS OF EXCHANGE, SOLUBILITIES AND CRITICAL MICELLAR CONCENTRATIONS OF CHOLESTEROL AND CHOLESTENONE

	Solubility in saline ($\mu g \cdot ml^{-1}$)	Apparent solubility after centrifugation at 20000 × g ($\mu g \cdot ml^{-1}$)	Critical micellar concentration (nM ± S.D.)	Rate constant for exchange (h^{-1} ± S.D.)
Cholesterol in saline	1.83	0.35	26 ± 4 (n = 8)	0.103 ± 0.003
Cholesterol in 1.5 M acetone	2.81	0.42	69 ± 8 (n = 8)	0.372 ± 0.012
Cholesterol in 1.5 M dimethyl-sulphoxide	2.82	0.93	68 ± 5 (n = 8)	0.36 ± 0.009
Cholesterol in 0.5 M tetraethyl-ammonium bromide	2.92	2.39 [a]	25 ± 4 (n = 8)	0.287 ± 0.070
Cholestenone saline	0.97	0.29	3 ± 1 (n = 3)	0.580 ± 0.120

[a] This high figure is attributed to the higher density of 0.5 M tetraethylammonium iodide.

almost 3-times this value (Table III). However, tetraethylammonium iodide which enhanced cholesterol exchange to a lesser extent did not show this effect. The critical micellar concentration of cholestenone was 3 nM, lower than that of cholesterol and was calculated from a plot with an expanded ordinate.

Discussion

It has been proposed that cholesterol exchange occurs via a water-soluble form of cholesterol which desorbs from lipoproteins or liposomes and is transferred to a site in the erythrocyte membrane or vice versa [3,22]. The rate of transfer may then depend on a number of factors: (1) the solubility of cholesterol in the medium, (2) the rate of desorption of cholesterol from the donor pool, (3) the absorption of the cholesterol into the acceptor pool. In this study the effect of agents which are known to accelerate exchange between lipoproteins and erythrocytes or, as shown above, between liposomes and erythrocytes was determined on the solubility of cholesterol, its critical micellar concentration and the activation energy of the exchange process. The same parameters were measured for cholestenone since this steroid exchanges more rapidly than cholesterol [13].

The solubility of cholesterol at 20°C in water, in which it forms rod-like aggregates of average molecular weight 162000, is 4.7 μM, in so far as these units are appropriate for aggregates [9]. In this study we found a similar value at 37°C (4.3 M) with evidence for the presence of large aggregates by centrifugation of these solutions. The free energy of micellization of cholesterol is -52.7 kJ · mol^{-1} [9] but the hydrophobicity of cholesterol is lower than predicted [23] with a calculated interaction between cholesterol monomers of 8–16 kJ · mol^{-1} favouring self association in a mixed liposome. The critical micellar concentration of cholesterol is, nevertheless, low at 25–40 nM [9], but comparable to that of phospholipid at 10 nM [24].

The apparent solubility of cholesterol in water is increased slightly by the presence of agents which cause the acceleration of exchange. If the larger aggregates are removed by centrifugation, dimethylsulphoxide appears to increase the number of smaller aggregates of cholesterol, whereas acetone does not. The apparent solubility of cholestenone is lower than that of cholesterol and yet cholestenone exchanges more rapidly.

These results do not at first sight fit with the proposal that the rate of lipid exchange is dependent on the concentration of lipid in solution [25]. However, the apparent solubility represents the greatest concentration of cholesterol aggregates that can be reached and which may far exceed the actual aqueous concentrations of cholesterol prevailing in the exchange experiments. This limit in overall solubility clearly has little bearing on the rate of exchange (Table III).

The rate of exchange of a series of fluorescent phospholipid derivatives was related to the monomeric concentrations of those lipids which were predicted from their critical micellar concentrations [25]. In the present study, both acetone and dimethylsulphoxide considerably increased the rate of exchange and the critical micellar concentration of cholesterol (Table III), which agrees with the results for phospholipid exchange. However, tetraethylammonium iodide, which also increases exchange, but to a lesser extent at the concentration employed, had no effect on critical micellar concentration. Acetone and dimethylsulphoxide are water structure-breakers, whereas tetraethylammonium iodide is a structure-maker, properties which may influence the aqueous concentrations of lipids. In contrast to the finding that structure-makers decrease the exchange rate for phospholipid derivatives [25], the rate of cholesterol exchange was increased.

Cholestenone was found to have a lower critical micellar concentration than cholesterol despite its relatively rapid rate of transfer. The actual aqueous concentration of cholestenone during exchange may still be far less than its optimum aggregate solubility, but it was recognised by Thilo (1977) that the exchange of lipids may involve small micelles and not only monomers [24]. The aqueous concentration of cholestenone during exchange may, therefore, be above or below its critical micellar concentration. The actual concentration may depend on the free energy changes in micellization of cholestenone and its desorption or reabsorption into the bilayer.

Cholestenone may dissociate from the bilayer more rapidly than does cholesterol because of the weaker interactions of this steroid with membrane phospholipids. Cholestenone has quite different properties from cholesterol at air/water interfaces exhibiting a greater mean molecular area, lower collapse pressure and reduced condensation effect with phospholipids [26]. It increases the permeability of erythrocytes and liposomes to solutes, when it partially replaces cholesterol in these membranes [27,28] and increases membrane fluidity [29]. However, the activation of energy of cholestenone transfer is of the same order as that of cholesterol. Similarly, dimethylsulphoxide, acetone or tetraethylammonium iodide do not affect the activation energy of cholesterol exchange even though they increase the rate of exchange. It has been shown that the activation energy of cholesterol exchange from liposomes containing phospholipids of different fatty acid composition is the same, even though the rate constant is greater when liposomes with unsaturated phospholipids are used [30].

In biological systems care must be taken in interpreting activation energy as the sole indicator of the rate at which process will occur at physiological temperatures. This depends both on the enthalpy and entropy changes which exist in the multi-step exchange of cholesterol molecules and, therefore, on the free energy of activation according to classical transition state theory. The activation energies for the exchange of a variety of phospholipid derivatives were found to be similar even though there was a very wide range of rate constants [25]. In general activation energies for the phospholipids were higher than that for cholesterol, although a different exchange system was used [31]. In similar studies it was calculated that there were big differences in the entropies of activation, but that the enthalpic component of the free energy of activation was more significant and more constant [32].

The nature of the acceptor may influence the exchange rate [33,34], in part, by variation in the thickness of the unstirred water layer which retards the diffusion of the desorbed cholesterol [35]. Dimethylsulphoxide, acetone and tetraethylammonium iodide may all disrupt this unstirred water layer and have the effect of facilitating diffusion.

Of particular interest is the finding that the rate of desorption of cholestenone from liposomes was almost equalled by the uptake of cholesterol from erythrocytes so that the rate constant for cholesterol influx into liposomes is several times its value in the absence of cholestenone. The rate of solubilization of cholesterol from the erythrocytes must not limit the influx into liposomes at least when there is a large excess of erythrocyte cholesterol, although the efflux of cholestenone was not quite matched by the cholesterol influx. However, the rate constant for cholesterol efflux from liposomes is much diminished by the presence of cholestenone. It is difficult to explain this retardation of cholesterol exchange on the basis of the independent desorption of the steroids into the aqueous medium. Another explanation is that the steroids are competing to fill a limited intermediate pool within the membrane through which exchange occurs.

The calculation of rates of cholestenone and cholesterol exchange assumes that they are independent steroid pools, although earlier work indicates that they occupy similar sites, at least in erythrocyte membranes [13]. The relative lack of cholesterol in liposomes containing cholestenone, compared with those including only cholesterol as a steroid, may give rise to non-equilibrium conditions which may reduce the desorption of cholesterol. Poznansky and Czekanski [36] noted a greater activation energy for exchange between liposomes and erythrocyte ghosts with a cholesterol/phospholipid ratio less than 1 : 2. This was not observed in exchange between liposomes of lower cholesterol content [29]. The observation of slow efflux of cholesterol cannot be used as an argument against the concept of desorption of cholesterol into the aqueous environment. Indeed Wharton and Green [14] use the different rates of transfer of cholesterol analogues as an argument against the concept of the collision theory and in favour of the existence of a water soluble pool of steroids as the mode of exchange.

In conclusion the data obtained in these experiments are consistent with a view that cholesterol exchange may occur as a result of the solvation of cholesterol in the aqueous medium for which we have direct evidence in the transfer of cholesterol between cells in multi-phase polymer systems (Bruckdorfer, K.R., unpublished observations).

The dissociation of the molecules must be the rate-limiting determinant in exchange.

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