

The Exchange of Unesterified Cholesterol between Human Low-Density Lipoproteins and Rat Erythrocyte 'Ghosts'

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1. The exchange of unesterified cholesterol molecules between rat erythrocyte 'ghosts' and human low-density lipoproteins has been studied under a number of different experimental conditions. 2. The process is pH-dependent, the rate being minimal at about pH 5. 3. Cholesterol exchange does not vary greatly with temperature, the rate at 50° being less than twice that at 2°. 4. Large variations in the ionic strength or Ca²⁺ concentration of the medium have little effect, but the exchange rate is greatly increased in the presence of a wide range of chemical compounds, e.g. urea, alcohols, acetone, dimethyl sulphoxide and tetra-alkylammonium salts. 5. Acetone and dimethyl sulphoxide have a much greater effect at 37° than at 8–10°. 6. It is proposed that hydrophobic bonding is of great importance in maintaining the structure of 'ghosts' and lipoproteins. 7. The results are discussed in relation to current theories of membrane and lipoprotein structure.

Unesterified cholesterol molecules readily exchange *in vivo* and *in vitro* between blood plasma and erythrocytes (Hagerman & Gould, 1951; Gould *et al.* 1955; Eckles, Taylor, Campbell & Gould, 1955; London & Schwarz, 1953; Porte & Havel, 1961; Sodhi & Kalant, 1963). Exchange takes place between erythrocytes and plasma obtained from animals of different species (Hagerman & Gould, 1951) and does not depend on any metabolic process (Murphy, 1962*b*). Few investigations (Murphy, 1962*b*; Spritz, 1965) have been made of the mechanism of the process, although this could be of importance in the elucidation of lipoprotein and membrane structure. The use of whole plasma presents difficulties because the amount of unesterified cholesterol in plasma changes during incubation (Murphy, 1962*a*), but this difficulty can be overcome by using purified high- and low-density plasma lipoproteins (Ashworth & Green, 1964; Basford, Glover & Green, 1964). We have now used the latter to study some of the factors that affect the rate of cholesterol exchange. A preliminary account of some of the findings has been given (Basford, Bruckdorfer & Green, 1966).

MATERIALS AND METHODS

[2-¹⁴C]Mevalonic acid was obtained from The Radiochemical Centre (Amersham, Bucks.). Neuraminidase (EC 3.2.1.18) and phospholipase C (EC 3.1.4.3) were from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.), and phospholipase D (EC 3.1.4.4) was from British Drug Houses Ltd. (Poole, Dorset). Phospholipase D was also prepared

from cabbage ('stage 3 precipitate') as described by Davidson & Long (1958). Urea, 1,4-dioxan and inorganic salts were of A.R. quality and other chemicals of reagent grade. Solvents were redistilled before use.

Rat erythrocyte 'ghosts'. Blood was obtained from rats by cardiac puncture 16 hr. after intraperitoneal injection of 2–3 μC of [2-¹⁴C]mevalonic acid. The cells were removed by centrifugation and washed three times with 0.15 M-NaCl, the buffy coat being discarded at each washing. 'Ghosts' were prepared by the method of Dodge, Mitchell & Hanahan (1963). To obtain maximal removal of haemoglobin, it was found necessary to reduce the centrifugation times from 40 min. to 10 min. throughout the procedure.

Neuraminidase-treated 'ghosts'. The 'ghosts' from 3 ml. of packed cells were incubated with 500 units of neuraminidase in sodium cacodylate buffer, pH 6.4 and I 0.15, for 1 hr. at 37°. (One unit of the enzyme liberates 1 μg. of *N*-acetylneuraminic acid from serum glycoprotein in 15 min. at 37°.) The reaction mixture also contained CaCl₂ (5 mM). Under these conditions, all of the neuraminic acid is liberated from intact erythrocytes and 'ghosts' (Eylar, Madoff, Brody & Oncley, 1962). Although quantitative measurements were not made, release of sialic acid into the medium was demonstrated by the method of Warren (1959). The 'ghosts' were sedimented and washed twice with phosphate buffer before use.

Human low-density lipoproteins. These were prepared from plasma by chromatography on hydroxyapatite (Hjertén, 1959). The finding of Basford *et al.* (1964) that such preparations consist mainly (80–90%) of lipoproteins of *S*₃₋₉ (*d*_{1.03}) was confirmed. The lipoproteins were dialysed for 24 hr. against large volumes of the appropriate buffer solutions.

Phospholipase C treatment of lipoproteins. The reaction mixture (6.5 ml.) contained low-density lipoproteins (1.05 mg. of unesterified cholesterol), tris-acetic acid buffer,

pH 7.5 (0.1 M), CaCl_2 (5 mM) and enzyme (1.25 units). (One unit of enzyme hydrolyses 1 μ mole of phosphatidylcholine/min. at 37°.) After only 2–3 min. incubation at 37°, the lipoprotein began to be precipitated. The precipitate did not redissolve on addition of sufficient EDTA to remove all of the Ca^{2+} .

Phospholipase D-treated lipoproteins. For this enzyme to act, it was found necessary to have ether present. To avoid exposing the lipoproteins to an ether/water interface, the lipoprotein solution was mixed with an equal volume of buffer that had previously been saturated with diethyl ether. A 4 ml. volume of lipoprotein solution, containing 630 μ g. of unesterified cholesterol, was incubated for 1 hr. at 25° with 14 units of phospholipase D. (One unit of enzyme liberates 1 μ mole of choline from egg phosphatidylcholine/hr. at 25°.) The reaction mixture also contained tris buffer, pH 7.1 (0.1 M) and CaCl_2 (0.25 M). The choline released was estimated by the method of Davidson & Long (1958). Assuming that 70% of the phospholipid of low-density lipoproteins is lecithin (Nelson & Freeman, 1960) it was calculated that 80–8% of the latter was hydrolysed. The lipoproteins were dialysed overnight against a large volume of phosphate buffer before use. Thus the enzyme, although inactive, remained in the lipoprotein solution.

Incubation conditions. Except where otherwise stated, incubations were carried out with gentle shaking at 37° in 0.1 M-phosphate buffer, pH 7.4, under air. The medium also contained ascorbic acid (5 mg./ml.) to prevent oxidation of the lipids (Ray, Davisson & Crespi, 1954), as well as benzylpenicillin (100 units/ml.) and streptomycin sulphate (100 μ g./ml.) to prevent bacterial contamination. The unesterified cholesterol content of the lipoproteins in the final incubation mixture was 93–310 μ g./ml. and of the 'ghosts' 58–132 μ g./ml. The ratio of the two was kept within the range 0.9–3.6.

Variation of pH. For the experiment shown in Fig. 2, borate buffers were used to give pH values above 7.4 and citric acid-phosphate buffers to give those below 7.4. Citric acid-phosphate buffers were also used for the longer experiments at pH 5.2 and 8 (Fig. 3). These buffers were prepared as described by Clark (1928). In all cases the final pH was checked with a pH-meter.

Variation of ionic strength. Na_2HPO_4 and NaH_2PO_4 were combined to give buffer solutions of pH 7.4 at various ionic strengths between 0.008 and 0.39.

Effects of Ca^{2+} . Incubations were carried out in sodium cacodylate buffer, pH 7.3 (26 mM) (Eylar *et al.* 1962), containing NaCl (0.15 M) and CaCl_2 (50 mM).

Effects of organic solvents. Because large variations in ionic strength did not affect the rate of exchange of cholesterol molecules, solvents were added to the standard phosphate buffer solution to give the required concentration without correction for the dilution of the buffer salts.

Determination of exchange rates. After incubation, 'ghosts' were sedimented by centrifuging at 20000g for 10 min. and washed three times with 0.15 M-NaCl. Lipid extraction of plasma and 'ghosts', precipitation of unesterified cholesterol with digitonin and determination of the cholesterol specific activity were carried out as described by Basford *et al.* (1964).

The extent of exchange was expressed as a percentage as follows.

If there are a mg. of unesterified cholesterol in the lipoproteins and b mg., containing initially x counts/min., in the

'ghosts', then, if complete exchange occurs, the specific activity of both 'ghost' and lipoprotein unesterified cholesterol will be $x/(a+b)$ counts/min./mg. If, at a given time, the unesterified cholesterol of the 'ghosts' has a specific activity of y counts/min./mg. and that of the lipoproteins is z counts/min./mg., the percentage of complete exchange is:

(a) from the low-density lipoproteins:

$$\frac{z(a+b)}{x} \times 100$$

(b) from the 'ghosts':

$$\frac{x/b-y}{x/b-x/(a+b)} \times 100$$

The mean of these two values was used except where otherwise stated. In most cases, the two differed by only 2–6%, although in some the difference was as great as 11–17%. Where small differences between controls and tests are being considered, both values are given. However, all conclusions are based on several results obtained with a number of different concentrations, times of incubation or chemical additives. Expression of results as percentages of a control value (Figs. 2 and 4) is only valid if the individual control values are close to each other. Where this was done, the individual controls differed by less than 5%.

RESULTS

The exchange of unesterified cholesterol between [^{14}C]cholesterol-labelled rat erythrocyte 'ghosts' and human low-density lipoproteins under standard conditions is shown in Fig. 1. As the specific activities of 'ghost' and lipoprotein cholesterol

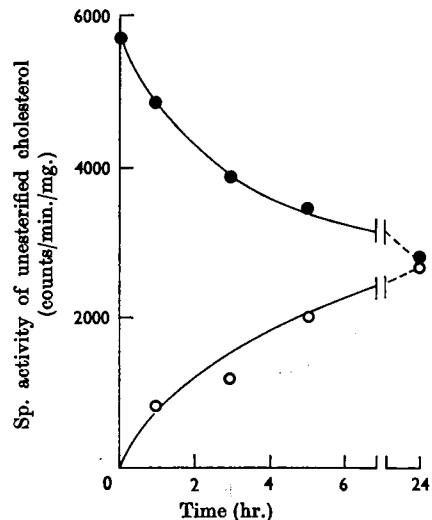


Fig. 1. Exchange of unesterified cholesterol molecules between rat erythrocyte 'ghosts' and human low-density lipoproteins. The conditions are described in the Materials and Methods section. O, Low-density lipoproteins; ●, erythrocyte 'ghosts'.

approach the same level, all of the unesterified sterol in both takes part in the process. The half-time is 160 min. and the exchange process appears identical with that seen with intact erythrocytes and lipoproteins (Basford *et al.* 1964).

It may be seen by comparing the control values obtained in all experiments that, even under standard conditions, the exchange rate varies. The reason is not known. It does not result from differences in the age of the erythrocytes used for 'ghost' preparation or in the ratio of 'ghosts' to lipoprotein (Basford *et al.* 1966). It may depend on differences in the composition of the lipoproteins from different individuals (Nelson & Freeman, 1960), since it is known that the cholesterol exchange rate does vary with the lipid composition of plasma lipoproteins (Spritz, 1965). In all experiments the same 'ghost' and lipoprotein preparations, in the same proportions, were used for tests and controls.

Effect of pH. Variation of exchange with pH is shown in Fig. 2. The rate is minimal at about pH 5 and increases more than twofold as the pH is raised to 11.1. To ensure that the results obtained by measurements of exchange at one time-interval were valid, a further experiment was carried out in which exchange was followed at two pH values over 24 hr. To rule out any differences that might have been caused by using different buffers for pH values above and below 7.4, the same buffer system (citric acid-phosphate) was used at pH 5.2 and 8.0. The results over the first 5 hr. are shown in Fig. 3. In

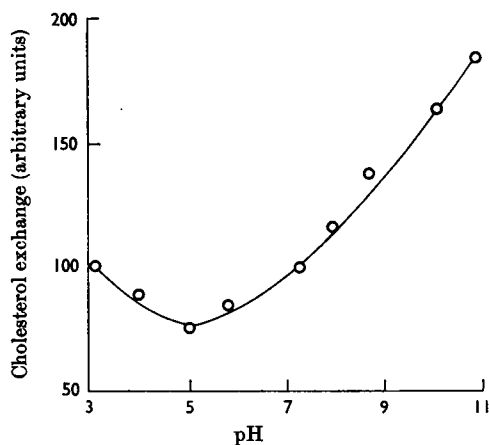


Fig. 2. Variation of cholesterol exchange with pH. The exchange was measured at 4 hr., the conditions being as described in the Materials and Methods section. To allow comparison of different experiments, the exchange is expressed as a percentage of that of the control (carried out at pH 7.4) for each experiment.

both cases exchange was about 95% complete in 24 hr.

Effect of ionic strength. As the effect of pH was studied at fairly high ionic strengths where electrostatic interactions, which could be important in the exchange process, are suppressed, exchange was studied at a number of different ionic strengths at pH 7.4. In addition, the effects of Ca^{2+} ions, which have profound effects on many properties of cell membranes, were investigated. The results (Table 1) show that there is no consistent variation in the rate of exchange over a large range of ionic strengths. Nor do Ca^{2+} ions have any marked effect even although the most concentrated solution has an

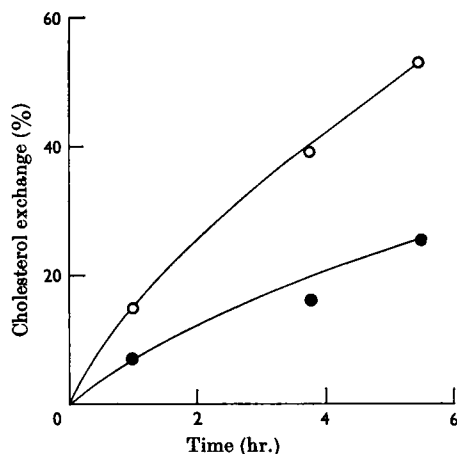


Fig. 3. Comparison of cholesterol exchange rates at pH 5.0 and 8.2. Details are given in the text. ●, pH 5.0; ○, pH 8.2.

Table 1. *Effect of increasing the concentration of (a) phosphate buffer and (b) calcium chloride on cholesterol exchange*

The CaCl_2 was added to sodium cacodylate buffer, pH 7.3 (26 mM), containing NaCl (0.15 M). Other conditions were as given in the Materials and Methods section and exchange was measured after 2 hr.

(a) In phosphate buffer		(b) In sodium cacodylate- CaCl_2	
Ionic strength	Exchange (%)	Concn. of CaCl_2 (M)	Exchange (%)*
0.0098	38.5	0	31.5
0.039	33.2	0.05	22.3
0.098	37.2	0.1	28.8
0.195	35.4	0.125	31.1
0.39	46.2	1.0	25.8

* Calculated from the specific activity of the low-density lipoprotein cholesterol only.

ionic strength greater than 3. It is thus unlikely that electrostatic forces play any significant role.

Basford (1965) found that hyperosmotic salt solutions caused inhibition of exchange when intact erythrocytes were used. This was due to the osmotic shrinking of the cells rather than to any specific ionic effect, since sucrose of the same osmoticity caused the same degree of inhibition. The 'ghosts' used in this work did not respond osmotically to changes in salt concentration, but did respond to

sucrose as determined by measurement of packed 'ghost' volume and extinction at 520 m μ . Exchange of cholesterol was measured in 0.27 M-sucrose solution at the same time as the experiments in Table 1 were performed. The exchange was 24.7% complete after 2 hr. incubation as compared with 33-46% in the buffer solutions. The packed 'ghost' volume in the sucrose was only 71% of that in 0.1 M-phosphate buffer.

Effect of modification of surface charge. Nearly all of the net charge on mammalian erythrocytes, including those of the rat, is due to sialic acid and this can be removed by treatment with neuraminidase (Eylar *et al.* 1962; Glaeser & Mel, 1964, 1966). The action of phospholipase D on isolated low-density lipoproteins has not been reported previously, although it is known to remove virtually all of the choline in the phosphatidylcholine of human high-density lipoprotein (Ashworth & Green, 1963). The effects of enzymic digestion of 'ghosts' and lipoproteins on cholesterol exchange are shown in Table 2. The lack of effect of removal of these charged groups from the two components of the system is consistent with the conclusions drawn from Table 1.

Effect of temperature. In this system, cholesterol exchange shows little temperature-dependence, since the rate over the first 2 hr. does not quite double when the temperature increases from 2° to 50° (Fig. 4). Murphy (1962b) studied the effects of temperature on the exchange of cholesterol between intact human erythrocytes and whole human serum. Although he did not measure specific activities, his results suggested that temperature affects the process much more in the more complicated system he used.

Effect of modification of solvent. The effects of a number of different chemical agents on exchange rates are shown in Table 3. All of the reagents were also tested against intact erythrocytes under the conditions used. None caused any significant haemolysis, indicating that there was no gross disruption of membrane structure. The dramatic increases in exchange rate confirm the findings of Basford (1965) with intact erythrocytes. He found

Table 2. *Effect of removal of neuraminic acid from 'ghosts' and of choline from low-density lipoproteins on cholesterol exchange*

Details are given in the Materials and Methods section. Exchange was measured after 4 hr.

Expt. no.	Treatment of 'ghosts'	Treatment of low-density lipoproteins	Exchange (%)
1	None	None	31.5
	Neuraminidase-treated	None	28.9
2	None	None	38.7
	None	Phospholipase D-treated	34.1

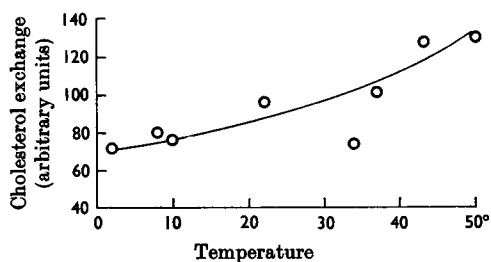


Fig. 4. Dependence of cholesterol exchange on temperature. Incubation was carried out for 2 hr. as described in the Materials and Methods section. To allow correlation of separate experiments, the exchange is expressed as a percentage of that of the control (performed at 37°) for each experiment.

Table 3. *Effect of organic compounds on cholesterol exchange*

The experiments were carried out as described in the Materials and Methods section, the exchange being measured after 4 hr.

Compound added	Concn. (M)	Exchange (%)	Compound added	Concn. (M)	Exchange (%)
None	—	62.7	None	—	37.2
Dioxan	1.5	91.9	Methanol	2.0	53.6
Urea	3.3	85.0	Ethanol	1.4	62.8
Guanidine hydrochloride	3.3	76.7	Ethylene glycol	3.0	62.2
			Acetone	2.1	100

Table 4. Comparison of the effects of acetone and closely related compounds on cholesterol exchange

The exchange was measured after incubation for 2 hr. Details are given in the text.

Expt.	Compound added	Concn. (M)	Exchange (%)
A	None	—	19.9
	Acetone	2	87.4
	Dimethyl sulphoxide	2	45.5
B	None	—	40.9
	Acetone	1	85.0
	Butan-2-one	1	92.8
	Propan-2-ol	1	66.0

that, in the presence of 15% (v/v) dimethyl sulphoxide, the half-time of exchange was lowered from 250 min. to 90 min. In neither investigation was any net transfer of cholesterol detected; apart from the great increase in rate, the process seemed to be the same as that in control experiments.

Because acetone appeared to be the most effective of the reagents tested, its action was compared with those of several closely related compounds (Table 4). Replacing the ketone group by a hydroxyl or sulphoxide group lowers the effectiveness. However if an extra methylene group is inserted, the effectiveness of the molecule is increased. This latter finding and the greater effect of ethanol than methanol (Table 3) recall the work of Hippel & Wong (1965) on the effect of inserting methylene groups into compounds that affect the thermal transition of ribonuclease.

In the foregoing experiments, the differences between the homologues were similar in magnitude to the differences between the exchange values calculated for each compound from the 'ghost' and lipoprotein cholesterol specific activities. Thus the extent of exchange calculated respectively from the 'ghost' and lipoprotein specific activities were: methanol, 55.6 and 50.5; ethanol, 65.9 and 59.7; acetone 82.4 and 87.6; butan-2-one 85.5 and 100%. Because of this, further study of the subject was necessary. In addition, since all of the compounds hitherto investigated were 'structure-breakers' (Frank & Evans, 1945), it was considered necessary to study some compounds that promote structure formation in water. Both of these factors were covered by studying the action of a homologous series of tetra-alkylammonium salts, which are all 'structure-formers' (Frank & Evans, 1945). The results (Fig. 5) demonstrate that these 'structure-forming' compounds have the same effect on cholesterol exchange as the 'structure-breaking' compounds and that increasing the alkyl chain length increases their effectiveness.

The above findings raised the possibility that the

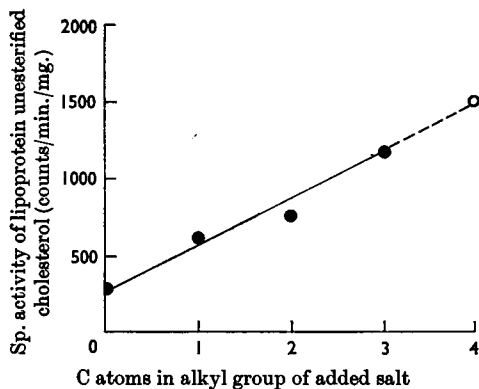


Fig. 5. Effect of tetra-alkylammonium salts on cholesterol exchange. Rat erythrocyte 'ghosts', labelled with $[^{14}\text{C}]$ -cholesterol, were incubated with human low-density lipoproteins in the presence and absence (control) of the salts. After 4 hr. incubation, the specific activity of the unesterified cholesterol in the lipoproteins was measured. The incubation conditions are described in the Materials and Methods section. Tetramethyl-, tetraethyl- and tetrapropylammonium iodide were added to a final concentration of 0.5 M and the results obtained are plotted directly. Tetra-butylammonium iodide was added to 0.125 M and the theoretical specific activity for the presence of 0.5 M-salt calculated by subtracting the control specific activity, multiplying by 4 and adding back the control value. The results are plotted in terms of the number of methylene groups present in the added salt (Hippel & Wong, 1965).

Table 5. Effect of temperature on the exchange of cholesterol in the presence of acetone and dimethyl sulphoxide

Acetone was present in 1 M concentration and dimethyl sulphoxide in 1.7 M. Other conditions were as given in the text, exchange being measured after incubation for 2 hr.

Expt.	Compound added	Temp.	Exchange (%)
A	None	10°	14.5
	None	37	21.1
	Dimethyl sulphoxide	10	20.8
	Dimethyl sulphoxide	37	54.0
B	None	8	35.3
	None	37	40.9
	Acetone	8	31.1
	Acetone	37	85.0

action of all of these chemical agents was on hydrophobic interactions. These are very much weaker at low temperatures (Scheraga, Némethy & Steinberg, 1962), and so the relative effectiveness of acetone and dimethyl sulphoxide at two different temperatures was determined (Table 5). It appears that the value for exchange in the presence of

acetone at 8° is too low, but it is obvious that both compounds have very much more effect at 37° than at the lower temperatures, indicating that their effect is indeed on hydrophobic bonding.

The normal temperature-exchange curve (Fig. 4) is thus the sum of two effects: (1) an increase in exchange rate with increasing temperature, presumably due to the raised kinetic energy of the molecules involved; (2) a decrease in exchange rate with increasing temperature due to the greater strength of hydrophobic bonding.

DISCUSSION

The view has been put forward by Vandenheuvel (1966) that the exchange of lipid between erythrocytes and lipoprotein molecules depends on certain lipid molecules acquiring sufficient kinetic energy, as a result of bombardment by water molecules, to escape from the erythrocyte membrane of plasma lipoprotein into the medium. These are then recaptured by the reverse process. If this were the case the process should be markedly temperature-dependent, and this is not so. A much more telling piece of evidence, which is not consistent with this theory, is the finding (Basford *et al.* 1964; J. M. Graham & C. Green, unpublished work) that cholesterol-depleted erythrocytes do not increase their sterol content on incubation with low-density lipoproteins, although the remaining cholesterol exchanges normally. Cholesterol-depleted lipoproteins, on the other hand, do replenish themselves at the expense of erythrocytes.

The variation in the rate of exchange of cholesterol between erythrocyte 'ghosts' and low-density lipoproteins with pH probably results from changes in the protein of the 'ghosts', or the lipoproteins, or both. It cannot depend on titration of the 'ghost' sialic acid since this group is completely ionized over most of the range studied. Nor does it appear to be the result of changes in the phospholipids, since phosphatidylcholine, and presumably sphingomyelin, is isoelectric over most of the relevant pH range (Anderson & Pethica, 1956). These phospholipids make up 80% of those of the rat erythrocyte and 94% of those of the low-density lipoproteins (DeGier & van Deenen, 1964; Nelson & Freeman, 1960). Measurement of the extinction of a suspension of 'ghosts' over the pH range 3-9 showed that the size of the 'ghosts' is minimal at about pH 5. It seems probable that the pH results are related to the expansion and contraction of the membrane of the 'ghosts', since it has been shown that osmotic shrinkage of whole cells (Basford, 1965) or of 'ghosts' (see above) results in a decreased exchange rate.

The interactions of the lipoproteins with erythrocyte 'ghosts' are not comparable with those

between emulsion particles stabilized by ionic amphipaths, since the latter are strongly dependent on ionic strength. Concentrated calcium chloride solution in particular should cause a big decrease in the size of the Gouy-Chapman layer around the particles and yet it has no effect on the cholesterol exchange rate. This lack of effect is not due to the presence of saturating concentrations of Ca^{2+} in 'ghosts' or lipoproteins (or both), as is demonstrated by a normal exchange picture in the presence of citrate (Figs. 2 and 3).

Many studies of biological processes similar to that shown in Table 3 have been made, and several explanations for the action of such series of compounds have been put forward. It can readily be shown from the results of Tables 3-5 that there is no correlation between alterations in exchange rate and: (1) the proportion of water in the final medium; (2) the viscosity of the medium; (3) the surface tension of the medium; (4) the dielectric constant of the medium; (5) the ether/water partition coefficient of the added compound; (6) whether the added compound is a 'structure-maker' or 'structure-breaker' (Frank & Evans, 1945). The finding that exchange is increased by urea and tetra-alkylammonium salts, which increase the dielectric constant of the medium, as well as by the organic solvents, which all decrease it, confirms the conclusion that electrostatic interactions are not important.

The one property that all of the compounds used have in common is a potent effect on the local structure of water. According to Hippel & Wong (1965) their action on the solvent structure results in fewer water molecules being available for incorporation into 'icebergs' around non-polar regions of lipoproteins and 'ghosts'. In consequence hydrophobic bonding is weakened and exposure of non-polar structures to the aqueous medium is increased. The effects of chain-lengthening in the tetra-alkylammonium salts (Fig. 5), and of employing acetone and dimethyl sulphoxide at different temperatures, strongly support this theory of action.

Most of the compounds used can act as hydrogen-bond donors or acceptors or both, but the concentrations used are, in most cases, below those needed to produce observable effects (Simpson & Kauzmann, 1953; Laskowski, 1966) and there is no correlation between the alterations in exchange rate and the relative abilities of the added compounds to form hydrogen bonds.

Since it appears that exchange of cholesterol molecules must be brought about by a collision of 'ghost' and lipoprotein (Gurd, 1960), it may be proposed that, on contact, small discrete areas of lipid fuse and so allow diffusion of cholesterol to occur. The main attractive forces involved in this interaction are probably van der Waals-London

dispersion forces. The areas of lipid that fuse are likely to be small, since the 'ghosts' and lipoproteins separate again, presumably under the influence of hydrodynamic and thermal forces, without any detectable disruption of structure or change in composition. Further, if large areas of lipid fused, then one would expect phospholipid molecules to exchange much more readily than they do. To account for the finding that sterol-depleted low-density lipoproteins replenish themselves at the expense of the erythrocyte whereas the converse does not occur, Basford *et al.* (1964) proposed that the fusion of lipid areas only occurs at sites on the erythrocyte containing cholesterol molecules. Once the latter are lost, fusion cannot occur. The rate of exchange depends on the tightness of packing of the molecules within the erythrocyte membrane, as shown by the effects of osmotic shrinking and pH. The effect of the weakening of hydrophobic bonds in increasing the rate of exchange could be because the increased exposure of hydrophobic regions of lipid molecules at the surface facilitates the interaction of 'ghost' and lipoprotein, or because individual molecules can move more freely as the bonds are weakened, or both.

Structure of low-density lipoproteins and cellular membranes. The results obtained in this study and those presented in a brief communication (Basford *et al.* 1964) bear directly on theories of the structure of cellular membranes and soluble lipoproteins.

The finding that low-density lipoproteins of *S*₃₋₉ are precipitated from solution on incubation with phospholipase C indicates that, even in this fraction where the protein/lipid ratio is highest, phospholipid ionic groups are needed to maintain the stability of the complex. As phospholipase D treatment removes most of the choline but causes no precipitation, it is the phosphoric acid residue that is needed for stabilization. Since the same treatment does not change the cholesterol exchange rate, internal charge neutralization within the surface phospholipid monolayer is not of significance in regulating the binding of the less-polar lipid molecules. The ease with which the unesterified cholesterol is esterified (Murphy, 1962*a*) and with which the phospholipids are attacked by phospholipases C and D, as well as by phospholipase A (Condrea, de Vries & Mager, 1962), strongly suggests that the lipid molecules are directly exposed to the aqueous medium as in the classical view of the lipoprotein structure (Gurd, 1960). Extensive coverage of the lipid by protein (Vandenheuevel, 1962) is unlikely, particularly since peptidases, in contrast with the above enzymes, have only a limited action on low-density lipoproteins (Banaszak & McDonald, 1962; Bernfeld & Kelley, 1966; Margolis & Langdon, 1966).

The results of this study also indicate that the

structure for the erythrocyte membrane proposed by Vandenheuevel (1965), which is maintained mainly by interactions between polar groups and in which hydrophobic bonds make no contribution (Vandenheuevel, 1966; Maddy & Malcolm, 1966), is improbable. The 'disk' structure of cellular membranes proposed by Kavenau (1965) is open to similar objections (Maddy & Malcolm, 1966), and his alternative 'pillar' form cannot be involved as the 'ghosts' have no source of energy to maintain it. Maddy & Malcolm (1965) have suggested that cholesterol molecules, held together by hydrogen bonds, dissolve in the region of the phospholipid hydrocarbon chains in the membrane. If this is so, it is difficult to explain why cholesterol esters do not enter this region and why cholesterol-depleted erythrocytes do not increase their sterol content during incubation with low-density lipoproteins.

It seems much more probable that the erythrocyte membrane is a mosaic of lipid and protein. This has been proposed several times (Ponder, 1949; Parpart & Ballentine, 1952) and is supported by chemical evidence (Moskowitz & Calvin, 1952; Maddy, 1966; Maddy & Malcolm, 1965) as well as by morphological studies (Hillier & Hoffman, 1953; Glaeser, Hayes, Mel & Tobias, 1966). It is also in line with modern views on the structure of other cellular membranes (Green & Perdue, 1966; Chance & Mela, 1966). If the membrane were made up of a mosaic of protein molecules and lipid patches, of different size and composition, it would explain the hydrophobic nature of the erythrocyte surface (Mudd & Mudd, 1926), the non-uniform distribution of cholesterol molecules over it (Murphy, 1965) and the great importance of hydrophobic bonding.

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