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THE TRANSFER OF PROTEINS AND LIPIDS FROM PLASMA TO LYMPH IN THE LEG OF THE NORMAL AND HYPERCHOLESTEROLAEMIC RABBIT

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Experimental evidence concerning the exchange of materials between the circulating plasma and the tissue fluid supports the view that in the capillary wall there are pores through which diffusion of the small molecules and ions is relatively free, whereas diffusion of larger molecules is restricted (cf. Pappenheimer, 1953; Yoffey & Courtice, 1956). Pappenheimer postulated that pores of 60–90 Å in diameter in the capillaries of the preparation that he used, the mammalian hind leg, would account for the observed rates of transfer across the capillary wall of water and of lipid-insoluble molecules of various sizes. The position of these pores has not been clearly defined, although Chambers & Zweifach (1947) gave evidence which suggested that they were confined to the intercellular substance. Pappenheimer's experiments supported this view in that the total area of the pores was such that they could be limited to this region.

This concept of capillary permeability has been challenged in recent years by several workers using the electron microscope to determine the fine structure of the vascular endothelium. Bennett, Luft & Hampton (1959) have classified capillaries, according to the structure of the endothelium, into three main classes as follows: first, those in the liver and spleen, where there are intercellular gaps up to several thousand Å across, secondly, those in such tissues as the small intestine, kidney, parathyroid and hypophysis, in which intracellular fenestrations of 300–600 Å in diameter have been observed, and finally those in which there are no intercellular or intracellular pores. The third class is found in cardiac and skeletal muscle, lung, nervous system and skin, and in the endothelial cells of these capillaries there are numerous vesicles of endoplasmic reticulum, 200–600 Å in diameter. The basement membranes lining these different types of capillaries may also vary considerably in thickness and differentially affect the passage of materials from plasma to lymph. These findings in general confirm the work of others, e.g. Palade (1953, 1956), Fawcett (1955), Pease (1955), and have given rise to further controversy

concerning the mechanisms of transfer of materials across the capillary membrane.

Interest has also recently been aroused in the passage of various lipid complexes through the vascular endothelium, for it is thought that this phenomenon is concerned in the deposition of lipids in the intima of arteries in atherosclerosis. Electron microscopy of this endothelium has revealed the absence of pores or fenestrations, but the presence in the endothelial cells of numerous vesicles of cytoplasmic membrane similar to those observed in the cells of capillary endothelium (Buck, 1958).

The lipid complexes of the plasma are all much larger than the pores postulated by Pappenheimer. They vary in size from 150 Å, the probable size of the high-density α-lipoprotein (Hayes & Hewitt, 1957) to 5000 Å or more, the size of some chylomicrons, and are stabilized in an aqueous medium by the hydrophilic properties of phospholipid and protein.

Experiments have shown that these complexes may pass through the capillary membrane (Kellner, 1954; Courtice & Morris, 1955; Morris & Courtice, 1956) and that the ease with which they are transferred may in some way be related to their size (Courtice, 1959*a*, *b*). In these latter experiments, it was shown that whereas the lipid complexes in the lipaemic plasma of the rabbit fed on cholesterol or given Triton WR-1339 intravenously appeared in the lymph of the paw, the larger lipid particles of chyle or of the artificial fat emulsion 'Lipomul' (Upjohn) given intravenously did not do so to any significant extent.

The present investigations are a continuation of these experiments. They have been devised to show whether, in the hypercholesterolaemic rabbit, the passage of the various lipoprotein complexes across the membrane of the skin capillaries is related to the size of the complex concerned.

METHODS

Hutch-bred rabbits of various strains were used as experimental animals. Hypercholesterolaemia was produced by feeding the rabbits on pollard (bran-type flour from wheat) with which had been mixed powdered cholesterol in a blender. Each rabbit was given daily 75 g of pollard containing 1 g of cholesterol for the first week, and thereafter the cholesterol level was usually maintained at a level of 500–1000 mg/100 ml. by giving either this mixture or the pollard without cholesterol; green vegetables were also given several times a week.

To determine the plasma–lymph gradients across the wall of the skin capillaries, the animals were anaesthetized with pentobarbital (Abbott's Veterinary Nembutal) intravenously. Blood samples were taken from a catheter in the carotid artery and lymph samples from a lymphatic vessel just below the popliteal fossa (Courtice, 1959*a*). When a sample of lymph had been collected over a period of approximately 1 hr, the paw was injured by immersion in water at 70° C for 1 min. Before this procedure the animal was very deeply anaesthetized with ether. Two samples of lymph were then collected during the subsequent hour. The paw was kept in a water-bath at a constant temperature of 43–45° C throughout lymph collection, so as to ensure a fairly uniform blood flow. Lymph flow was promoted by passive movement of the leg and massage along the pathway of the duct.

Analytical methods. Total protein in plasma and lymph was determined by micro-Kjeldahl digestion, distillation and Nesslerization. The individual proteins were determined by paper electrophoresis on Whatman No. 1 paper, elution and determination of the dye, and measurement of the areas of the dye curves with a planimeter. As the globulins bind less dye than albumin, the areas of the dye curves for globulins were multiplied by the following factors: γ-globulin plus fibrinogen, 1·76; β-globulin, 1·40; and α-globulin, 1·50, before the protein concentrations were calculated. The detailed procedure adopted for these determinations is described elsewhere (Courtice, 1960). Total cholesterol in plasma and lymph was determined by the method of Abell, Levy, Brodie & Kendall (1952) and phospholipid by the method of Zilversmit & Davis (1950).

RESULTS

The lipid complexes in the plasma of normal and hypercholesterolaemic rabbits

In the normal rabbit fed on the pellets used in this laboratory, the cholesterol and phospholipid levels in the plasma were on an average 60 and 143 mg/100 ml. respectively and somewhat higher, 162 and 174 mg/100 ml., when fed on pollard. Havel, Eder & Bragdon (1955) by means of the preparative ultracentrifuge showed that these lipids were divided mainly between the high-density or α-lipoprotein and the lipoproteins within the density range 1·019–1·063 or β-lipoprotein. Hayes & Hewitt (1957) determined the size of these lipoproteins in human plasma by the electron microscope and found the diameter of the hydrated molecule to be 150 Å for the α-lipoprotein and 350 Å for the β-lipoprotein.

With increased cholesterol levels in the plasma of the cholesterol-fed rabbit, Gofman, Lindgren, Elliott, Mantz, Hewitt, Strisower, Herring & Lyon (1950) showed by the analytical ultracentrifuge that while there was a moderate increase in the concentration of these smaller lipoproteins, the main increase was in less dense complexes which dispersed light to make the plasma appear opalescent or milky. Under dark-ground illumination of the light microscope, very small particles up to approximately 0·2 μ (2000 Å) in diameter can be seen. The range in size of these lipoproteins is not known at present, but preliminary investigations by electron microscopy show that in such plasma the variation is from 150 Å to 1500 Å, with some larger particles.

In composition these lipid complexes in the plasma of the hypercholesterolaemic rabbit consist mainly of cholesterol and cholesterol esters stabilized with phospholipid and protein, and the density varies directly with the proportion of protein to lipid. In the lipid portion of the molecule or complex the ratio of cholesterol to phospholipid varies inversely with the density. In the high-density or α-lipoprotein fraction this ratio is on an average 0·4–0·5; as the complexes become less dense, it increases considerably to reach values of 3·0 or even higher. The relation between the cholesterol:phospholipid ratio and the total cholesterol in the plasma is

shown in Fig. 1. When the total cholesterol in the plasma was less than 100 mg/100 ml. the cholesterol:phospholipid ratio was on the average 0·49 and the lipid was mainly in the high-density lipoprotein. With levels between 100 and 200 mg/100 ml. the ratio rose to a mean of 0·93 and more of the lipid was present as β-lipoprotein, as determined qualitatively by zone electrophoresis. Above this level the ratio rose more gradually to values of 3·00 and the lipid was present mainly as the low-density complexes which gave the plasma a milky appearance. While the actual size of the

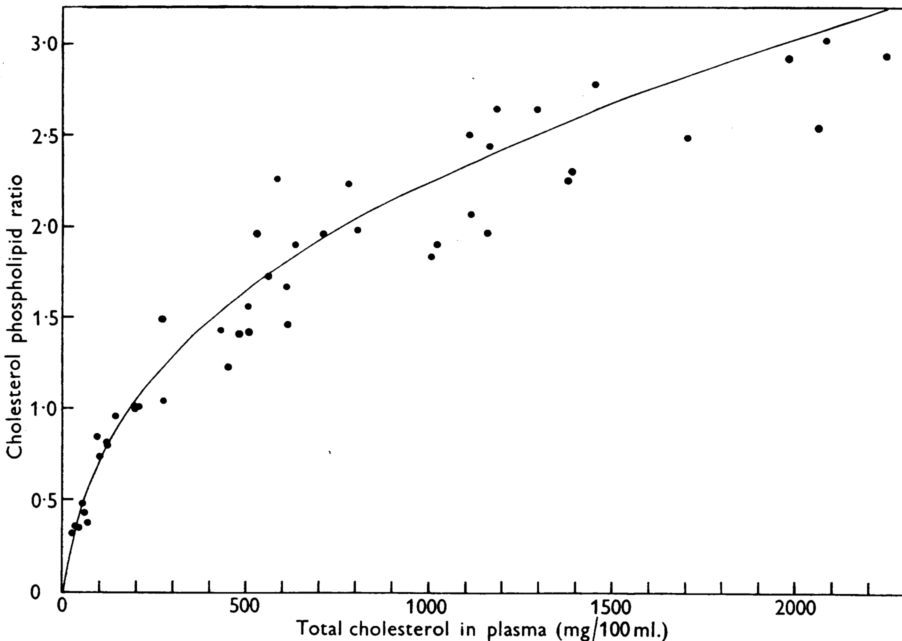

Fig. 1. The relationship between the level of cholesterol and the cholesterol: phospholipid ratio in the plasma of normal and hypercholesterolaemic rabbits.

different lipoproteins in the plasma has not yet been accurately determined, preliminary experiments suggest that this runs roughly parallel with the cholesterol:phospholipid ratio.

The plasma–leg lymph gradients of protein and lipids in normal and hypercholesterolaemic rabbits, before and after thermal burn to leg

In order to determine the protein and lipid gradients from plasma to lymph it was necessary to ascertain whether any changes in the composition of the plasma occurred while the lymph was being collected. Blood samples were obtained before Nembutal anaesthesia (from ear vein) and under anaesthesia (from the carotid artery) while lymph was being

collected, both before injury and at the end of the experiment after injury. The average figures, given in Table 1, showed a slight fall in the total plasma proteins throughout the experiment and a slight rise in cholesterol and phospholipid. In relating the levels in the lymph to those in the plasma, the figures for the plasma during anaesthesia before injury were used. Any changes in the levels in the plasma would be reflected in the

TABLE 1. Total protein, cholesterol and phospholipid concentration in plasma taken before Nembutal anaesthesia and during anaesthesia before and after thermal injury to the paw. Expressed as mean and standard error of the mean. The figures in parentheses give the numbers of observations

		Before anaesthesia	Under Nembutal anaesthesia	
			Before injury	After injury
Total protein (g/100 ml.)	(14)	6·86 ± 0·17	6·70 ± 0·16	6·47 ± 0·18
Total cholesterol (mg/100 ml.)				
Normal	(9)	143 ± 16	143 ± 17	140 ± 20
Hypercholesterolaemic	(8)	929 ± 214	1005 ± 224	1022 ± 213
Phospholipid (mg/100 ml.)				
Normal	(9)	168 ± 12	154 ± 12	153 ± 13
Hypercholesterolaemic	(8)	422 ± 55	446 ± 61	454 ± 44

TABLE 2. The protein and lipid levels in the plasma and leg lymph of normal and of hypercholesterolaemic rabbits, expressed as the mean and standard error of the mean. The figures in parentheses give the number of observations

	Total protein (g/100 ml.)	Albumin (g/100 ml.)	α-globulin (g/100 ml.)	β-globulin (g/100 ml.)	γ-globulin and fibrinogen (g/100 ml.)	Total cholesterol (mg/100 ml.)	Phospholipid (mg/100 ml.)
Normal (6)							
Plasma	6·76	3·70	0·72	0·96	1·37	149	143
	± 0·18	± 0·11	± 0·05	± 0·08	± 0·17	± 22	± 16
Lymph, before	2·81	1·75	0·28	0·39	0·40	35	47
injury	± 0·30	± 0·16	± 0·03	± 0·05	± 0·09	± 6	± 4
Lymph, after	5·54	3·14	0·56	0·80	1·04	90	106
injury	± 0·21	± 0·16	± 0·06	± 0·05	± 0·15	± 16	± 12
Hypercholesterolaemic (9)							
Plasma	6·66	3·49	0·70	1·19	1·28	798	407
	± 0·25	± 0·23	± 0·06	± 0·08	± 0·17	± 117	± 36
Lymph, before	2·78	1·68	0·27	0·36	0·47	98	83
injury	± 0·26	± 0·25	± 0·03	± 0·04	± 0·06	± 11	± 10
Lymph, after	4·97	2·71	0·47	0·77	1·02	371	209
injury	± 0·34	± 0·23	± 0·05	± 0·06	± 0·13	± 53	15

values for lymph after a lag for mixing in the tissue-fluid pool. This mixing would be relatively slow before injury and rapid after injury. As there is little difference in the levels of protein and lipids in the two samples taken during anaesthesia, it was thought that these levels should be used for comparison with the values for lymph.

While the plasma–lymph gradients for the lipids were determined in a larger series, the individual plasma proteins, together with the lipid levels

were measured in six normal and nine hypercholesterolaemic animals. The results are given in Table 2. The mean levels of each individual protein in the plasma and in the lymph before injury were approximately the same in both groups of animals. In each group the blood pressure, determined by the capsule method in the ear artery, was approximately the same, and the limb was kept at a constant temperature while lymph was collected. The fact that the plasma–lymph gradients for the different proteins were approximately the same in both groups under these conditions would

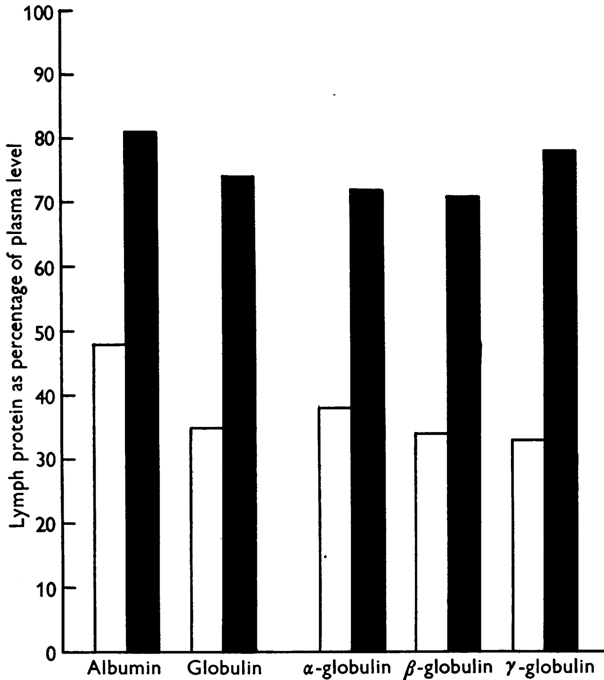

Fig. 2. The mean levels of the various protein fractions in the lymph from the leg, expressed as percentages of the corresponding levels in the plasma of a group of 15 rabbits. □, before injury; ■, after thermal injury to leg.

suggest that there was no appreciable change in permeability of the skin capillaries to the plasma proteins due to the increased lipid levels. When the leg was injured by a thermal burn, however, gross oedema rapidly developed and the lymph flow increased greatly (cf. Courtice, 1946). The concentrations of each of the protein fractions and of the lipids in the lymph rose considerably.

When expressed as a percentage of the plasma levels, the concentrations of the proteins in the lymph were as shown in Fig. 2. In lymph from the leg before injury the plasma–lymph gradient for albumin was less than for

total globulin or for any of the individual globulins. The mean concentration of albumin in the lymph in these experiments was 48 % while that of globulin was 35 % of the corresponding levels in the plasma. The relative levels of each of the individual globulin fractions did not vary appreciably from that of the mean total globulin. In lymph after injury the difference in the gradients for albumin and globulin was less marked, but still evident. The mean concentration of albumin in the lymph was 81 % while that of the total globulin was 74 % of the corresponding levels in the plasma.

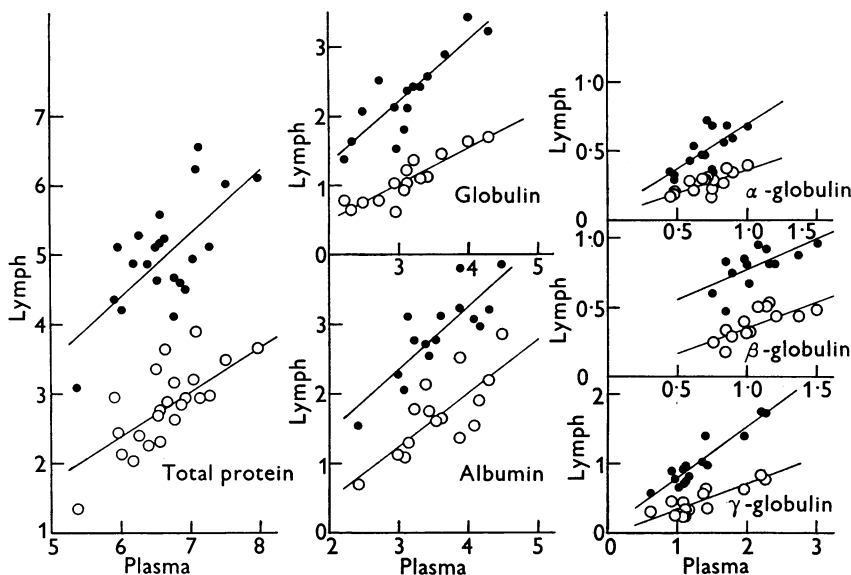

Fig. 3. The relation between the concentration of protein in lymph from the leg and that in the plasma; figures are g/100 ml. ○, before injury; ●, after thermal injury to leg.

When the individual protein levels in the lymph were plotted against those in the plasma, the regression equations showed that the concentrations in the lymph were related to those in the plasma, $P < 0{\cdot}001$ (Fig. 3). The slope of the regression line for albumin was steeper than those for the globulins, $P < 0{\cdot}05$. After injury the slope of the regression line for globulin increased to show no significant difference from that for albumin. These findings are all in conformity with the classical 'Pore Theory' of capillary permeability.

With regard to the lipid complexes, in the normal animal the levels of cholesterol and phospholipid in the lymph relative to those in the plasma were slightly less than in the α- and β-globulin (Fig. 4). In this series the levels of cholesterol and phospholipid in the lymph were respectively 24 and 33 % of the plasma levels before injury and 60 and 74 % after injury.

This is probably due to the fact that α- and β-lipoprotein molecules are larger than other globulins that migrate electrophoretically at the same rate. The finding that the relative levels of cholesterol and phospholipid varied also shows that in the normal animal there must be different plasma–lymph gradients for α- and β-lipoproteins.

In hypercholesterolaemic animals the levels of cholesterol and phospholipid in the lymph relative to those in the plasma were lower than in normal animals, i.e. the plasma–lymph gradients were greater. The average for

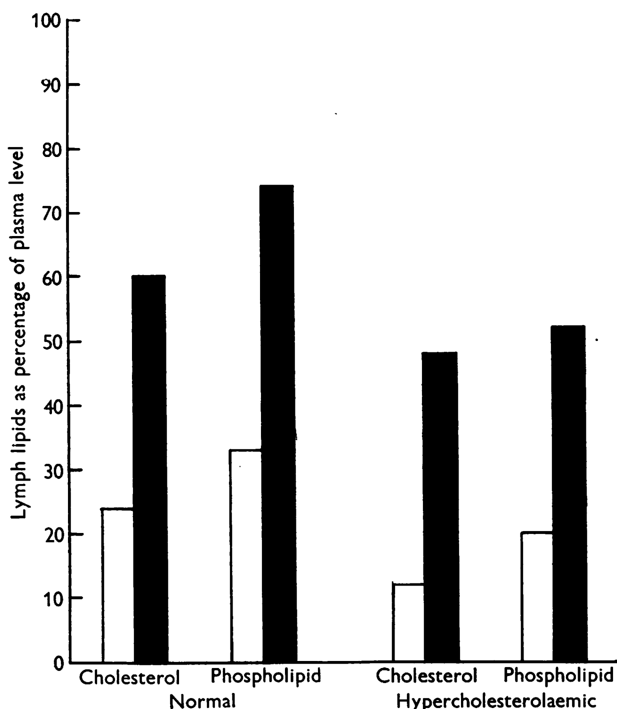

Fig. 4. The mean levels of cholesterol and phospholipid in the lymph from the leg, expressed as percentages of the corresponding levels in the plasma in a group of 6 normal and 9 hypercholesterolaemic rabbits. □, before injury; ■, after thermal injury to leg.

the experiments represented in Fig. 4 shows that the cholesterol and phospholipid levels in the lymph were 12 and 20% of the plasma levels respectively before injury and 48 and 52% after injury. These differences again show the differential filtration of the various lipid complexes in hypercholesterolaemic plasma.

In a larger series of animals in which plasma and lymph lipids have been measured, the results are depicted in Fig. 5. This shows that when the plasma cholesterol increases from 40 to 200 mg/100 ml., the lymph

cholesterol rose much more steeply than with changes in the plasma level from 500 to 2200 mg/100 ml. The changes in plasma–lymph gradient with rising plasma cholesterol are more clearly depicted in Table 3. Fourteen animals on a diet to which no cholesterol was added have been divided into two groups, with ranges of plasma cholesterol of 0–100 and 101–200 mg/100 ml. Similarly twenty-two animals on a diet to which cholesterol was

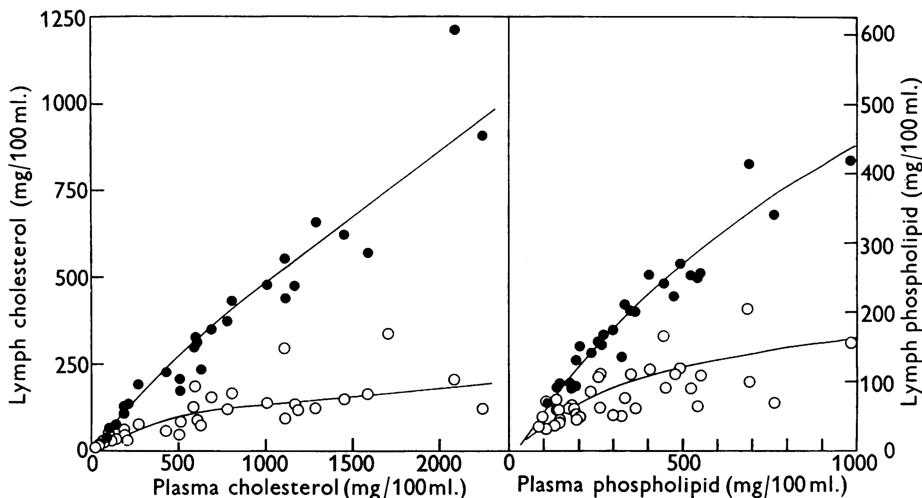

Fig. 5. The relation between the levels of cholesterol and phospholipid in the lymph from the leg and the corresponding levels in the plasma of normal and hypercholesterolaemic rabbits. ○, before injury; ●, after thermal injury to leg.

TABLE 3. The total cholesterol and the cholesterol:phospholipid ratios in plasma and leg lymph in four groups of rabbits with different plasma cholesterol levels

Plasma cholesterol range (mg/100 ml.)	No. of animals	Total cholesterol (mg/100 ml.)		$\frac{\text{Lymph cholesterol}}{\text{Plasma cholesterol}} \times 100$	Cholesterol:phospholipid ratio	
		Plasma	Lymph		Plasma	Lymph
0–100	8	60 ± 9	23 ± 5	38 ± 4	0·49 ± 0·07	0·42 ± 0·06
101–200	6	162 ± 18	37 ± 5	23 ± 2	0·93 ± 0·04	0·77 ± 0·12
201–1000	11	585 ± 46	108 ± 14	18 ± 2	2·02 ± 0·17	1·29 ± 0·10
1001–2000	11	1450 ± 126	171 ± 24	12 ± 2	2·45 ± 0·13	1·47 ± 0·10

added have been divided into two groups, with ranges of plasma cholesterol of 201–1000 and 1001–2000 mg/100 ml. In the first group, with a mean plasma cholesterol of 60 mg/100 ml., most of the cholesterol is present as α-lipoprotein. The level of cholesterol in the lymph was 38 % of the plasma level and the cholesterol:phospholipid ratio was nearly the same as that in the plasma. As the level of cholesterol in the plasma rose, the relative

concentration in the lymph fell and the difference between the cholesterol:phospholipid ratios of plasma and lymph became progressively greater.

When the skin capillaries were injured by heat, however, larger lipid complexes more readily passed into the lymph, with much less differentiation according to the size of the complexes (Fig. 5). The lymph, which was opalescent before injury, became quite milky in appearance. It would

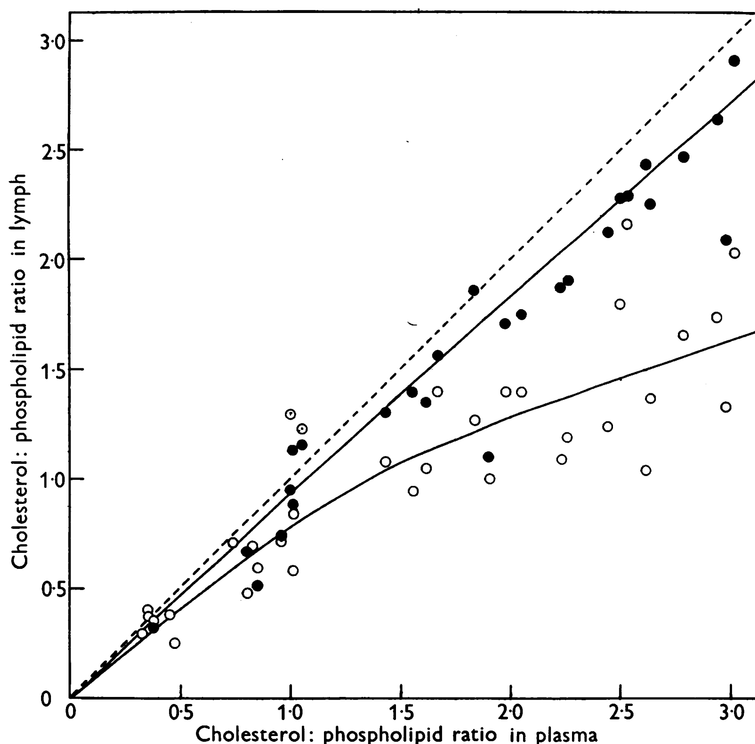

Fig. 6. The relation between the cholesterol: phospholipid ratio in the lymph to that in the plasma in normal and hypercholesterolaemic rabbits. The interrupted 45° line represents identity of ratios. ○, before injury; ●, after thermal injury to leg.

seem, therefore, that in the uninjured capillaries there is a considerable differentiation in the plasma–lymph gradients between the various lipid complexes, while in the injured capillaries this differentiation, though still detectable, is much less.

These conclusions can also be drawn when the cholesterol:phospholipid ratio in the lymph is plotted against the cholesterol:phospholipid ratio in the plasma (Fig. 6). In the uninjured limb the ratio in the lymph was approximately the same as in the plasma at levels below 0·5, when

most of the lipids were present as α-lipoprotein. As the plasma ratio rose, the lymph ratio rose less steeply, indicating that the larger, less dense complexes enter the lymph less readily than the smaller molecules. In the injured limb, however, this differentiation was much less marked, but still evident to some slight extent.

DISCUSSION

The mechanisms concerned in the transfer of macromolecules from plasma to tissue fluid and lymph are as yet far from clear. Whether these molecules are unchanged during the process of transfer is not known. Plasma lipoproteins are not inert substances; the different components to some extent exchange with other lipoproteins in the plasma and in cells. It is, therefore, conceivable that in the skin of the rabbit's paw there is a continual exchange of cholesterol and phospholipid between the lipoproteins in the plasma and lymph and those of the adjacent cells. When the same lipoproteins are found in the lymph as in the plasma, therefore, it cannot be stated for certain that these lipoproteins actually passed through the walls of the blood and lymph capillary as such. Nor can it be stated that the proportion of the different lipoproteins in the lymph is the same as that in the capillary filtrate, because the exchanges between the cells in the skin and the tissue fluid may modify this.

In the experiments in the normal and hypercholesterolaemic rabbits described here, however, the transport of cholesterol approximated to a steady state as closely as such a state can be attained. The hypercholesterolaemic rabbits had received no cholesterol in their diet for from 2 to 14 days and during the course of the experiment there was no appreciable change in the level of plasma cholesterol, and probably none in the cells of the skin, although this was not measured. It seems that in these circumstances the exchanges of cholesterol between tissue fluid and cells would be in equilibrium during the experiment and would not significantly modify the proportions of the different lipoproteins in the tissue fluid.

If this is so, the levels of the various proteins and lipoproteins in the tissue fluid, though actually higher than in capillary filtrate, would be in the same relative proportions, since these molecules do not diffuse back into the blood stream. As lymph has the same composition as tissue fluid from any region (Yoffey & Courtice, 1956), the plasma–lymph gradients of the proteins and lipoproteins will, therefore, give a relative measure of the plasma–capillary filtrate gradients.

The great increase in the rate of transfer of the various proteins and lipoproteins from plasma to lymph when the skin is injured by a thermal burn suggests that this is mainly a transfer of the complexes as they exist in the plasma. Within seconds after injury the lymph may become more

opalescent. The sudden change in the composition of the lymph and the large bulk transfer of plasma, together with the finding that the gradients for proteins and lipoproteins vary with the size of the molecule, support this view. The results show clearly that the plasma–lymph gradient progressively increases from the smallest protein molecule, albumin, to the largest lipoprotein complex found in the plasma. The experiments of Courtice (1959*a*, *b*) show further that lipid complexes which are even larger, such as the particles in chyle or the artificial fat emulsion 'Lipomul', are not transferred from plasma to lymph, even after injury, to any measurable extent. Although the diameter of all these molecules and complexes has not yet been accurately determined, it seems that the plasma–lymph gradient runs roughly parallel with their size.

If it is true, then, that these complexes pass through the capillary membrane as such, it is clear that they cannot pass through pores 60–90 Å in diameter without being grossly distorted. Electron microscopy has failed to show any fenestrations or pores in the intercellular substance except in the liver and spleen. It may be that in the living capillary the intercellular substance is in a continually changing state, as the pressure on capillaries in any region is continually changing with vasomotion. If this were so, the intercellular substance might at times be pulled somewhat apart causing fenestrations, whereas in the fixed preparation observed with the electron microscope there is a shrinkage. It is well known that in the living state large blood cells may pass between endothelial cells if they stick on the endothelium, so that large gaps may for a time be produced. Until a technique is devised for obtaining the requisite high magnifications in living capillaries, however, this question will not be settled conclusively.

If the lipoproteins do not pass through the intercellular substance, they must pass through the cells. Here the vesicles of endoplasmic reticulum may be implicated. Alksne (1959) has shown that the endothelial cell layer of the capillaries in the skin of the mouse varies in thickness from 4·0 to 0·25 μ, being thickest in the part of the cell containing the nucleus. Particles of colloidal mercuric sulphide 50–250 Å in diameter introduced into the blood-stream passed through the cytoplasm and were found within intracellular membrane structures such as the vesicles. When the capillaries were injured by histamine, there was still no evidence of pores in the intercellular substance but the vesicles increased in size, some becoming as large as 2000 Å in diameter, giving the cells a foamy or vesicular appearance. Alksne postulated an active transfer across the endothelial cells; he envisaged engulfment of material on the luminal side through the 'pinching off' of membrane-lined caveolae, the vesicles so formed being then conveyed across the endothelial cells to discharge their contents into the

extracellular space outside the endothelium. The basement membrane then forms a further barrier which affects the further transfer of any substance.

The sizes of the lipid complexes in the plasma of the hypercholesterolaemic rabbit are in conformity with the diameters of the vesicles observed in the endothelial cells of skin capillaries of the mouse before and after histamine application. The capillaries in the skin of the rabbit's paw before and after thermal injury have not yet been similarly investigated, but if the vesicles change in size, as in the mouse, it is conceivable that they might be implicated in the transfer of the lipid complexes. No one has yet reported, however, the finding of such lipid particles in these vesicles. In the endothelium of the aorta, over a plaque in the hypercholesterolaemic rabbit, Poole & Florey (1958) have shown lipid in the cytoplasm of the cell. Whether this was in the vesicles was not determined. Hence, although the experimental results suggest that the lipid complexes of the hypercholesterolaemic rabbit are filtered through a sieve-like arrangement, the mechanisms cannot as yet be clearly defined.

SUMMARY

1. The concentrations of the protein fractions, albumin and α-, β- and γ-globulins and also cholesterol and phospholipid were determined in the plasma and leg lymph of groups of rabbits fed on a normal diet and on a diet to which cholesterol was added before and after thermal injury to the leg.

2. The plasma–lymph gradients thus obtained showed that in the lymph from the uninjured leg the level of albumin was 48 % and total globulin 35 % of the corresponding concentrations in the plasma. The level of cholesterol in the lymph was on an average 38, 23, 18 and 12 % of the plasma level respectively when the plasma cholesterol was within the ranges 0–100, 101–200, 201–1000 and 1001–2000 mg/100 ml.

3. After injury the plasma–lymph gradients of all these substances were much less, and, while there was still a progressive change in the gradient from albumin to the largest lipid complex, this differentiation was less marked than in the uninjured leg.

4. The results indicated that the plasma–lymph gradient of the proteins and lipoproteins in normal and hypercholesterolaemic rabbits depended, to some extent at least, on the size of the molecule or complex concerned. Possible mechanisms of transfer of these macromolecules or complexes across the capillary wall are discussed.

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