Movement of cholesterol in vitro in rat blood and quantitation of the exchange of free cholesterol between plasma and erythrocytes

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Abstract After administration of [4-^{14}C]cholesterol to rats, blood was obtained and incubated for 6 hr or less. Incubation resulted in a net loss of erythrocyte cholesterol and, simultaneously, in an increase of esterified cholesterol in plasma and α-lipoproteins. Erythrocyte labile cholesterol was shown to be the sole precursor of esterified cholesterol. However, the relation between loss and esterification was not absolute. Loss of erythrocyte cholesterol could be inhibited without affecting esterification and vice versa. A catenary turnover model is proposed, which links in vivo erythrocyte labile cholesterol and plasma esterified cholesterol. Free cholesterol also exchanged between erythrocytes and lipoproteins. The topological model, as tested by analog computer, appears to be a bicompartmental system governed by nonconstant exchange fluxes. They are exponential functions of time and vary from 0.065 to 0.020 mg/hr/g of blood. The fitting of the curves obtained by analog computer analysis to the experimental curves requires esterification as described above. Variation of the exchange fluxes would be the consequence of lipoprotein structural alterations. If this is true, the initial value of the measured flux in vitro is identical with the in vivo value, and the turnover time of erythrocyte cholesterol is 9.2 hr. Initial exchange flux is not dependent on plasma cholesterol level or on the age of the rats, but it is temperature dependent. Addition of amphotericin B to the plasma does not modify exchange fluxes, but erythrocyte cholesterol loss is increased.

Supplementary key words flux · cholesterol esterification · lecithin:cholesterol acyltransferase · amphotericin B

THE EXCHANGE of free cholesterol between erythrocytes and plasma has been established in a number of in vivo and in vitro studies (1–9). London and Schwarz (2) demonstrated a relatively insignificant degree of synthesis of cholesterol by erythrocytes. They observed an active turnover of cholesterol by erythrocytes and concluded the existence of dynamic exchange of cell cholesterol with that of the plasma. Hagerman and Gould (1) have studied the in vitro kinetics of this interchange. Their conclusions were: (*1*) only the free cholesterol is exchangeable; (*2*) all the erythrocyte cholesterol is available for exchange; (*3*) free cholesterol specific activities approach equilibrium in 4 hr; and (*4*) plasma free cholesterol turnover time is 1.44 hr.

For the calculation of the value of 1.44 hr, Hagerman and Gould (1) used the relation $t_{1/2} = T \log 2$, which is only applicable to a single-compartment turnover. This relation cannot be applied to a double-compartment system (erythrocytes, plasma) or to a multicompartment system (10). Consequently, if the authors' observations are correct, their numeric results are disputable. Porte and Havel (7) also have determined in vivo the cholesterol turnover time by erythrocytes, using the same calculation as Hagerman and Gould.

The purpose of the present study is to determine the free cholesterol turnover time of rat erythrocytes under suitable conditions. It is known that incubation of plasma is followed by cholesterol esterification. This reaction can affect isotope exchange kinetics. Thus a preliminary study of the changes in cholesterol concentrations during incubation was carried out. An investigation of cholesterol movements between α and β plasma lipoproteins and between each lipoprotein and erythrocytes has been published previously (11).

MATERIALS AND METHODS

All experiments were carried out on male adult Wistar rats weighing 370 g. They were injected with

Abbreviations: CPIB, ethyl *p*-chlorophenoxyisobutyrate; LDL, low density lipoproteins.

[4-^{14}C]cholesterol solution (1 μCi, 0.5 ml), which was obtained by adding 5 ml of 0.9% NaCl to 1 mg of labeled cholesterol (10 μCi) dissolved in 0.1 ml of Tween 80. The animals were killed 1.5 hr later by intraaortic punction. Blood was collected in a heparinized syringe and centrifuged immediately at 2200 *g* for 30 min at 5°C.

Heated plasma was prepared by the immediate incubation of plasma in a water bath at 57°C for 30 min.

Incubations were generally carried out at 37°C in a closed flask with gentle shaking unless otherwise mentioned. The course of incubation never exceeded 6 hr in order to prevent osmotic lysis.

For experiments A and B, cholesterol-labeled erythrocytes were mixed with unlabeled plasma (A) and cholesterol-labeled plasma with unlabeled erythrocytes (B); incubation was at normal rat hematocrit levels (40%). For eight experiments, however, blood obtained from the labeled animals was incubated directly. This process was more physiological than that used in experiments A and B. Finally, in five other experiments 1 ml of plasma obtained from a rat that received 10 μCi of [4-^{14}C]-cholesterol was mixed with the blood from a normal animal. Concentration and specific activity measurements of cholesterol were as previously described (11).

RESULTS

Changes in cholesterol concentrations

Incubation of blood. The mean values of cholesterol concentrations determined during the incubation are shown in Table 1. Plasma free cholesterol concentration was constant regardless of time of incubation (2.25, 4, or 6 hr). In contrast, concentration of esterified cholesterol increased 6, 13, and 12%, respectively, and that of erythrocyte cholesterol decreased 5, 10, and 9% during the same times. This decrease is called "erythrocyte loss" as defined previously (11). The sum of the mean concentration values of each component was comparable to 1% at any period of incubation. In each incubation, erythrocyte cholesterol decrease was equal to plasma esterified cholesterol increase. The representative curve of the changes in cholesterol concentration in erythrocytes during incubation was compared with the exponential function: $a\ (1-e^{-Kt})$ (Fig. 1). The constants a and K were determined graphically.

In two experiments, plasma α- and β-lipoproteins were separated after 2.25 hr of incubation. Increase of esterified cholesterol was observed only for α-lipoproteins.

An attempt to lessen the loss of erythrocyte cholesterol was made by incubating blood at 20°C. Under these conditions the magnitudes of all concentration variations were reduced, and therefore the incubation was continued for 24 hr. Streptomycin (2 mg/ml) was added to the incubated blood. Previous incubations at 37°C showed that the antibiotic had no effect on cholesterol concentrations (Table 1). After 24 hr of incubation at 20°C, erythrocytes had lost only 3.7% of the cholesterol. In the plasma, we observed a decrease of 26% in free cholesterol and an increase of 24% in esterified cholesterol (Table 1).

TABLE 1. Changes in cholesterol concentrations under different experimental conditions

Temp.	Hm[b]	N[b]	T[b]	Cholesterol Concentrations[a]			
				Plasma		Erythrocytes	
				Free	Ester	Free	Total
°C	%		hr	mg/g of blood			
37	40	9	0	0.136	0.415	0.608	1.159
			2.25	0.138	0.440	0.578	1.156
			Δ%[c]	+1.14 ± 1.87	+5.77 ± 2.4	−5.27 ± 1.18	−0.43
37	41.4	16	0	0.123	0.392	0.645	1.160
			4	0.121	0.442	0.582	1.165
			Δ%	−1.92 ± 3.8	+12.76 ± 2.25	−9.79 ± 1.41	+0.43
37	40.1	8	0	0.132	0.424	0.617	1.173
			6	0.138	0.475	0.558	1.171
			Δ%	+4.81 ± 6.50	+12.08 ± 3.20	−9.44 ± 2.64	−0.17
37	40.7	5 Plus streptomycin	0	0.136	0.433	0.611	1.180
			6	0.151	0.479	0.546	1.176
			Δ%	+12.02 ± 7.0	+8.08 ± 3.79	−10.79 ± 1.91	−0.34
20	41	4 Plus streptomycin	0	0.139	0.400	0.610	1.149
			24	0.102	0.495	0.588	1.185
			Δ%	−26.47 ± 10.3	+23.75 ± 7.1	−3.65 ± 1.31	+3.14
37	45.5	8 Preheated (57°C)	0	0.136	0.381	0.671	1.188
			4	0.176	0.388	0.633	1.197
			Δ%	+29.5 ± 3.42	+1.98 ± 1.95	−5.70 ± 2.13	+0.76

[a] Mean values of the indicated number of experiments.
[b] Hm, hematocrit level: N, number of experiments; T, incubation time.
[c] Δ%, concentration change during incubation expressed as percentage of initial concentration ± SE.

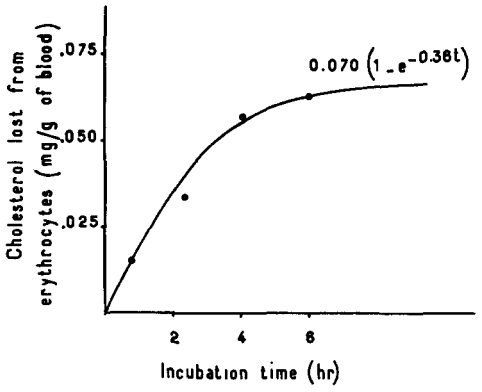


FIG. 1. Amount of cholesterol lost from erythrocytes as a function of incubation time of blood at 37°C.

Heating plasma at 57°C for 30 min inhibits the process of esterification. This plasma was incubated with fresh erythrocytes for 4 hr at 37°C. No change in esterified cholesterol was noted (Table 1). In contrast, erythrocytes lost 5.7% of their cholesterol, and plasma free cholesterol increased by 30%.

Incubation of plasma. An additional study of plasma cholesterol esterification was undertaken to compare the rate of this process with that observed in blood.

The percentage esterification of free cholesterol was estimated by two different methods in relation to the incubation time. For long incubations (6 and 24 hr), concentration changes were large, and so the determination of free and esterified cholesterol concentrations at time 0 and at 6 or 24 hr allowed the percentage esterification to be calculated. However, for short incubations, changes were small, and accuracy of the results was enhanced by use of plasma from rats that had been injected with [4-^{14}C]cholesterol 1.5 hr before they were killed. In this plasma, free cholesterol specific activity was about 10-fold greater than that of esterified cholesterol. The percentage of free cholesterol that was esterified during this period was calculated from the increase in specific activity of the esterified cholesterol. The results are given in Fig. 2. 15 and 22% of free cholesterol was esterified during 2.25 and 4 hr of incubation, respectively. This percentage reached 50% after 24 hr. Some experiments were performed at 10, 20, and 45°C. At 20°C, the percentage esterification was only 33%. At 45°C, the amount esterified was very low after both 2.25 and 24 hr (Fig. 2). Relative values of cholesterol esterified after 24 hr of incubation at different temperatures are shown in Fig. 3. The reference value is 100 at 37°C.

Changes in cholesterol specific activities

Time course of specific activity of plasma and erythrocyte cholesterol is shown in Fig. 4 for experiments A and B (cholesterol-labeled erythrocytes incubated with unlabeled plasma and vice versa), in Fig. 5 for the eight experiments where blood obtained from labeled animals was incubated directly, and in Fig. 6 for the five experiments where 1 ml of labeled plasma was incubated with unlabeled blood. Mean value curves are also drawn on Figs. 5 and 6. These mean curves were used for the exchange flux determination (see below). Regardless of initial experimental conditions, specific activities of erythrocyte and plasma free cholesterol approached an equilibrium value. Moreover, esterified cholesterol specific activity increased in all cases with but a single exception, in experiment B, where it decreased (Fig. 4).

Quantitation of free cholesterol exchange flux between erythrocytes and plasma

Computer analyses were performed on a PACE 231 R analog computer (Electronic Associates, West Long Branch, N.J.).

Fig. 7 shows a schematic diagram of the movements of cholesterol in rat blood in vitro. It was used as a basic

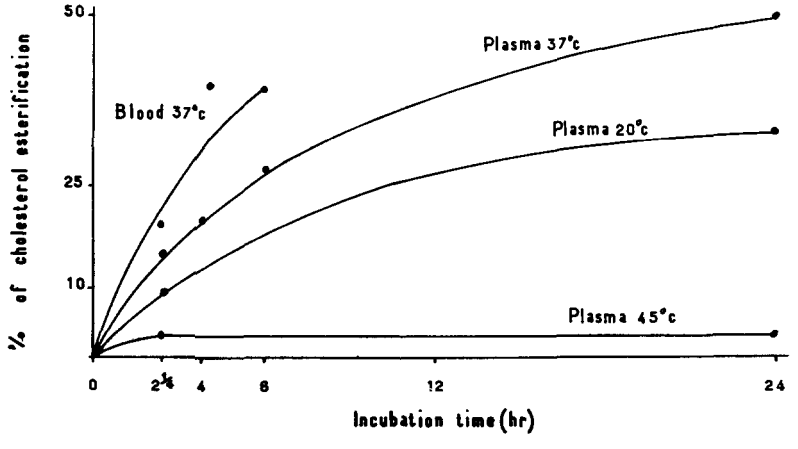


FIG. 2. Percentage of cholesterol esterification in plasma and in blood as a function of time and temperature of incubation.

model in the computer analyses, assuming that: (*1*) the fluxes of free cholesterol from plasma to erythrocytes and from erythrocytes to plasma were equal; (*2*) the outflux of cholesterol from erythrocytes (loss) was the same as that measured experimentally; and (*3*) cholesterol lost from erythrocytes was esterified, the reaction occurring directly without mixing of erythrocyte cholesterol with that of the plasma. The latter condition was suggested by the experimental data as discussed below.

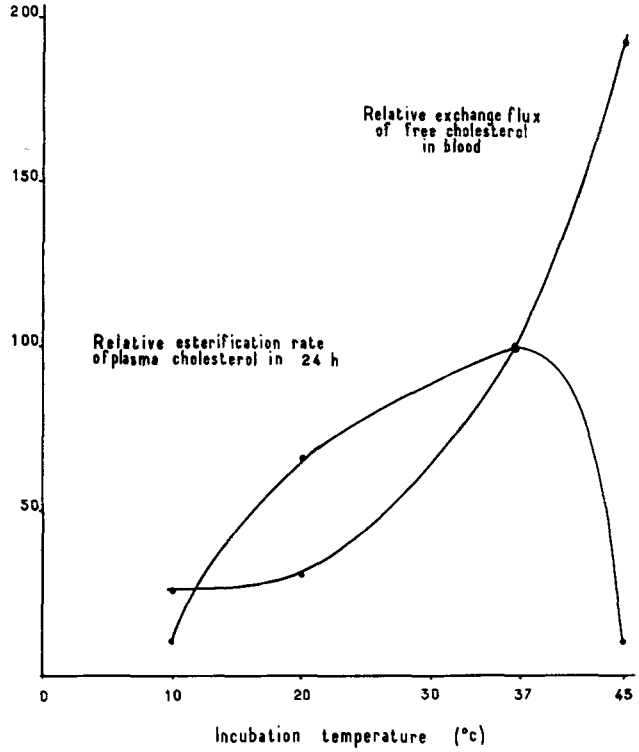


FIG. 3. Esterification rates after 24 hr of incubation in plasma, and free cholesterol exchange fluxes in blood, as a function of incubation temperature. For the two processes, at 37°C, 100 is the reference value.

Such a study required (*1*) a mathematical model which represented the biological system and was described by a system of differential equations (see Appendix); (*2*) the constants and parameters of the model (amounts of cholesterol in each compartment and their changes as function of time [Fig. 1]); and (*3*) the mean experimental curves of cholesterol specific activities in each compartment (Figs. 5 and 6).

The exchange flux was determined by solving the system of differential equations by electrical simulation. Solutions were considered acceptable when the discrepancy between the computer-derived curve and the experimental curve was less than 1% (see Figs. 5 and 6).

The simplified model suggested by the changes in concentration (Fig. 7) was not adequate for reproduction of experimental curves by analog computer. It was therefore logical to test a more complex model with three compartments: erythrocytes, α-lipoproteins, and β-lipoproteins. Exchanges take place from each of them to the two others. This triangular model, even if structurally justified, cannot explain the curves obtained for blood. The hypothesis distinguishing two compartments in the erythrocyte pool (loss and exchange) appeared also to be negative, as in the case of erythrocytes and lipoprotein exchanges. A model containing four compartments was next considered, as heterogeneity can

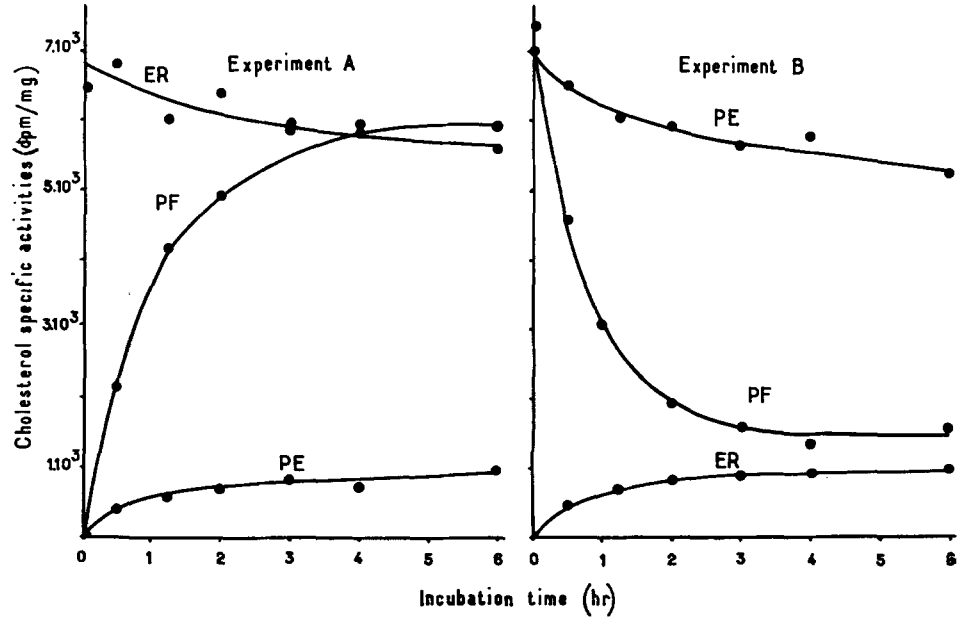


FIG. 4. Changes in specific activities of plasma free (*PF*) and esterified (*PE*) cholesterol and erythrocyte cholesterol (*ER*) during incubations of blood at 37°C. Experiment *A*, erythrocytes contain labeled cholesterol at time 0. Experiment *B*, plasma contains labeled cholesterol at time 0.

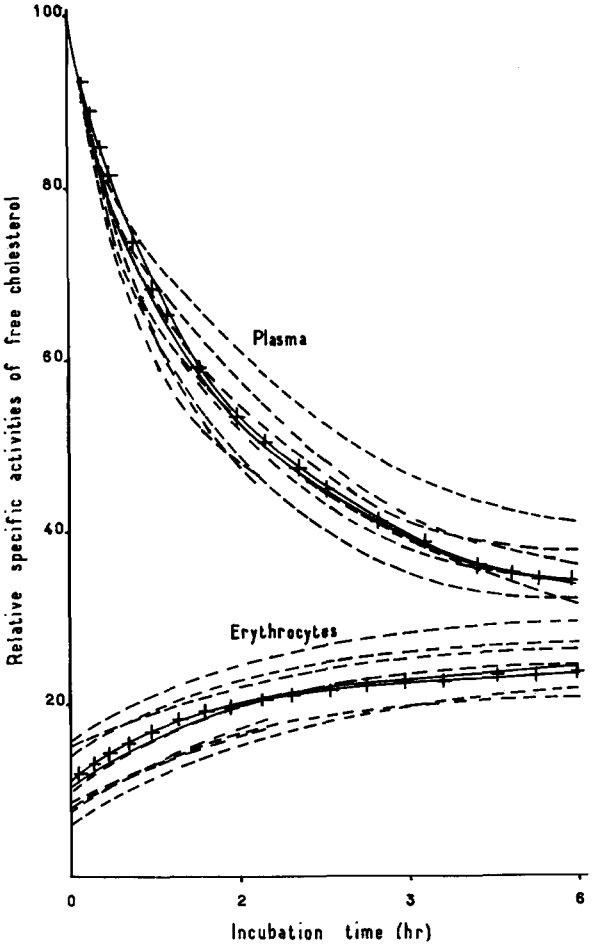


FIG. 5. Changes in relative specific activities of erythrocyte and plasma free cholesterol during incubation of blood at 37°C. Labeled cholesterol was intraperitoneally injected 1.5 hr before killing the animals. ---, individual experiment curves; ——, mean experimental curve; +—+, analog computer-derived curve.

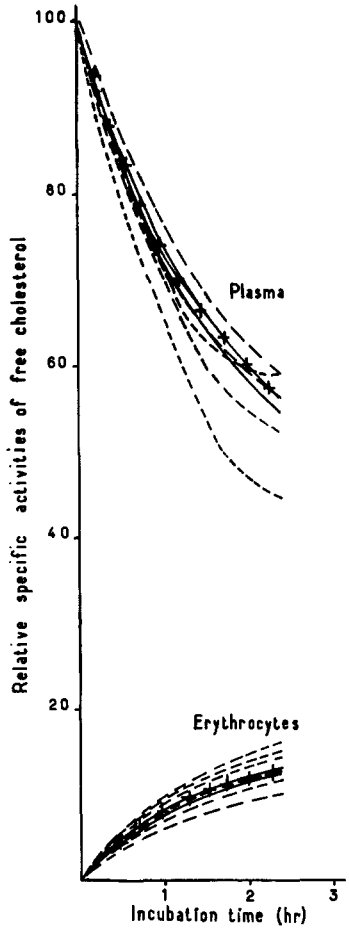


FIG. 6. Changes in relative specific activities of erythrocyte and plasma free cholesterol during incubation of blood at 37°C. 1 ml of plasma obtained from an animal that was injected intraperitoneally with labeled cholesterol was mixed with normal blood. ---, individual experiment curves; ——, mean experimental curve; +—+, analog computer-derived curve.

exist in both the erythrocyte pool and the plasma pool. Even with these four exchange fluxes, results were always poor.

By analogy with the similar study carried out with lipoproteins and erythrocytes, an additional parameter was introduced in the simplified model of Fig. 6 (11). In the new model, the exchange fluxes of cholesterol between plasma and erythrocytes were not constant, but decreased exponentially to a final value. It was then possible to reproduce precisely the experimental curves of Figs. 5 and 6. Exchange fluxes in 1 g of blood were, respectively, equal to: $(0.07–0.02)\ e^{-0.5t} + 0.02$ mg/hr and $(0.06–0.02)\ e^{-0.5t} + 0.02$ mg/hr.

This difference in results between the two types of experiments was not significant. We have considered only the mean value of the flux: $(0.065–0.02)\ e^{-0.5t} + 0.02$ mg/hr.

It was checked, moreover, that loss of erythrocyte cholesterol by esterification is an imperative condition.

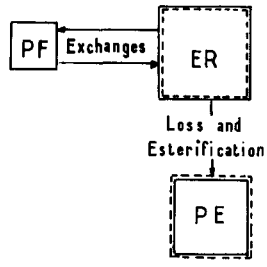


FIG. 7. Model of cholesterol movements during incubation of blood at 37°C. *PF*, plasma free cholesterol; *PE*, plasma esterified cholesterol; *ER*, erythrocyte free cholesterol. Areas within solid lines represent initial sizes of compartments. Areas within broken lines represent compartment sizes at the end of incubation. Areas of the squares are proportional to the amount of cholesterol in the compartments.

In fact, when we use a model in which a cholesterol labile fraction (one that can be modulated from 0 to 100%) is mixed with plasma free cholesterol before it is esterified, and in which exchange flux is equal to that

determined as above, curves are displaced toward lower values as compared with experimental curves.

As the system is bicompartmental, the exchange flux calculations can be resolved easily by supposing that the two compartments are stationary and the exchange flux is constant. This calculation was undertaken in order to find the error made on the exchange flux. The procedure was as follows. Erythrocyte and plasma free cholesterol specific activities are exponential functions of time ($r_{M'}$ and r_M). It can be shown, when plasma cholesterol was initially labeled, that

$$r_{M'} = r_{Eq}\,(1 - e^{-Kt})$$

and

$$r_M = r_{Eq}\left(1 + \frac{M'}{M}\,e^{-Kt}\right)$$

K (exponential coefficient) is the same for the two functions and is proportional to the exchange flux (ρ) and to exchangeable cholesterol pools (M for plasma and M' for erythrocytes). We have

$$K = \rho\,\frac{M + M'}{MM'}$$

Equilibrium specific activity (r_{Eq}) can be determined experimentally if the experiment time is long enough, or it can be calculated knowing r_0, specific activity at time 0 of the labeled compartment:

$$r_{Eq} = \frac{r_0 M}{M + M'}$$

The latter exponential relations can be transformed thus:

$$r_{Eq} - r_{M'} = r_{Eq}\,e^{-Kt}$$

$$r_M - r_{Eq} = (r_0 - r_{Eq})\,e^{-Kt}$$

This method transforms these exponential functions into linear functions whose characteristics are easier to determine. The representative lines are obtained by plotting against time on a semilogarithmic scale the values ($r_{Eq} - r_{M'}$) and ($r_M - r_{Eq}$). These lines have the same slope P, equal to 0.43 K. Knowing M and M', and after having determined P graphically, ρ can be calculated. Equilibrium specific activity calculations have been made from experimental specific activities, including concentration values of each time and not only with values of time 0; a mean equilibrium value is calculated. It takes into account not only concentration variations but also variability of results. As the exchange flux was not constant in our experimental conditions, the results plotted on a semilogarithmic scale form a curve and not a straight line. Nevertheless, the closest possible straight lines were obtained by joining the experimental points. Their slopes allow calculation of a value of "approximate exchange flux." This value is underestimated from that of the initial exchange flux determined after analog computer analysis. To minimize this difference, we have used for these calculations only short experiments (2.25 hr). For the eight experiments of Fig. 5, the initial exchange flux determined by analog computer analysis was 0.070 mg/hr; the value calculated with the slope was 0.055 mg/hr. The error on the initial exchange flux is about 20% less when this method is applied. It can be used particularly when the number of experiments is not sufficient to achieve valuable calculations by computer, and it allows good comparisons.

Influence of some factors on cholesterol movements

In this section, only "approximate exchange fluxes" were calculated as described above.

The exchange flux determined on blood from young rats (100 g, 1 month old) was the same as that found with blood from adult animals (0.055 mg/hr).

In order to examine the influence of the free cholesterol level of plasma, blood from rats that had ingested a diet containing 0.5–2% cholesterol was incubated. The range examined was about 0.1–0.7 mg of plasma free cholesterol/g of blood. Whatever the initial free cholesterol concentration in plasma, it remained practically constant during incubation. Cholesterol loss and esterification were not affected by high levels of plasma free cholesterol (Table 2). In Fig. 8, the reciprocal values of the half-life of free cholesterol ($1/t_{1/2}$) are plotted against the reciprocals of its plasma concentration for 20 experiments. Assuming a constant exchange flux of free cho-

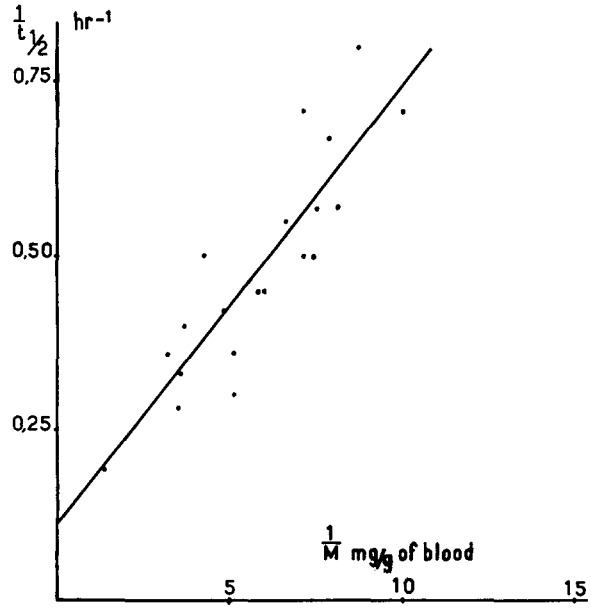


FIG. 8. Reciprocals of half-life of free cholesterol in blood in vitro as a function of the reciprocals of its plasma concentration.

TABLE 2. Influence of free cholesterol level of plasma on the exchange flux of cholesterol between plasma and erythrocytes

Number of Experiments	Incubation Time	Cholesterol Concentrations				Cholesterol Exchange Flux
		Plasma		Erythrocytes		
		Free	Ester	Free	Total	
	hr	*mg/g of blood*				*mg/hr/g of blood*
10	0	0.132[a] (0.099–0.165)[b]	0.433	0.640	1.205	
	2.25	0.137	0.461	0.605	1.203	0.047
9	0	0.237 (0.171–0.313)	1.040	0.601	1.878	
	2.25	0.235	1.100	0.568	1.903	0.044
1	0	0.714	2.750	0.920	4.384	
	2.25	0.732	2.902	0.764	4.398	0.051

Incubations were carried out at 37°C with blood obtained from animals that were fed diets containing 0.5–2% cholesterol.
[a] Average.
[b] Range.

lesterol between erythrocytes and plasma whatever the plasma cholesterol level, experimental points must fall on a straight line. We have seen that:

$$K = \rho \frac{M + M'}{MM'}$$

and by definition

$$t_{1/2} = \frac{0.69}{K}$$

thus

$$t_{1/2} = \frac{0.69\ MM'}{(M + M')}$$

and

$$\frac{1}{t_{1/2}} = \frac{\rho}{0.69} \frac{1}{M} + \frac{\rho}{0.69\ M'}$$

There is, therefore, a linear relationship between $1/t_{1/2}$ and $1/M$. The values of the slope ($\rho/0.69$) and of the intercept ($\rho/0.69\ M'$) obtained from the calculated regression line permit a determination of M' and ρ. The values obtained are close to the experimental ones (calculated from each individual experiment for ρ, and determined chemically in each experiment for M'). Therefore, the exchange flux of free cholesterol between plasma and erythrocytes is not dependent on plasma free cholesterol level and is equal to 0.046 ± 0.008 mg/hr/g of blood.

Experiments were carried out at 10, 20, and 45°C to study the effects of incubation temperature. At 10°C, the rates of loss and esterification were too low for measurement. At 45°C, an increased rate of loss of cholesterol from the erythrocytes together with the inhibition of esterification described previously resulted in a great increase in plasma free cholesterol (30% in 2.25 hr). At 10 and 20°C, exchange fluxes were much slower than at 37°C (0.013 and 0.015 mg/hr). Conversely, at 45°C an acceleration of cholesterol exchange flux was observed (0.092 mg/hr) (Fig. 3).

Preliminary investigations were made of the effects of amphotericin B (Fungizone [Squibb]) and CPIB (Ayerst Laboratories). Amphotericin B was added directly to incubated blood at concentrations of 0.03 and 0.10 mg/g of blood. The concentration of 0.10 mg/g produced an acute loss of erythrocyte cholesterol (20% instead of 5% in 2.25 hr). A larger increase of esterified cholesterol (44% instead of 12%) was associated with an increase of plasma free cholesterol (23%). Exchange flux was unchanged (0.049 and 0.042 mg/hr). In other experiments, CPIB was added to the diet at 2.5 and 7.5 g/kg. The total plasma cholesterol decreased (0.56 and 0.45 mg/g of plasma, respectively, compared with 1 mg/g for the controls). The esterified cholesterol concentration was more affected. It was halved on the low dose and reduced by a factor of 3.5 on the high dose. The percentage ratio of free to esterified cholesterol in plasma increased from 50 to 86% in the first case and from 50 to 67% in the second. The exchange rate did not appear to change very much (0.044 and 0.039 mg/hr).

DISCUSSION

In a previous work we studied cholesterol movements between isolated lipoproteins (α and β) and erythrocytes (11). Conclusions from this study are taken into account in the present discussion (Table 3).

Loss of erythrocyte cholesterol and its fate

In recent years, several authors have described the loss of cholesterol observed during incubation of blood at 37°C (12–15). Reed and Swisher (12, 13) who studied

TABLE 3. Comparison of cholesterol amounts, exchange fluxes, and their changes during different incubations

Incubation[a]	Initial Amount of Cholesterol[b]			Changes in Amount of Cholesterol			Initial Exchange Flux[b]	Change in Exchange Flux
	Erythrocytes	Plasma or Lipoproteins		Erythrocytes	Plasma or Lipoproteins			
	Free	Free	Ester	Free	Free	Ester		
	mg/g of erythrocytes			%	%	%	*mg/g of erythrocytes/hr*	%
Erythrocytes + α-lipoproteins	1.5	0.15	2.70	−42	+206	+14	0.070	−75
Erythrocytes + β-lipoproteins	1.5	0.86	1.22	−24	+23	+8	0.064	−20
Blood	1.5	0.33	1.03	−10	0	+12	0.163	−70

[a] All incubations were conducted for 6 hr at 37°C.
[b] Cholesterol amounts and exchange fluxes are expressed relative to the same initial cholesterol amount in erythrocytes (1.5 mg, mean quantity per gram of erythrocytes).

erythrocyte lipids in hereditary spherocytosis, found a 24% decrease of cholesterol after 24 hr of incubation, but these authors did not find this lipid loss in normal cells in vitro. Cooper and Jandl (14), in contrast, showed that this loss affects normal and pathological erythrocytes. Murphy (15) reported a decrease of erythrocyte cholesterol after 24 hr of incubation only under special experimental conditions. He used human plasma in which the free cholesterol concentration had been reduced by esterification during a 24-hr preincubation. Murphy (15) established a double correlation, first between the osmotic cell fragility and cholesterol loss and secondly between the loss and decrease in concentration of plasma free cholesterol during preincubation. Moreover, Gomperts (16) has demonstrated a relationship between erythrocyte osmotic fragility and the course of incubation, as the fragility increased progressively up to 24 hr. We observed that the loss of erythrocyte cholesterol is completed before hemolysis begins. If there is a real relation between the two events, loss and hemolysis are not synchronous. The first correlation suggested by Murphy (15) between osmotic fragility and cholesterol loss is not direct; the second also is disputable. Our previous study concerning α- and β-lipoproteins indicates that preesterification is not necessary for the initiation of loss (11), and the same is true for incubation of blood.

If esterification is prevented in incubations with preheated plasma, the loss is reduced but not inhibited (5.7% in 4 hr instead of 10%) (Table 1). Cholesterol that leaves erythrocytes is mixed with plasma free cholesterol, the concentration of which consequently increases. In contrast, if loss is reduced by altering incubation temperature (3.7% in 24 hr at 20°C), esterification continues with a decrease in free cholesterol of 26%. Therefore, these two phenomena, loss and esterification, are not interdependent, as one of them can be inhibited without affecting the other. However, the rates of the two processes are similar, since the plasma free cholesterol concentration remains constant in blood incubated at 37°C. Lastly, erythrocyte cholesterol loss is more temperature dependent than esterification.

To know if the esterification rate is the same in incubated blood as in incubated plasma, we have expressed for blood the increase in concentration of esterified cholesterol in percentage of plasma free cholesterol concentration, which remains constant. Results show increases of 18% in 2.25 hr and 40% in 4 and 6 hr, values which are higher than those observed in isolated plasma (Fig. 2). Therefore, esterification is greater in blood than in plasma. This can be explained by the presence of additional lecithin:cholesterol acyltransferase bound to erythrocytes. This hypothesis was also stated to explain the esterification observed in incubations of erythrocytes and β-lipoprotein (11). On the other hand, the higher esterification rate in blood could be the result of substrate supply, erythrocyte cholesterol being more accessible to enzymatic action than plasma free cholesterol. Both mechanisms may be important, as is discussed below.

The isotope results permit us to define the origin of cholesterol that has been esterified. There are three possibilities. Esterification can result directly from erythrocyte cholesterol loss from a special compartment not affected by exchanges (hypothesis 1) or from a compartment of erythrocyte total cholesterol (hypothesis 2). It is also possible that cholesterol loss occurs into plasma and that the cholesterol mixes with plasma free cholesterol before esterification (hypothesis 3). Calculations were performed from results of experiments A and B, which are shown in Table 4. The amount of cholesterol esterified is calculated hour by hour by dividing changes in its activity by a specific activity which is different for each hypothesis. Therefore, specific activity of cholesterol to be esterified is equal to erythrocyte cholesterol specific activity at time 0 (hypothesis 1), or to erythrocyte cholesterol specific activity at each time (hypothesis 2), or to plasma free cholesterol specific activity at each time (hypothesis 3). Changes in concentration of erythrocyte cholesterol (loss) and of plasma esterified cholesterol (esterification) are also reported in

TABLE 4. Experimental and calculated values of amount of cholesterol esterified during incubation of blood

		Experiment A	Experiment B
Experimental values[a] (mg/g of blood)	ΔER[b]	0.080	0.060
	ΔPE[c]	0.077	0.053
Calculated values according to three hypotheses[d] (mg/g of blood)	Hypothesis 1	0.060	0.095
	Hypothesis 2	0.071	0.083
	Hypothesis 3	0.250	0.256

[a] Blood was incubated for 6 hr at 37°C. Cholesterol-labeled erythrocytes were mixed with unlabeled plasma (expt. A), and cholesterol-labeled plasma with unlabeled erythrocytes (expt. B).
[b] ΔER, change in concentration of erythrocyte cholesterol.
[c] ΔPE, change in concentration of plasma esterified cholesterol.
[d] See text for statement of the three hypotheses.

Table 4 in order to compare the experimental with the calculated values. Hypothesis 3 gives values that are not compatible with those obtained experimentally. In contrast, the two other hypotheses give favorable results. Therefore, increase of plasma esterified cholesterol is correlated with direct esterification of erythrocyte cholesterol, and cholesterol lost from the cells does not mix with the plasma free cholesterol. But, when esterification is blocked, erythrocyte cholesterol loss results in an increase of the pool of plasma free cholesterol. In conclusion, cholesterol lost from erythrocytes is esterified before plasma free cholesterol. This result was confirmed by analog computer analysis.

Exchange of free cholesterol between plasma and erythrocytes

The examination of cholesterol specific activity curves as a function of time of incubation confirms the free cholesterol exchanges between erythrocytes and plasma (Figs. 4, 5, and 6). The simplest model to describe free cholesterol exchange between erythrocytes and plasma is a two-compartment one. This conclusion may appear to be surprising because, structurally, plasma free cholesterol consists of at least two pools (α- and β-lipoproteins) (11). This will lead to a three-compartment model. In fact, pool magnitudes and kinetic characteristics of the system are such that it can be converted to a bicompartment model. First, α- and β-lipoprotein free cholesterol pools are approximately the same size (0.043 and 0.070 mg/g of blood), and second, initial exchange fluxes of cholesterol determined independently between erythrocytes and lipoproteins and related to a same pool of erythrocyte cholesterol (0.6 mg, quantity contained in g of blood) are close (0.029 and 0.025 mg/hr) (11). For these two reasons, plasma free cholesterol can be considered to be a single compartment which exchanges with the erythrocyte cholesterol compartment. In this view, the exchange flux between erythrocytes and plasma must be equal to the sum of individual fluxes between each lipoprotein class and erythrocytes. This relationship is valid because the exchange flux determined in blood for a similar quantity of erythrocyte cholesterol is 0.065 mg/hr compared with 0.054. This examination is valuable if the two following hypotheses are kept in mind. First, free cholesterol molecules of each lipoprotein class exchange in a noncompetitive manner with those of the cellular membrane. Moreover, lipoprotein free cholesterol concentrations (absolute values or relative values) are different in lipoprotein–erythrocyte incubations and in blood incubations. The second hypothesis postulates that exchange fluxes are not dependent on cholesterol concentration, at least in the range considered. Our results showed that this hypothesis is correct (Table 3). In addition, Quarfordt and Hilderman (17) have demonstrated recently that despite a large range of cholesterol concentrations in lipoproteins the exchange flux of cholesterol between erythrocytes and lipoprotein was relatively constant.

The decrease of the exchange flux is observed regardless of the type of incubation (erythrocyte with α- or β-lipoproteins, or whole blood) (Table 3). Two explanations are available for this finding: structural heterogeneity of at least one compartment, or change in biological material. We conclude for blood that the better model to explain isotope kinetics contains only two compartments (erythrocyte and plasma) in which exchange flux is decreasing during the incubation. A study carried out by analog computer and reported in a previous paper (11) showed that it was possible to obtain the same specific activity curves with the two models, the first with two compartments exchanging with a flux decreasing with time and the second, catenary, with n compartments exchanging with constant fluxes. In the second model the $(n - 1)$ first compartments represent one compartment of the first model. The decrease in exchange flux could be attributed to the heterogeneous distribution of free cholesterol among different lipoproteins or on the erythrocyte membrane.

Changes of biological material during the course of incubation could explain this variation of exchange flux with time. We think that this second explanation is the best. In this view, one could imagine that a decrease in erythrocyte labile cholesterol is the determining factor for the decrease in exchange flux. But we see that a 75% decrease in exchange flux is associated with a high loss in the case of α-lipoproteins and that a large decrease is associated with only a small loss in the case of whole blood (Table 3). Moreover, during incubation, changes in plasma free cholesterol do not seem to be determining factors. In blood, indeed, these variations are zero and we observe nevertheless a markedly decreasing exchange

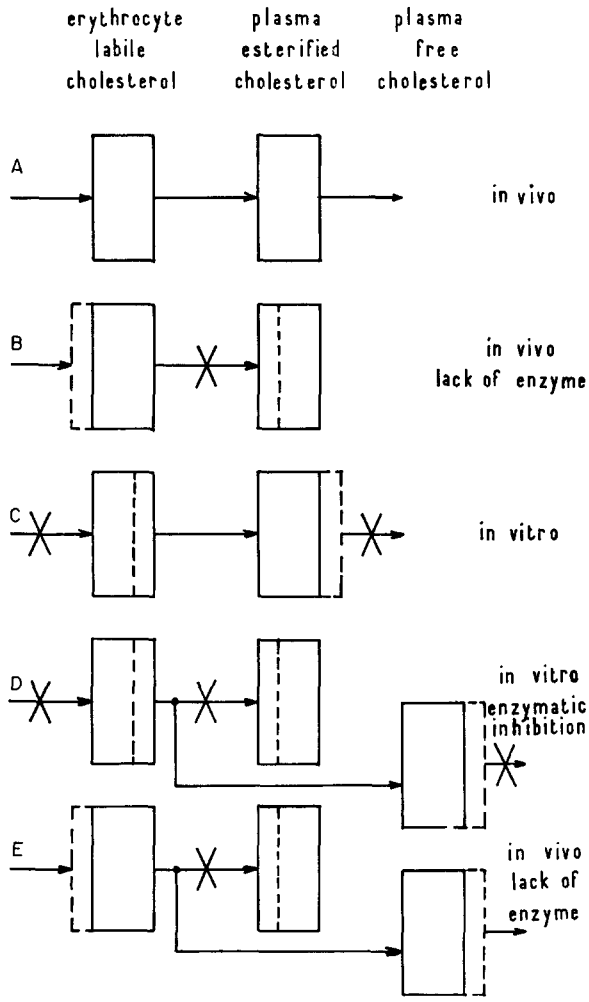


FIG. 9. Model of cholesterol movements between erythrocyte labile cholesterol and plasma esterified or free cholesterol in several situations in vivo and in vitro. Solid lines represent initial sizes of compartments and broken lines represent the final sizes.

flux. Finally, in two cases where the decrease is large (blood and α-lipoproteins–erythrocytes), we observe higher percentages of esterified cholesterol increase. It is then possible that lipoprotein esterified cholesterol enrichment in vitro could result in a progressive change in the accessibility or mobility of free cholesterol molecules.

If the decrease in exchange rate is explained by degradation of the biological material, the initial value represents the physiological one. The exchanges of free cholesterol between erythrocytes and plasma in vivo could be achieved at a constant rate (0.065 mg/hr/mg of blood) if one agrees that it is not altered by removal of blood from the animal. The turnover time for free cholesterol in erythrocytes is about 9.2 hr, a value which is consistent with that determined by Quarfordt and Hilderman for human red cells (8 hr) (17).

The great temperature dependence of the exchange flux of cholesterol is in agreement with the conclusions of Murphy (8), but seem to be inconsistent with the results of Bruckdorfer and Green (18). The latter authors, who incubated rat erythrocyte ghosts with human serum LDL, observed only a slight effect of temperature on the exchange. Lastly, it is interesting to note the difference in temperature dependence between esterification and exchange (Fig. 9). Inhibition of the first at 45°C probably explains the decrease of esterified cholesterol in fever and infectious diseases. In contrast, at this temperature the exchange flux is increased twofold. These observations must be kept in mind when considering the pathophysiology of these diseases.

Cholesterol movements in blood in vitro are summarized in Fig. 10. Free and esterified cholesterol exchanges between lipoproteins are included in the diagram. These movements were reported in a previous paper, but the esterified cholesterol exchanges between lipoproteins are only hypothetical (11).

Turnover model of erythrocyte cholesterol

A fraction of erythrocyte cholesterol is labile. This fact is demonstrated by cholesterol loss when blood is incubated. During the first hours of incubation, all cholesterol that becomes esterified comes from the erythrocytes, without mixing with plasma free cholesterol. This observation suggests the following turnover model in blood in vivo. Erythrocyte labile cholesterol and plasma esterified cholesterol constitute a catenary system in a steady

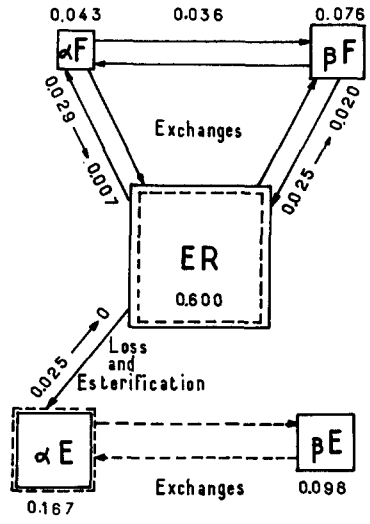


FIG. 10. Movements of cholesterol between α and β plasma lipoproteins and erythrocytes at 37°C in vitro. αF, α-lipoprotein free cholesterol; αE, α-lipoprotein esterified cholesterol; βF, β-lipoprotein free cholesterol; βE, β-lipoprotein esterified cholesterol; *ER*, erythrocyte free cholesterol. Areas within solid lines represent initial compartment sizes, and areas within broken lines represent compartment sizes at the end of incubation. Areas of the squares are proportional to the cholesterol masses of compartments. These masses are indicated in mg/g of blood near the squares. Exchange flux values are indicated in mg/hr near the arrows representing the exchanges. Esterified cholesterol exchanges between lipoproteins, being hypothetical, are indicated by dashed lines.

state (Fig. 9, *A*). The influx of cholesterol (of indeterminate origin) into the compartment "erythrocyte labile cholesterol" is equal to the esterification rate, which is equal to the disappearance rate of ester from plasma. Observations in vivo and in vitro can be explained easily with this model. In patients who lack plasma lecithin:cholesterol acyltransferase, plasma cholesteryl ester level is low, but erythrocyte cholesterol level is elevated 60–100% (19). In other pathological cases in which the esterification reaction is inhibited (obstructive jaundice) or the quantity of enzyme is low (chronic hepatitis and cirrhosis), erythrocyte cholesterol is also increased (14, 20). The outflux from the erythrocyte labile cholesterol compartment being reduced or zero, a new steady state occurs, characterized by a higher concentration (Fig. 9, *B*). In vitro in blood, the influx into the "labile compartment" is suppressed. Persistence of esterification leads to another equilibrium state characterized this time by a low erythrocyte cholesterol concentration and by an increase of esterified cholesterol because this cannot be removed (Fig. 9, *C*). In fact, two other observations in vitro allow completion of this first model. The absence of influx into the erythrocyte labile compartment leads to a decrease of its concentration, as was demonstrated above in vitro. When erythrocytes are incubated with α-lipoproteins, or with β-lipoproteins to a lesser extent, lipoprotein free cholesterol concentration increases simultaneously with direct esterification of erythrocyte labile cholesterol. In these cases the esterification rate is less than that of loss, resulting in an increase of lipoprotein free cholesterol (11). A similar situation is found in incubated blood if the action of esterifying enzyme is inhibited by preheating. These facts show that the erythrocyte cholesterol can take a secondary route in vitro when enzymatic activity is low or artificially reduced. A more complete model can then be elaborated (Fig. 9, *D*). It is possible to apply this latter model in vivo. If one admits that the rate of cholesterol esterification is less than the influx into the labile compartment, the new steady state which will be established will be characterized by high levels of erythrocyte labile cholesterol and of plasma free cholesterol. This seems to be the case; Gjone and Norum (20) have shown that if there is a deficiency of enzyme, erythrocyte cholesterol concentration is proportional to plasma free cholesterol. All these facts, therefore, support the initial model. But its validity will be proved when it is shown in a direct way in vivo that erythrocyte labile cholesterol leaves erythrocytes by the process of esterification.

Action of drugs

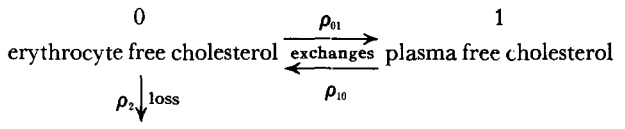
Amphotericin B is a polyene antibiotic that has an antifungal but not an antibacterial action. This action is inhibited by the presence of sterols (21). Moreover, this antibiotic has been shown to stimulate sodium, potassium, and chloride ion transport across bladder (22), and it is agreed that the site of polyene action is the cell membrane and that its selective toxicity is the consequence of linkage with a sterol membrane constituent (23). It seems that amphotericin B does not fix onto the erythrocyte membrane, as some authors have suggested (22). Increased loss from erythrocytes shows, on the other hand, that the linkage of antibiotic with cholesterol accelerates the sterol liberation from the cell membrane. Therefore, an increase in permeability to ions could be related to erythrocyte cholesterol loss. This conclusion agrees with that previously stated by Jacob (24).

CPIB has been described as a hypocholesterolemic drug in man and in laboratory animals (25–27). Our results confirm the previous observations and point out that cholesteryl esters are decreased much more than free cholesterol. The exchange flux was unchanged.

APPENDIX

Kinetic equations for exchange between two compartments (erythrocyte and plasma free cholesterol) in a non-steady state (erythrocyte cholesterol is decreasing with time).

1. Schema of the system



2. Definitions and notations

S_0 amount of cholesterol in erythrocytes
S_1 amount of free cholesterol in plasma
S_{00} initial value of S_0
ΔS_0 amount of cholesterol lost from compartment 0 (erythrocyte cholesterol loss)
ΔS_{00} maximal value of ΔS_0

} (mg/g of blood)

$\rho_{01} = \rho_{10} = \rho_1$ flux of exchange between compartments 0 and 1
ρ_0 initial value of ρ_1
ρ_∞ final value of ρ_1
ρ_2 rate of loss from compartment 0

} (mg/hr/g of blood)

a_0, a_1 specific activities in compartments 0 and 1 (dpm/mg)
t time (hr)

3. Equations

$$\frac{da_0}{dt} = \frac{\rho_1}{S_0}(a_1 - a_0) - \frac{\rho_2}{S_0}a_1$$

where S_0 and ρ_1 are varying with time and defined by:

$$S_0 = S_{00} - \Delta S_0$$

$$\Delta S_0 = \Delta S_{00}(1 - e^{-\alpha t}) \text{ (see Fig. 1)}$$

$$\rho_2 = \frac{d\Delta S_0}{dt}$$

and $\rho_1 = (\rho_0 - \rho_\infty)e^{-\gamma t} + \rho_\infty$

$$\frac{da_1}{dt} = \frac{\rho_1}{S_1}(a_0 - a_1)$$

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