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# THE KINETICS OF THE *IN VITRO* CHOLESTEROL UPTAKE AT THE ENDOTHELIAL CELL SURFACE OF THE RABBIT AORTA

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SUMMARY

The kinetics of [4-$^{14}$C]cholesterol uptake by intima-media layers of thoracic aortas from normal rabbits have been studied by use of an *in vitro* method.

1. The uptake of labelled cholesterol was followed during a 4-h period. The uptake curve shows an initial steep rise (estimated from the 15-min uptake value) followed by a step which has a less steep, obviously rectilinear course.

2. The initial uptake of labelled cholesterol was found not to depend significantly on temperature (4–38°). The initial uptake is probably due to a superficial fixation of labelled cholesterol.

3. The rate of [$^{14}$C]cholesterol uptake after the initial 15 min is, on the contrary, temperature dependent, presumably due to two processes having different temperature dependences. One of the hypothetical processes is assumed to have a temperature optimum at 20°; the other has an "activation energy" of about 18 kcal/mole. Judging from the temperature dependence, the [$^{14}$C]cholesterol uptake corresponding to the second phase of the uptake is thought to represent transport of cholesterol across the endothelial cell membrane. No causal explanation of the course of the uptake–temperature curve in the interval about 20° can be given at the present.

4. The transfer rate of cholesterol from serum to intima-media is of the same order of magnitude as that found *in vivo*.

5. The relationship between the rate of cholesterol transfer from serum to intima-media and total cholesterol concentration within the normal range (27 to 107 mg per 100 ml serum) is similar to the relationship between the velocity of an enzymatic reaction, showing substrate saturation phenomenon, and substrate concentration. In the model the initial uptake corresponds to the formation of enzyme–substrate complex. Other interpretations of the relationship between cholesterol transfer rate and cholesterol concentration are discussed.

6. Finally, the similarity of the kinetics found in this study with those for protein uptake by Ehrlich ascites-tumor cells and *Amoeba proteus*, assumed to be due to pinocytosis, is emphasized.

## INTRODUCTION

In order to throw light on the mechanism of cholesterol uptake by the intima-media of the thoracic aorta of normal rabbits an *in vitro* method for the measurement of serum cholesterol uptake at the intimal surface was previously developed[1]. The method made it possible to establish control conditions for the study of the influence of various parameters on the cholesterol uptake by the use of hemisegments from the same thoracic aorta.

The present paper is concerned with the kinetics of the cholesterol uptake process. By the use of [4-$^{14}$C]cholesterol—incorporated into serum lipoprotein by a biosynthetic route[1]—the rate of cholesterol transfer across the intimal surface from serum to intima-media has been evaluated. Furthermore, the dependence of cholesterol transfer rate on serum cholesterol concentration and temperature is considered.

## MATERIALS AND METHODS

### *Animals*

The animals used in the experiments were obtained from uniform breeds of adult white female rabbits (from The Danish State Serum Institute). They were used at an age of 4–6 months. Previous to the experiments the rabbits were fed an ordinary vegetable diet. Before exsanguination the serum donor rabbits were deprived of food for 24 h.

### *Methods*

The preparation of aortic hemisegments, the serum-labelling procedure, the incubation technique and the analytical details have been described previously[1]. Intima-media layers were prepared from the central parts of right and left hemisegments, cut out at two levels of the thoracic aorta. One of the two corresponding intima-media layers from the same level of the thoracic aorta was used as a control for the other.

The label used was $^{14}$C in the biologically stable 4-position of the cholesterol molecule[2]. The [$^{14}$C]cholesterol-containing serum from one donor rabbit was sufficient for 20–24 incubations comprising aortic segments from 5–6 animals.

The net $^{14}$C activities found in the aortic intima-media layers ranged from 10% to 300% of the background. For each sample 4000–10 000 counts were recorded. The background value was determined almost daily. Blind samples from non-incubated tissue ensured that the low activities were not simply due to contamination during the preparation procedure.

The intima-media uptake of labelled cholesterol was studied relative to the following 3 parameters:

1. *Time.* The dependence of intima-media uptake of [$^{14}$C]cholesterol on time was estimated from experiments of 15, 60, 120, 180 and 240 min duration at 38°. To eliminate individual variations the uptake is expressed relatively (as a fraction of control uptake, *i.e.* the uptake value for the corresponding intima-media following a 240-min incubation at 38°). To obtain sufficient observations, sera with various cholesterol concentrations were inevitably used.

2. *Serum cholesterol concentration.* For the description of the relationship

between [$^{14}$C]cholesterol uptake or cholesterol transfer rate and serum cholesterol concentration use has been made of the various series of control uptake values at 38° (240 min). The latter were obtained from the experiments performed to determine the relationship between [$^{14}$C]cholesterol uptake and time or temperature as well as the elimination experiments, in which one of the two corresponding intima-media layers served as control for the other. The cholesterol concentration of the sera used ranged from 27 to 107 mg per 100 ml.

3. *Temperature.* The intima-media uptake of labelled cholesterol was measured in 240-min incubations at 4°, 15°, 20°, 26°, 32° and 38° and in 15-min incubations at 4° and 38°. The final $CO_2$ equilibration took place at the temperature in question. In the 240-min experiments, the uptake is expressed relative to a control, *viz.* the uptake value for the corresponding intima-media following a 240-min incubation at 38°. In the 15-min experiments the uptake is expressed relative to a control, *viz.* the uptake value for the corresponding intima-media following a 15-min incubation at 38°. By means of a conversion factor found for the uptake-time relationship the 15-min uptake at 4° is also expressed relative to the uptake value at 38° for $t =$ 240 min.

*Elimination of labelled cholesterol from intima-media.*

Aortic hemisegments previously incubated for 15 or 240 min at 38° in serum containing labelled cholesterol were washed in a slightly modified Krebs–Ringer solution[1] and then transferred to serum without labelled cholesterol, but having almost the same cholesterol concentration. The incubations in serum without labelled cholesterol were carried out at 38° for 15 min and followed by washing and preparation of central intima-media layers as described earlier[1].

## RESULTS

*The intima-media uptake from serum of lipoprotein-bound [$^{14}$C]cholesterol as a function of time*

The results primarily originate from 3 main groups of experiments: the cho-

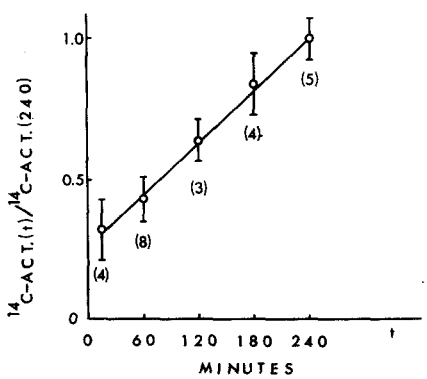



Fig. 1. The uptake of [$^{14}$C]cholesterol by intima-media layers from the upper part of the thoracic aorta as a function of time (the uptake is expressed as a fraction of the 240-min uptake). Temp., 38°; pH 7.35 ± 0.04 (S.D.). Serum cholesterol concentration, 43–61 mg per 100 ml. Each point represents the mean of the number of samples indicated in parentheses ± 1 S.E.

lesterol concentration in Group I ranged from 43 to 61 mg per 100 ml ($n = 24$), in Group II it was 27 mg per 100 ml ($n = 6$), and in Group III it was 80 mg per 100 ml ($n = 9$). The 24 results from Group I are shown in Fig. 1.

As the samples in Group I are grouped with regard to time it is possible to test the linearity of the regression function by comparing the variance of the group means at different times about the regression line with the variance within the groups at different times[3]. The test quotient is 0.047, and the value of the significance limit $F(3, 19)$ for $P = 0.05$ is 3.13. The statistical test therefore does not disprove the linearity of the regression function in Fig. 1. The equation of the regression line in Fig. 1 is:

$$\frac{^{14}\text{C activity } (t)}{^{14}\text{C activity } (240)} = 0.188t + 0.25$$

By an analysis of covariance[4] it was then tested whether the relative uptake–time relationship at different cholesterol concentrations may be described by the same regression line. The results of the analysis are shown in Table I. As the mean squares in lines 1, 2 and 3 in Table I are not significantly different, homogeneous

TABLE I

ANALYSIS OF COVARIANCE

| | *Cholesterol concentration* | | | *Deviations from regression* | | |
|---|---|---|---|---|---|---|
| | | $f$ | $b_{y,x}$ | $f$ | $\Sigma y^2 - (\Sigma xy)^2/\Sigma x^2$ | *Mean square* |
| 1 | 27 mg% | 5 | 0.234 | 4 | 0.0822 | 0.0206 |
| 2 | 43–61 mg% | 23 | 0.188 | 22 | 0.8217 | 0.0374 |
| 3 | 80 mg% | 8 | 0.264 | 7 | 0.2478 | 0.0354 |
| 4 | Within | | | 33 | 1.1517 | 0.0349 |
| 5 | Regression coefficients | | | 2 | 0.0627 | 0.0314 |
| 6 | Common | 36 | 0.208 | 35 | 1.2144 | 0.0347 |
| 7 | Adjusted means | | | 2 | 0.0086 | 0.0043 |
| 8 | Total | 38 | 0.205 | 37 | 1.2230 | 0.0331 |

variance will be assumed. The ratio between the mean square for regression coefficients (line 5) and the mean square within samples (line 4) is 0.90; *d.f.*: 2, 33. Thus the sample regression coefficients are found not to differ significantly. The ratio between the mean square for adjusted means (line 7) and the mean square for common regression (line 6) is 0.24; *d.f.*: 2, 35. The value lacks significance at the 5% level. Thus the elevation of the regression lines is found not to differ significantly.

The conclusion is that the relative uptake as a function of time may be described by the same regression line in the 3 main groups at different cholesterol concentration. The regression line calculated from all observations has the following equation:

$$\frac{^{14}\text{C activity } (t)}{^{14}\text{C activity } (240)} = 0.205t + 0.22$$

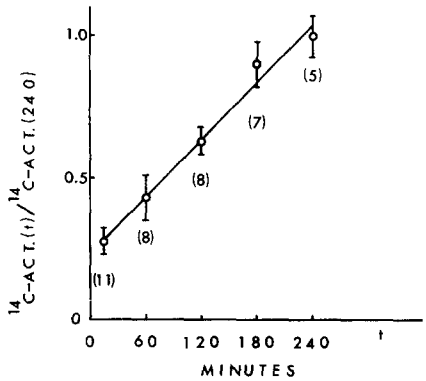



Fig. 2. The uptake of [$^{14}$C]cholesterol by intima-media layers from the upper part of the thoracic aorta as a function of time (the uptake is expressed as a fraction of the 240-min uptake). Pooled observations from 3 main groups at various cholesterol concentrations (for further explanation see text). Temp., 38°; pH 7.35 ± 0.04 (S.D.). Each point represents the mean of the number of samples indicated in parentheses ± 1 S.E.

The standard error of the slope 0.205 is 0.022. The last term of the equation is different from zero ($t = 4.53$, $P < 0.001$).

Fig. 2 shows the pooled results from all 3 main groups at different cholesterol concentration. The mean values ± 1 S.E. are shown and the regression line is presented. Figs. 1 and 2 show that at 38° the relative rate for the [$^{14}$C]cholesterol uptake process is initially high. Following the first 15 min of incubation the rate is lower and apparently constant within the experimental time. Rearranging the equation of the common regression line we get the equation:

$$^{14}\text{C activity}(t) = {}^{14}\text{C activity}(240) \times (0.205t + 0.22)$$

The rate at 38° for the [$^{14}$C]cholesterol uptake process corresponding to the second phase is: $^{14}$C activity(240) × 0.205. Thus we are able to calculate this rate from the 240-min uptake value by multiplication with the slope of the regression line shown in Fig. 2, if the conditions are the same as in these experiments with regard to temperature, pH and factors which otherwise might influence the slope.

As the rate for the [$^{14}$C]cholesterol uptake process corresponding to the second phase seems to be constant within the experimental time, it is reasonable to assume that the calculated rate for this phase is the transfer rate for [$^{14}$C]cholesterol from serum to intima-media, because any significant transport of [$^{14}$C]cholesterol in the opposite direction would make the curve flatten off (see later: DISCUSSION). The rate of cholesterol transfer ($\mu$g per cm$^2$ intimal surface per h) from serum to intima-media following the initial phase can therefore be calculated by dividing the transfer rate for [$^{14}$C]cholesterol by the specific activity of the cholesterol found in the serum used for incubation.

In the experiments mentioned above, the intima-media layers originated from the upper part of the thoracic aorta. For intima-media layers from the lower half of the thoracic aorta a similar qualitative uptake–time relationship is reasonable to assume. From 11 experiments with "lower" intima-media layers in which the ratio $^{14}$C activity($t$): $^{14}$C activity(240) was determined for $t = 15$ min ($n = 5$), $t = 120$ min ($n = 1$) and $t = 240$ min ($n = 5$), the slope of the second step of the relative uptake

curve was calculated to be 0.235 ± 0.014 (S.E.) (Fig. 5). This value is not significantly different from that calculated for upper intima-media layers (0.205 ± 0.022).

The [$^{14}$C]cholesterol uptake by intima-media layers from segments of the upper and lower part of the same thoracic aorta was measured following incubation at 38° for 240 min. The upper and lower segments from the same thoracic aorta were incubated in the same serum. On an average, the intima-media layers from the upper part had a 12% greater uptake per cm$^2$ intimal surface than those from the lower part. The actual average difference was 2.7 ± 1.1 (S.E.) counts/min; $n = 39$; $P < 0.02$. As the regression coefficient found above for intima-media layers from the upper part of the thoracic aorta is 13% lower than that for intima-media layers from the lower part, the calculated transfer rates for cholesterol corresponding to the second phase of the uptake process do not differ for upper and lower intima-media layers. This indicates that the initial uptake of [$^{14}$C]cholesterol is greater for upper than for lower intima-media layers, other things being equal.

*The influence of serum cholesterol concentration upon transfer rate of serum cholesterol*

Before presentation of the relationship between cholesterol transfer rate and concentration of total cholesterol it will be mentioned that the ratio between the concentrations of esterified cholesterol and total cholesterol was determined for various sera and found not to change as a function of the concentration of total

TABLE II

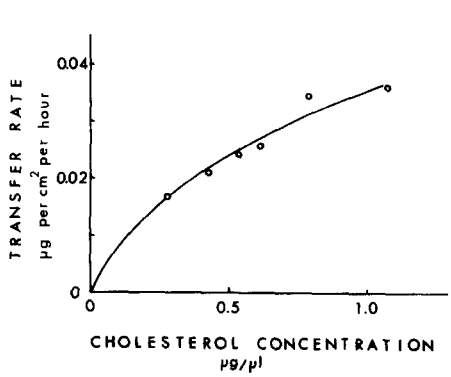
THE RATIO BETWEEN ESTERIFIED CHOLESTEROL AND TOTAL CHOLESTEROL FOUND IN SERA WITH VARIOUS CONCENTRATIONS OF TOTAL CHOLESTEROL (WITHIN THE NORMAL RANGE)

The ratio was determined by chemical as well as radioactive analysis.

| *Rabbit No.* | *Concentration of total cholesterol in serum (mg/100 ml)* | *Esterified /total cholesterol* | |
|---|---|---|---|
| | | *Chemical determination* | *Radioactive determination* |
| 316 A | 40.4 | 0.75 | 0.76 |
| 357 A | 40.8 | 0.81 | 0.82 |
| 429 D | 46.9 | 0.84 | 0.84 |
| 623 A | 53.7 | 0.77 | 0.75 |
| 751 A | 54.3 | 0.72 | 0.71 |
| 359 D | 73.5 | 0.77 | 0.76 |
| 407 E | 77.1 | 0.79 | 0.77 |
| 324 E | 107 | 0.76 | 0.75 |

cholesterol (Table II). The separation of free end esterified cholesterol was performed as earlier described[1] by the method of CARROLL[5].

Fig. 3 shows the cholesterol transfer rate (μg per cm$^2$ intimal surface per h) at 38° *versus* serum cholesterol concentration. The transfer rates were calculated as described above. Each point is a mean value for intima-media layers from the lower part of 3–6 thoracic aortas. In Fig. 4 is shown the relationship between the reciprocal cholesterol concentration and the reciprocal transfer rate for the same observations as in Fig. 3. The plot in Fig. 4 is similar to a LINEWEAVER–BURK plot[6], known from enzymology, and often used in studies on biological transport mechanisms for the demonstration of saturation of the transport mechanism.



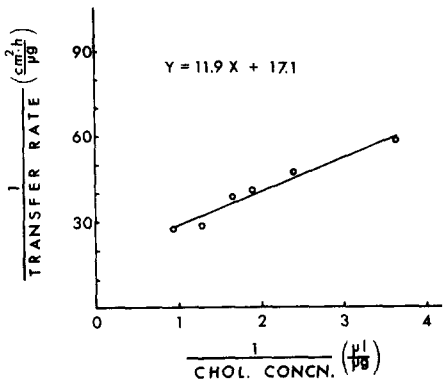




Fig. 3. Rate of cholesterol transfer from serum to intima-media (from the lower part of the thoracic aorta) *versus* total cholesterol concentration. Temp., 38°; pH 7.35 ± 0.04 (S.D.). The points are group mean values from 3–6 samples.

Fig. 4. Reciprocal rate of cholesterol transfer *versus* reciprocal cholesterol concentration (Lineweaver–Burk plot). The same observations as in Fig. 3. The regression line calculated from all single observations is presented ($n = 29$).

In Figs. 3 and 4 normal distribution within each group of reciprocal transfer rates is assumed. In Fig. 4 the ratio between the variance of the group means about the regression line and the variance within the groups is 0.20. The value of the significance limit $F$ (4, 23) for $P = 0.05$ is 2.80, *i.e.* the statistical test does not disprove the linearity of the regression function. The regression line in Fig. 4 has the following equation:

$$\frac{1}{v} = 11.9 \frac{1}{[\mathrm{Ch}]} + 17.1$$

where $v$ is the transfer rate for the second step in the cholesterol uptake process and [Ch] is the total cholesterol concentration. The regression coefficient is different

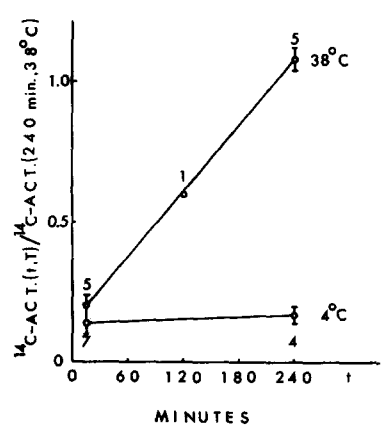



Fig. 5. The uptake of [$^{14}$C]cholesterol by intima-media layers from the lower part of the thoracic aorta at 4° and 38° (expressed as a fraction of the 240-min uptake at 38°).

from zero ($t = 4.72$, $P < 0.001$). The last term (17.1) of the equation is different from zero, too ($t = 3.01$, $P < 0.01$).

*The dependence of [$^{14}$C]cholesterol uptake on temperature*

In 4 experiments the 240-min uptake of [$^{14}$C]cholesterol at 4° was 17 ± 3 (S.E.)% of the uptake at 38°. However, in 7 experiments, the 15-min uptake of [$^{14}$C]cholesterol at 4° was 71 ± 16 (S.E.)% of the uptake at 38°. Thus the effect of lowering the temperature on the 240-min uptake is significantly different ($P < 0.05$) from the effect on the 15-min uptake of labelled cholesterol.

Fig. 5 shows the relative [$^{14}$C]cholesterol uptake at 4° and 38° as a function of time. The second step in the uptake curve can reasonably be described as rectilinear according to Figs. 1 and 2. At 4° no significant uptake of labelled cholesterol is found following the initial 15-min period (the relative uptake values being 0.14 ± 0.04 at 15 min and 0.17 ± 0.03 at 240 min).

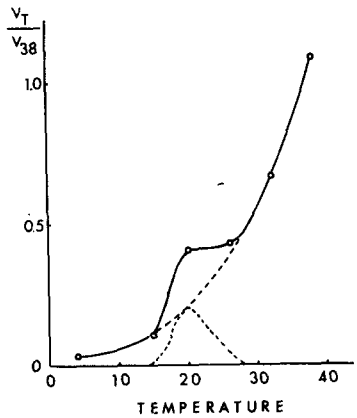



Fig. 6. The calculated relative rate of cholesterol transfer from serum to "lower" intima-media layers (following the initial 15-min period of incubation) as a function of temperature. The points are mean values calculated from 4–7 observations at the temperature in question (including 38°). For curve analysis see text.

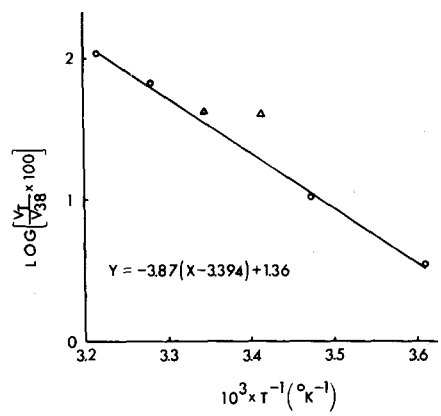



Fig. 7. Arrhenius plot of the results shown in Fig. 6. The regression line for the mean values at $T = 277°$, 288°, 305° and 311°K is presented. For further explanation see text.

Extrapolation of the curves in Fig. 5 to zero time gives the same intercept values on the ordinate axis. Therefore, in calculation of the transfer rates at various temperatures from serum to lower intima-media layers, correction for initial uptake has been done by means of this intercept value (0.14), irrespective of the temperature in question between 4° and 38°.

Fig. 6 shows rate of cholesterol transfer *versus* temperature. It is tempting to consider the curve as consisting of 2 "simpler" curves, shown in the figure as dotted lines, which by interference give the observed curve: the temperature relationship found might be due to 2 temperature-dependent processes interfering with each other. It is assumed that one of the two processes has a temperature optimum at about 20° and that the process showing a temperature optimum prevails only in a limited temperature interval. How great this temperature interval is cannot be

exactly shown from the observations. However, beyond the interval 15–32° the influence of this process is assumed to be negligible. In Fig. 7 the logarithm of the transfer rate (as in Fig. 6 expressed as a fraction of control) is plotted *versus* reciprocal temperature. To evaluate the temperature dependence of the process not showing a temperature optimum, only the ordinate values corresponding to temperature values of 277°, 288°, 305° and 311° K have been used. For these values it is reasonable to assume a rectilinear regression function. The regression line calculated from the mean values shown in the figure has the following equation:

$$\log \frac{v_T}{v_{311}} \times 100 = -3.87 \left( \frac{10^3}{T} - 3.394 \right) + 1.36$$

The slope is different from zero ($P < 0.001$). The correlation coefficient $r = -1.00$. The activation energy for the process is about 18 kcal/mole, and $Q_{10}$ is about 2.6 for the temperature range 28–38°.

*Elimination*

After 15 min of incubation at 38° in serum containing labelled cholesterol the elimination of labelled cholesterol now present in the intima-media layers was studied during a second incubation in non-labelled serum for 15 min at 38°. The elimination per cent amounted to $17 \pm 22$ (S.E.), a value not significantly different from zero ($n = 6$).

Similarly, after 240 min of incubation at 38° in serum containing labelled cholesterol a second incubation in serum without labelled cholesterol was undertaken for 15 min ($n = 6$) at 38°. The elimination per cent amounted to $-4 \pm 7$ (S.E.). This value is not different from zero either.

## DISCUSSION

Figs. 1 and 2 show a biphasic [$^{14}$C]cholesterol uptake curve having an initial steep rise followed by a less steep, obviously rectilinear course. The actual course of the initial step is unknown.

The uptake curves found here are essentially similar to those found by CONNER AND UNGAR[7] for the incorporation of cholesterol by Tetrahymena and by RYSER *et al.*[8,9] and SCHUMAKER[10] for the uptake of [$^{131}$I]albumin by Ehrlich ascites-tumor cells and ribonuclease and cytochrome *c* by *Amoeba proteus*, respectively. In all of these studies an initial, rapid, temperature-independent uptake is seen followed by a slower, temperature-dependent uptake. The authors consider the initial uptake to be due to a non-metabolic phase, probably involving surface adsorption. As pointed out by RYSER[9] the two-step process is reminiscent of the kinetics of virus fixation by host cells and thus may illustrate certain similarities in the process of macromolecular and supramolecular penetration observed with various types of cells.

The significant temperature dependence found for the 240-min uptake and the absence of a demonstrable temperature dependence for the 15-min uptake together with the two-step uptake curve indicate a need for a separate consideration of two phases in the uptake process.

Relative to the mechanism of the initial uptake of labelled cholesterol, two

proposals should be mentioned. From the point of view that the washing previous to the incubation had removed lipoprotein adsorbed to the intimal surface *in vivo*, one proposal is the possibility of adsorption of lipoprotein containing labelled cholesterol. The washing previous to the incubation of the aortic segments in serum containing labelled cholesterol is, however, not so extensive as is the standardized washing following incubation. The final washing also includes a slight brushing. Therefore, the 15-min labelling can hardly be due to adsorption of labelled lipoprotein substituting for adsorbed lipoprotein, which was removed during the washing of the thoracic aorta previous to incubation.

Another proposal relative to the mechanism of the initial uptake is that exchange of [$^{14}$C]cholesterol occurs between serum and a superficial endothelial cell-membrane pool or a pool of adsorbed lipoprotein (the last possibility implies that adsorbed lipoprotein is not removed by any of the washing procedures used). An exchange of cholesterol between serum and erythrocytes as first described by HAGERMAN AND GOULD[11] is a well-known phenomenon. Two facts seem to indicate that, in the case of exchange, equilibrium has been reached, *viz.* the course of the uptake curves in Figs. 1 and 2 and the lack of a temperature dependence such as that found by MURPHY[12] in studies on the exchange of cholesterol between serum and erythrocytes. If, however, the initial uptake is due to an exchange process, which has reached equilibrium, a practically total loss of labelled cholesterol from intima-media is expected during a second 15-min incubation in serum without labelled cholesterol. No significant loss of label has been found during a second 15-min incubation, which therefore seems to indicate that simple exchange of cholesterol initially is less probable. The initial uptake must represent a net uptake, but its nature is still unknown.

The rectilinear course of the [$^{14}$C]cholesterol uptake curve following the initial 15 min indicates that no significant amount of labelled cholesterol has moved from intima-media to serum during the interval from $t = 15$ to $t = 240$ min, *i.e.* that the [$^{14}$C]cholesterol uptake rate represents the rate of [$^{14}$C]cholesterol transport from serum to intima-media. As the specific activity of serum cholesterol has been shown to remain almost constant during the incubation[1], the rate of cholesterol transport from serum to intima-media can be calculated from the rate of [$^{14}$C]cholesterol uptake per cm$^2$ intimal surface, dividing the latter by the specific activity of the serum cholesterol. However, from these data nothing can be concluded about the possibility of a transport of cholesterol from intima-media to serum since the specific activity of the intima-media cholesterol pool is unknown. The specific activity of this pool may well be too low to enable the detection of any transport of cholesterol from intima-media to serum during the experimental period.

The transfer rates found here are of the same order of magnitude as those found by CHRISTENSEN AND JENSEN[13] *in vivo* for cholesterol transfer across the intimal surface of the thoracic aorta in rabbits.

The rate of cholesterol transfer from serum to intima-media corresponding to the second phase is temperature dependent (Fig. 6). Beyond the temperature interval about 20°, in which the transfer rate–temperature relationship may be assumed to be due to two different temperature-dependent phenomena, the temperature dependence may reasonably be described by a rectilinear relationship between transfer rate and the logarithm of $1/T$. The estimated $Q_{10}$ and the "activation energy" for

this one of the two temperature-dependent processes seem to indicate that cholesterol molecules are transferred across some energy barrier, presumably the endothelial cell membrane, although it is not possible to state the definite mechanism behind the temperature dependence. According to DAVSON AND DANIELLI[14], "$Q_{10}$ values of the order of 3 or more are not uncommon, and it was at one time thought that these high $Q_{10}$ values indicated that specific chemical reactions were responsible for the transport of many types of molecules across the cell membranes". As emphasized by DANIELLI AND DAVSON[15], any type of potential energy barrier might demand a certain minimum of kinetic energy for the particles to let them pass through, and it is a simple consequence of diffusion kinetics that a slowly penetrating molecule will have a high $Q_{10}$ and a rapidly penetrating molecule a low $Q_{10}$. Furthermore, the membrane itself (*in casu* the endothelial cell membrane) might structurally change as the result of changes in temperature, which might lead to an altered permeability. A $Q_{10}$ value (2.6) or an "activation energy" (18 kcal/mole) as found in this study for the cholesterol transfer is therefore not in itself a proof that the endothelial cells participate actively in the transport process.

As to the hypothetical second process, which is assumed to have a temperature optimum at about 20° (Fig. 6), no causal explanation can be given at present.

Figs. 3 and 4 indicate that a parallelism may be drawn between an enzymatic reaction following the so-called MICHAELIS–MENTEN equation[16] and the mechanism involved in the transfer of cholesterol from serum to intima-media. If the serum cholesterol could be considered as a "homogeneous substrate", Figs. 3 and 4 would exclude diffusion as the only mechanism by means of which cholesterol is transported, because these figures indicate a limitation in transport capacity. An assumption of a carrier mechanism as suggested, *e.g.*, for glucose transport through the red-cell membrane[17,18] is, on the other hand, conceivable. The application of Michaelis–Menten kinetics allows the calculation of the maximal velocity and the Michaelis constant ($K_m$) for the process of cholesterol transport. This procedure may perhaps be reasonable but would seem to be hampered by the fact that serum cholesterol chemically is a multicompartment system (free and esterified cholesterol exist distributed over various lipoproteins).

In studies on the uptake of labelled cholesterol by the intima-media of the intact rabbit aorta[19], by the rat aorta *in vitro*[20] and by tissue-culture cells[21,22], it has been tentatively assumed that the finding of more labelled cholesterol in the free fraction of the aortic tissue or culture cells than could be accounted for on the basis of the distribution of labelled cholesterol between free and esterified serum cholesterol, was an indication for a preferential uptake of the non-esterified form. However, in certain studies[19,21] it was admitted that hydrolysis of esterified cholesterol after uptake might explain the observations. This problem is not important for the interpretation of the kinetics in this study, especially the relationship between transfer rate and "substrate" (total cholesterol), because no significant change was found in the ratio esterified/total cholesterol within the concentration range of total cholesterol (Table II).

If the transport of cholesterol is due to a transport of lipoprotein molecules with various transfer rates, a relative increase in the cholesterol content of the larger lipoprotein molecules might result in a concentration dependence for the cholesterol transfer rate such as that shown in Figs. 3 and 4. Moreover, if cholesterol molecules

are transported separately as such, the same result might emerge, if, at increasing serum cholesterol concentration, the cholesterol were bound in increasing amounts to proteins from which it was released with greater difficulty. No solution to these problems can be reached from the experiments in this study.

From Figs. 1 and 2 and the equations related to these figures it appeared that the rate of transfer of labelled cholesterol during the second phase was $^{14}C$ activity of the intima-media at 240 min multiplied by $\alpha$, and that the intercept of the extrapolated regression line on the ordinate axis was the $^{14}C$ activity of the intima-media at 240 min multiplied by $\beta$. Neither $\alpha$ nor $\beta$ could be shown to change with a 3-fold change in total cholesterol concentration, the temperature and pH being constant. Thus the initial uptake of cholesterol as well as the rate of cholesterol transfer during the second phase depend on the cholesterol concentration in the same way, given by the dependence of the $^{14}C$ activity of the intima-media at 240 min on the cholesterol concentration. A reasonable interpretation might be that it is primarily the initial uptake of cholesterol which is influenced by variation in the cholesterol concentration and that the rate of cholesterol transfer during the second phase is determined by the extent of initial uptake. The following model would fit the above considerations:

$$Ch_S + M \rightleftharpoons ChM \underset{?}{\rightleftharpoons} M + Ch_{I-M}$$

$Ch_S$ (serum cholesterol), M (endothelial cell membrane), ChM (cholesterol bound to the endothelial cell membrane) and $Ch_{I-M}$ (cholesterol taken up by intima-media — ChM). The initial uptake corresponds to ChM in the model.

The kinetics presented here are—as mentioned earlier—in several respects similar to those found for the uptake of [$^{131}I$]albumin by Ehrlich ascites-tumor cells[11] and for the uptake of ribonuclease and cytochrome *c* by *Amoeba proteus*[12], presumed to be due to pinocytosis.

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REFERENCES

1 J. JENSEN, *Biochim. Biophys. Acta*, 135 (1967) 532.
2 I. L. CHAIKOFF, M. D. SIPERSTEIN, W. G. DAUBEN, H. L. BRADLOW, J. F. EASTHAM, G. M. TOMKINS, J. R. MEIER, R. W. CHEN, S. HOTTA AND P. A. SRERE, *J. Biol. Chem.*, 194 (1952) 413.
3 K. DIEM, *Documenta Geigy, Scientific Tables*, J. R. Geigy, Basel, 1960, p. 170.7.
4 G. W. SNEDECOR, *Statistical Methods*, The Iowa State University Press, Ames, Iowa, U.S.A., 1956.
5 K. K. CARROLL, *J. Lipid Res.*, 2 (1961) 135.
6 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
7 R. L. CONNER AND F. UNGAR, *Exptl. Cell Res.*, 36 (1964) 134.
8 H. RYSER, J. C. AUB AND J. B. CAULFIELD, *J. Cell Biol.*, 17 (1962) 437.
9 H. RYSER, *Lab. Invest.*, 12 (1963) 1009.
10 V. N. SCHUMAKER, *Exptl. Cell Res.*, 15 (1958) 314.

11 J. S. HAGERMAN AND R. G. GOULD, *Proc. Soc. Exptl. Biol. Med.*, 78 (1951) 329.
12 J. R. MURPHY, *J. Lab. Clin. Med.*, 60 (1952) 571.
13 S. CHRISTENSEN AND J. JENSEN, *J. Atherosclerosis Res.*, 5 (1965) 258.
14 H. DAVSON AND J. F. DANIELLI, *The Permeability of Natural Membranes*, Cambridge, 1952, p. 296.
15 J. F. DANIELLI AND H. DAVSON, *J. Cellular Comp. Physiol.*, 5 (1934) 495.
16 L. MICHAELIS AND M. L. MENTEN, *Biochem. Z.*, 49 (1913) 333.
17 W. WILBRANDT, S. FREI AND T. ROSENBERG, *Exptl. Cell Res.*, 11 (1956) 59.
18 T. ROSENBERG AND W. WILBRANDT, *J. Gen. Physiol.*, 41 (1957) 289.
19 H. A. I. NEWMAN AND D. B. ZILVERSMIT, *J. Biol. Chem.*, 237 (1962) 2078.
20 S. HASHIMOTO AND S. DAYTON, *Circulation*, 30, Suppl. III (1964) 13.
21 J. M. BAILEY, *Proc. Soc. Exptl. Biol. Med.*, 107 (1961) 30.
22 G. H. ROTHBLAT, R. W. HARTZELL, H. MIALHE AND D. KRITCHEVSKY, *Biochim. Biophys. Acta*, 116 (1966) 133.