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# DIFFERENTIAL UPTAKE OF CHOLESTEROL AND OF DIFFERENT CHOLESTEROL ESTERS BY ATHEROSCLEROTIC INTIMA *IN VIVO* AND *IN VITRO*

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SUMMARY

Cholesterol-fed rabbits were intubated with a single dose of [$^3$H]cholesterol and the entry of the resultant labelled free cholesterol, cholesterol ester and of the individual cholesterol esters in the serum into the aortic intima and media was determined 4 days after ingestion of the cholesterol. The entry of cholesterol into the aortic intima exceeded that of cholesterol ester; the entry of monounsaturated cholesterol ester exceeded that of polyunsaturated cholesterol ester. The greater entry of cholesterol relative to cholesterol ester was confirmed in experiments *in vitro* in which atherosclerotic aortas were incubated with hypercholesterolaemic serum labelled with [$^3$H]cholesterol ester and $^3$H/$^{14}$C-labelled free cholesterol in the lipoprotein. It was not possible, however, to demonstrate any significant increase in entry of monounsaturated cholesterol ester over polyunsaturated cholesterol ester in these experiments *in vitro*. By using hypercholesterolaemic serum containing [$^3$H]cholesterol ester and $^3$H/$^{14}$C-labelled free cholesterol in the lipoprotein, as incubation medium, it was possible to demonstrate in the experiments *in vitro* that hydrolysis of the labelled cholesterol ester entering the intima could not account for the greater relative influx of free over ester cholesterol. The factors associated with the entry and accumulation of cholesterol and of various cholesterol esters in the atherosclerotic lesion are discussed.

Key words: *Atherosclerosis – Cholesterol – Cholesterol ester (polyunsaturated, monounsaturated, saturated, hydrolysis) – Cholesterol-fed rabbits*

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## INTRODUCTION

The atherosclerotic lesion is characterised by the deposition in the intima of considerable quantities of both cholesterol and cholesterol ester, and over recent years much work has been directed at determining the mechanism of deposition of these two lipid fractions. Cholesterol can be activiely synthesised by the intima[1,2], but the observations of NEWMAN *et al.*[3,4] and of DAYTON[5] have indicated that most of the cholesterol in the atherosclerotic intima arises from the plasma, rather than by synthesis in the wall. The entry of lipoprotein cholesterol into the arterial intima may not, however, be a simple filtration process. Both NEWMAN AND ZILVERSMIT[4,6] and HASHIMOTO AND DAYTON[7,8] have demonstrated that the influx of radioactively labelled cholesterol into the intima from the plasma is greater relative to cholesterol ester than one would expect on the basis of their relative concentrations in the plasma. One explanation of this data is that the plasma cholesterol exchanges more readily with the respective fractions in the arterial intima than does the plasma cholesterol ester. Hydrolysis of cholesterol ester in the atherosclerotic intima has been reported, however, by PATELSKI *et al.*[9] and by DAY AND GOULD-HURST[10]. Increased accumulation of labelled cholesterol compared with cholesterol ester may therefore be accounted for by partial hydrolysis of cholesterol ester following its entry. One aspect of the present paper is concerned with comparing the accumulation from the plasma of cholesterol with that of cholesterol ester under both *in vivo* and *in vitro* conditions, and assessing the role of exchange and of active hydrolysis in the wall in effecting such accumulation.

The deposition of cholesterol ester in the atherosclerotic arterial intima presents additional problems. It has been demonstrated in atherosclerotic lesions that [$^{14}$C]-acetate is incorporated *in vitro* into fatty acids which are in turn incorporated into cholesterol esters[11–13], and that this process is accelerated in the atherosclerotic lesion as compared with the normal intima. $^{14}$C-labelled fatty acids have also been shown to be taken up *in vitro* by the atherosclerotic intima and incorporated into cholesterol esters[14,15]. Cholesterol esterification in the wall may therefore account in part for some of the cholesterol ester deposition in this site. The composition of early atherosclerotic lesions in man[16–19] and experimental animals[20,21] — characterised as it is by a higher cholesterol oleate and a lower cholesterol linoleate content than is present in the plasma — would reinforce this possibility. While differential esterification may be important, differential entry or differential removal of cholesterol esters deposited from the plasma may also account for the high level of cholesterol oleate. SWELL *et al.*[22] have reported that the influx of monounsaturated cholesterol esters labelled with [4-$^{14}$C]cholesterol into the whole aorta of the cholesterol-fed rabbit *in vivo* is greater than that of saturated cholesterol esters or of cholesterol linoleate. However, this might reflect the differences in concentration of the individual cholesterol esters in the serum. In the present paper, the influx of individual cholesterol esters into atherosclerotic intimas is also investigated both *in vivo* and *in vitro* and the influx of these esters in relation to their concentration in the serum assessed.

## MATERIALS AND METHODS

*In vivo experiments*

Male New Zealand white rabbits fed a diet containing 1 % cholesterol and 3 % peanut oil for 3–4 months were used. They were intubated with an infant feeding tube under light ether anaesthesia and given 500 $\mu$Ci of cholesterol-T (G) (2370 mCi/m*M* Radiochemical Centre, Amersham, Great Britain) in 2 ml corn oil, followed by 100 mg sodium taurocholate in 0.9 % sodium chloride solution. Blood samples taken at 12, 24, 48, 72 and 96 h, were centrifuged at 3000 rev./min for 15 min and portions of the serum obtained extracted according to the method of FOLCH *et al.*[23]. The animals were killed by ether anaesthesia at 96 h and their thoracic aortas removed. The severity of atherosclerosis was assessed macroscopically and ascribed a grade from 0–V, according to the method of DAY AND WILKINSON[24]. Aortas used had grades from III–V. The adventitia was freed of superficial fat and the aorta thoroughly washed in 0.9 % sodium chloride solution. The vessel was then divided longitudinally into halves, one half being reserved for efflux studies. The other half was separated into intima and media/adventitia. The plane of separation was generally along the internal elastic lamina, but "intima" did include a small amount of the media. Intima and media/adventitia were extracted separately with chloroform–methanol (2 : 1, v/v) by the method of FOLCH *et al.*[23]. Lipid extracts of serum and aortic fractions were separated into cholesterol and cholesterol ester and then the cholesterol ester was separated into individual cholesterol esters for determination of the specific activity of these fractions as set out below. In order to calculate the influx of each component into the aortic intima or media an expression was required to indicate mean exposure of the aorta to $^3$H-labelled cholesterol (or cholesterol ester) over the 96 h period. To this end the median specific activity of each serum component was determined using Simpson's rule where the curve of changing specific activities is broken up into polygons.

$$\text{Median specific activity} = \frac{\frac{1}{2}t_1\,x + \frac{1}{2}(t_1 + t_2)\,y + \frac{1}{2}t_2\,z}{\Sigma t}$$

Where $x$ = the specific activity at time 0 h

$y$ the specific activity after time $t_1$

$z$ the specific activity after a further interval of time $t_2$, etc.

A Digital Equipment Corporation Data Processor PDP-8 was programmed to derive this information.

The half of the thoracic aorta reserved for efflux studies was incubated with shaking for 4 h at 37°C in 5 ml of medium containing Hank's–hypercholesterolaemic rabbit serum (50 : 50, v/v) in an atmosphere of air. The serum used was between 2 and 8 weeks old having been kept at 4°C with added penicillin and streptomycin (approximately 0.1 mg/ml of each). After 4 h the aorta was thoroughly washed in 0.9 % sodium chloride solution and treated in the same manner as for the influx experiments.

In one experiment, the rabbit was intubated with 3.5 mCi cholesterol-T (G), in order to obtain [$^3$H]serum for the *in vitro* experiments set out below. The thoracic aorta of this rabbit was removed 2 days after ingestion of cholesterol, divided longitudinally into halves and the efflux of cholesterol over a 4 h period determined. In this experiment, however, one-half was incubated in Hank's solution and the other half in Hank's–hypercholesterolaemic serum (50 : 50, v/v). The medium was changed at 10–30 min intervals in order to determine efflux in relation to time. The intima and media and the series of incubation media were extracted and processed as set out above.

*In vitro experiments*

Hypercholesterolaemic serum containing lipoprotein doubly labelled with [$^{14}$C]- and [$^3$H]cholesterol and cholesterol ester was obtained as follows. 3.5 mCi of [$^3$H]cholesterol was fed by intubation to a male New Zealand white rabbit fed cholesterol for 4 months. 42 h after intubation, the animal was exsanguinated by cardiac puncture, the serum separated and ethylenediaminetetraacetic acid (1 mg/ml) and penicillin and streptomycin added. The thoracic aorta from this animal was used to study efflux in relation to time as described above. The serum obtained labelled with [$^3$H]cholesterol both in the ester and free form was then incubated with normal rabbit erythrocytes[7] in order to exchange the [$^3$H]cholesterol, leaving the serum predominantly labelled with [$^3$H]cholesterol ester. This serum was then incubated *in vitro* for 3 h with [$^{14}$C]cholesterol (55.8 mCi/m*M*) (Radiochemical Centre, Amersham, Great Britain) suspended with Tween 20 (ref. [25]) in order to label the free cholesterol in the lipoprotein with [$^{14}$C]cholesterol. In this way a preparation of hypercholesterolaemic serum was obtained in which the lipoprotein cholesterol ester was labelled almost exclusively with $^3$H and lipoprotein free-cholesterol labelled with both $^{14}$C and $^3$H. This serum was used as incubation medium for the *in vitro* experiments and its content of free and ester cholesterol and their specific activities ($^3$H and $^{14}$C) were determined. In addition the distribution of the $^3$H and $^{14}$C in the free cholesterol among the lipoprotein fractions was determined after ultracentrifugation in a Spinco Model L Preparative Ultracentrifuge using densities of 1.006, 1.019, 1.063, 1.21. Electrophoresis was performed on cellulose acetate using 0.1 *M* barbitone–acetate buffer (Oxoid) pH 8.6 at 200 V for 2 h. This verified that both the $^3$H and $^{14}$C were present as a single peak with a migration corresponding to the pre-$\beta$-lipoprotein region as demonstrated by Oil Red O staining.

The influx *in vitro* of cholesterol and cholesterol esters was determined using whole thoracic aortas obtained from rabbits fed cholesterol for 4 months. The aorta was removed following killing by ether anaesthesia, cleaned and then incubated for 4 h at 37°C in 4.5 ml of the $^3$H/$^{14}$C doubly labelled serum prepared as above. Serum was incubated alone, also, to observe possible esterification and hydrolysis in the medium. After incubation, the intima and media/adventitia of the aorta and the incubation medium were extracted with chloroform–methanol and separated into cholesterol and cholesterol esters as for the *in vivo* experiments.

*Column chromatography*

Free and ester cholesterol were separated on alumina columns by the method described by DEYKIN AND GOODMAN[26]. With each batch of samples, a standard hypercholesterolaemic serum extract was run, the cholesterol ester collected in 4 fractions and the free cholesterol in 3 fractions in order to check separations at the loadings used. Column fractions were made up to a known volume and samples taken for counting and for cholesterol determination. Whenever possible, duplicate determinations were done. The cholesterol ester was further fractionated by thin layer chromatography on silver nitrate impregnated silicic acid.

*Thin-layer chromatography*

Cholesterol ester was separated into polyunsaturated, mono-unsaturated and saturated classes by thin-layer chromatography on silver nitrate impregnated silicic acid according to the method of MORRIS[27]. The spots, visualized using a spray of 0.2 % dichlorofluorescein in ethanol, were then scraped from the plates and the cholesterol esters eluted with three washes of 5–6 ml of chloroform–light petroleum (9 : 1, v/v). Each eluant was made up to a known volume and aliquots taken for radioassay and for cholesterol determination.

Cholesterol ester was separated from other lipid fractions prior to gas–liquid chromatography, by neutral lipid thin-layer chromatography on Silica Gel G (Merck) using diethyl ether–acetic acid–*n*-hexane (38 : 3 : 100, v/v/v) as developing solvent.

*Radioassay*

Counting was done using 10 ml scintillator containing 4 g PPO and 100 mg POPOP per l toluene in a Packard Tricarb Model 3375 Spectrometer with conditions adjusted to count either $^{3}H$ alone or $^{3}H$ and $^{14}C$ together.

*Cholesterol assay*

Cholesterol was determined in the extracts or in the column eluants by the method of ZLATKIS *et al.*[28] following saponification by the method of ABELL *et al.*[29].

*Gas–liquid chromatography*

Aliquots of the cholesterol ester separated by thin-layer chromatography from serum or aorta were methyl esterified at 65°C with 5 % $H_2SO_4$ in methanol in sealed ampoules and the fatty acid composition determined by gas–liquid chromatography using diethylene glycol succinate columns at 185°C in an F & M Model 5750 Gas Chromatograph. Quantitative results with fatty acid standards KA, KB, KC and KD from Applied Science Laboratories, State College, Pa. agreed with the stated comparison data, with a relative error less than 5 % for major components (> 10 % of total mixture) and less than 10 % for minor components (< 10 % of total mixture).

This data was used in the *in vivo* experiments to calculate the amount of mono-, poly- and saturated cholesterol esters present in the total cholesterol ester.

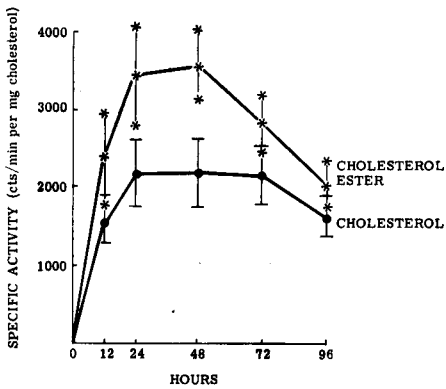


Fig. 1. Specific activities of serum free and ester cholesterol following ingestion of [$^3$H]cholesterol. Means of six experiments and standard errors of means are plotted.

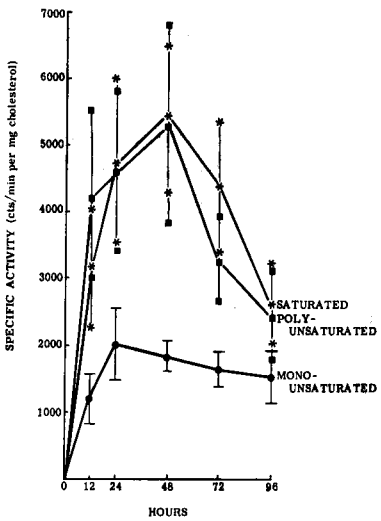


Fig. 2. Specific activities of saturated, monounsaturated, and polyunsaturated cholesterol esters in the serum following ingestion of [$^3$H]cholesterol. Means of four experiments and standard errors of the means are plotted.

## RESULTS

The ingestion of 500 $\mu$Ci of $^3$H-labelled free cholesterol is followed by labelling of both free and ester cholesterol in the serum. The change in specific activity of these fractions is shown in Fig. 1 for the four day period studied in the *in vivo* experiments. There is appreciable labelled cholesterol and cholesterol ester present in the serum at 12 h, and the specific activity of both fractions rises together reaching a maximum 48 h after ingestion. Over the whole period of the experiment the specific activity of the cholesterol ester exceeds that of the cholesterol.

The changes in specific activity of the individual groups of cholesterol esters over the 4 day period are shown in Fig. 2. The pattern of labelling with respect to time of the saturated, mono- and polyunsaturated cholesterol esters is similar, and resembles that of the total cholesterol ester shown in Fig. 1. The specific activities of the polyunsaturated and saturated cholesterol ester exceed that of the monounsaturated group. The amount of monounsaturated cholesterol ester present in the serum, however, is more than twice that of the other two fractions (see Table 1 below).

The median specific activities of the serum cholesterol and cholesterol ester and of the individual cholesterol esters were derived from the specific activities of these fractions at the different time intervals, as described in METHODS. This median specific activity was then used to calculate the influx of the various fractions into the arterial wall as follows. It was assumed that the fractions were passing into the intima from common plasma pools at the respective median specific activities over the 4 day period studied. The influx of cholesterol or of the various cholesterol esters into the aorta was then calculated by dividing the counts/min of the respective fraction, which accumulated over the 4 day period in the intima or media, by the median specific activity in the serum. The influx was expressed as $\mu$g/day.

The influx of free and ester cholesterol into the intima, together with the influx of the individual cholesterol esters, saturated, monounsaturated and polyunsaturated, is given in Table 1. This table also contains data regarding the content of free cholesterol and of cholesterol esters in the serum and the intima, together with the median specific activities of the various fractions in the serum and in the intima 4 days after the ingestion of the cholesterol. The influx of free cholesterol and of cholesterol ester into the intima from the serum is approximately the same. However, about twice as much cholesterol ester as free cholesterol is present in the serum and a more comparable figure can be derived by comparing the influx of the different fractions entering in relation to their concentration in the serum. The influx per mg/ml of the respective fraction in the serum is given in Table 1, together with the relative influx calculated on this basis for the individual experiments. On this basis it can be seen that the entry of free cholesterol relative to cholesterol ester into the intima is about twice as much as might be expected in relation to their respective concentrations in the serum.

The influx of the individual cholesterol esters, saturated, monounsaturated and polyunsaturated, into the intima is also given in Table 1. Most of the cholesterol ester entering the artery is monounsaturated. However, the concentration of monounsaturated cholesterol ester in the serum is much greater than that of the other two groups. When this is taken into account the relative influx of monounsaturated cholesterol ester is greater than that of saturated or polyunsaturated. The difference between monounsaturated and saturated, however, is not statistically significant.

In four of the six experiments, the influx of free and ester cholesterol into the aortic media was also determined, and this data is provided in Table 2, together with the mean data for the chemical content of the serum and its median specific activity over the time period studied. The influx of cholesterol and cholesterol ester into the

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TABLE 1

INFLUX OF LIPOPROTEIN [$^3$H]CHOLESTEROL AND CHOLESTEROL ESTERS INTO CHOLESTEROL-FED RABBIT INTIMAL HALVES *in vivo*

| | *Cholesterol content* | | *Specific activity (counts/min/mg)* | | *Influx* | | |
|---|---|---|---|---|---|---|---|
| | *serum (mg/ml)* | *intima (mg in toto)* | *serum* | *intima* | *μg/day* | *μg/day per mg/ml* | *relative influx*[c] |
| Free cholesterol[a] | 5.10 ± 0.41 | 5.35 ± 0.95 | 1881 ± 322 | 269 ± 74 | 179.1 ± 46.3 | 34.1 ± 6.8 | 1 |
| Cholesterol ester[a] | 11.61 ± 1.30 | 10.36 ± 1.62 | 2797 ± 397 | 256 ± 80 | 224.8 ± 76.2 | 18.5 ± 4.2 | 0.54 ± 0.02[d] |
| Cholesterol ester[b] | | | | | | | |
| saturated | 2.23 ± 0.23 | — | 4091 ± 938 | — | 33.0 ± 5.1 | 15.4 ± 3.1 | 0.78 ± 0.11[e] |
| monounsaturated | 5.82 ± 0.78 | — | 1631 ± 294 | — | 108.0 ± 4.4 | 20.1 ± 3.8 | 1 |
| polyunsaturated | 2.28 ± 0.22 | — | 3880 ± 908 | — | 30.6 ± 5.1 | 14.0 ± 4.5 | 0.68 ± 0.06[f] |

[a] Means and standard errors of means of 6 experiments.
[b] Means and standard errors of means of 4 experiments.
[c] Relative influx is the ratio of the influx per mg/ml of serum cholesterol and cholesterol ester with respect to free cholesterol; or of the individual cholesterol esters with respect to monounsaturated cholesterol ester.
[d] Significant at $< 0.1$ % level.
[e] No significant difference.
[f] Significant at $< 2$ % level.

TABLE 2

INFLUX OF LIPOPROTEIN [$^3$H]CHOLESTEROL AND CHOLESTEROL ESTERS INTO CHOLESTEROL-FED RABBIT AORTIC MEDIAS *in vivo*[a]

| | *Cholesterol content* | *Specific activity counts/min/mg* | *Influx* | | |
|---|---|---|---|---|---|
| | *serum mg/ml* | *serum* | *μg/day* | *μg/day per mg/ml* | *relative influx* |
| Free cholesterol | 4.95 ± 0.40 | 1590 ± 359 | 40.8 ± 7.5 | 8.6 ± 2.0 | 1 |
| Cholesterol ester | 10.40 ± 1.13 | 2529 ± 553 | 26.5 ± 8.6 | 2.5 ± 0.7 | 0.29[b] ± 0.02 |

[a] Means and standard errors of means of 4 experiments.
[b] Significant at < 0.1 % level.

TABLE 3

PERCENTAGE DISTRIBUTION OF SERUM AND INTIMAL CHOLESTEROL ESTER FATTY ACID PATTERNS OF INDIVIDUAL RABBITS USED FOR *in vivo* STUDIES

| | *16 : 0* | *16 : 1* | *18 : 0* | *18 : 1* | *18 : 2* | *20 : 0* |
|---|---|---|---|---|---|---|
| Serum | 19.1 | 7.2 | 3.9 | 51.9 | 18.0 | — |
| Intima | 18.9 | 5.2 | 5.2 | 58.1 | 9.6 | 2.3 |
| Serum | 19.7 | 5.7 | 5.4 | 44.2 | 27.5 | — |
| Intima | 15.0 | 3.2 | 4.8 | 56.7 | 18.6 | 1.8 |

media was calculated assuming that it comes directly from the serum rather than the intima. The influx of both free cholesterol and cholesterol ester is considerably less than that into the corresponding intima. The relative influx, however, indicates that the entry of free cholesterol exceeds that of cholesterol ester by an even greater margin than that for the intima. In view of the very small influx of cholesterol ester into the media in these *in vivo* experiments, it was not possible to calculate the influx of the individual cholesterol esters into the media. The chemical content of the individual cholesterol esters in the media is low and was not determined in these experiments.

Since the relative influx of $^3$H-labelled monounsaturated cholesterol ester into the arterial intima over the 4 day period exceeded that of the polyunsaturated ester, an attempt was made to confirm by gas–liquid chromatography whether there were differences in the cholesterol ester fatty acid composition of the aortic intima relative to the serum. This data is shown for 2 rabbits in Table 3. In both rabbits the percentage of cholesterol oleate in the intima exceeds that in the serum, whereas that of cholesterol linoleate in the intima is less than that in the corresponding serum.

The efflux of cholesterol and cholesterol ester from the intima to the incubation medium over a 4 h period is presented in Table 4. In these experiments, the other aortic halves were used to study the efflux of cholesterol and cholesterol ester. The efflux

TABLE 4

EFFLUX INTO HANK'S–HYPERCHOLESTEROLAEMIC SERUM OF [$^3$H]CHOLESTEROL FROM CHOLESTEROL-FED RABBIT AORTIC HALVES LABELLED *in vivo*[a]

| | *Cholesterol content* | *Specific activity counts/min/mg* | | *Efflux*[c] | |
|---|---|---|---|---|---|
| | *intima (mg in toto)* | *intima* | *μg/day* | *μg/day per mg in toto* | *relative efflux* |
| Free cholesterol | 4.47 ± 0.69 | 256 ± 70 | 7401 ± 1448 | 1925 ± 466 | 1 |
| Cholesterol ester | 10.01 ± 1.63 | 209 ± 46 | 9873 ± 3453 | 915 ± 216 | 0.54 ± 0.11[b] |

[a] Means and standard errors of means of 6 experiments.
[b] Significant at 1 % level.
[c] Values are calculated assuming efflux from a single pool. This assumption is probably incorrect as discussed in the text.

from the artery into the incubation medium may have been derived from both intima and media, but since a mean of 81.6 % of the $^3$H-labelled total cholesterol in these aortic halves was present in the intima, the effluxes shown in Table 4 can be considered to come predominantly from the intima. The efflux is calculated making the assumption that the free and ester cholesterol are removed from a single pool at the specific activity of the intima and is obtained from the counts/min of free and ester cholesterol which appear in the incubation medium divided by the specific activity of the intima. The efflux is then expressed as μg/day. The efflux calculated in this way is considerably in excess of that for the calculated influx *in vivo* in the other aortic half. It appears likely, therefore, that there may be more than one intimal pool. However, if one can assume that the relative specific activities of the free and ester cholesterol in the more active pool are similar to that for the whole intima then the comparison of efflux data is valid. A greater relative efflux of cholesterol than cholesterol ester is apparent from Table 4.

In order to study whether a more active pool may exist in the intima, an additional experiment was carried out in which the efflux of cholesterol *in vitro* was examined at 10–30 min intervals over a 4 h period from an artery labelled *in vivo* following ingestion of [$^3$H]cholesterol. The efflux of [$^3$H]cholesterol into incubation medium containing either Hank's–hypercholesterolaemic serum (50 : 50, v/v) or Hank's solution was compared using halves of the same artery, and this data is presented in Fig. 3. The [$^3$H]cholesterol was assumed to be coming solely from the intima since more than 90 % of the total $^3$H in the artery was in the intima. In Fig. 3A the total counts/min remaining in the intima of each half of the aorta have been plotted on a logarithmic scale against time; this represents a disappearance curve of [$^3$H]-cholesterol from the intima. This curve can be resolved into two separate components suggesting two separate pools in the intima. After 120 min both curves are essentially straight and represent the exit of [$^3$H]cholesterol from the less active pool. The half life of this pool, determined from the gradient of this line is 25 h in the case of the Hank's–serum incubation medium (50 : 50, v/v) and 50 h in the case of the Hank's–

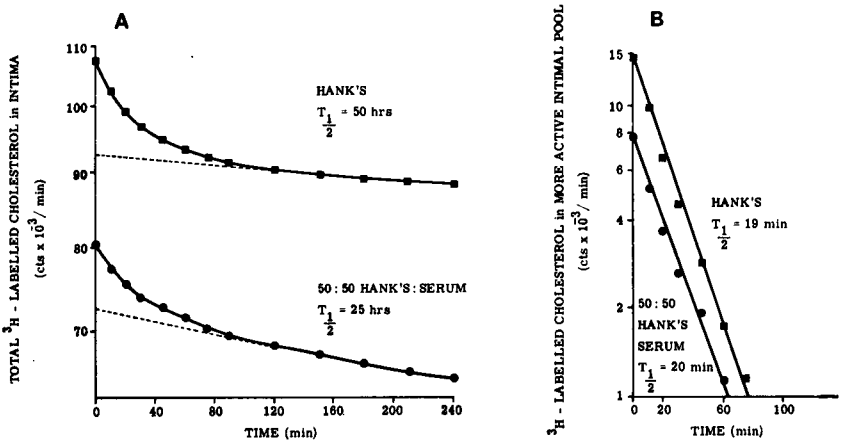


Fig. 3. Efflux of [³H]cholesterol from atherosclerotic intimal halves into either an incubation medium of Hank's solution or Hank's–hypercholesterolaemic serum (50 : 50, v/v). Counts/min remaining in the intima are plotted logarithmically in both A and B. Curves in A have been resolved into two components. The more slowly removed component (dotted line) has been subtracted from the composite curves to yield a plot of the more rapidly removed component in B.

incubation medium. Extrapolation of this line back to the ordinate and subtraction from the disappearance curve, resolves the second component (Fig. 3B). That this component can be represented by a straight line suggests that it is a single more active pool, the half life of which is 19 min for Hank's solution and 20 min for Hank's serum (50 : 50, v/v). 19.1 % of the [³H]cholesterol present initially in the artery was removed during the 4 h into the medium containing hypercholesterolaemic serum, and 17.9 % of the [³H]cholesterol present in the artery into the medium containing Hank's solution, so that in both cases over 80 % of the label was still present in the artery at this time.

The characteristics of the ³H/¹⁴C-labelled serum used as incubation medium for the *in vitro* influx experiments are shown in Table 5. Most of the [³H]cholesterol was

TABLE 5

*In vivo* [³H]CHOLESTEROL ESTER/*in vitro* [¹⁴C]CHOLESTEROL LABELLED LIPOPROTEIN FROM CHOLESTEROL-FED RABBITS

| | *Cholesterol (mg/ml)* | *Specific activity (disint./min ³H/mg)* | *Specific activity (disint./min ¹⁴C/mg)* | *³H/¹⁴C ratio* |
|---|---|---|---|---|
| Free cholesterol | 1.9 | 255,300 | 199,000 | 1.26 |
| Cholesterol ester | 8.4 | 576,800 | 717 | 804 |
| Cholesterol ester | | | | |
| saturated | 1.8 | 716,200 | — | — |
| monounsaturated | 4.4 | 413,500 | — | — |
| polyunsaturated | 2.1 | 744,000 | — | — |

TABLE 6

INFLUX OF LIPOPROTEIN $^{3}H/^{14}C$-LABELLED CHOLESTEROL AND CHOLESTEROL ESTERS INTO CHOLESTEROL-FED RABBIT AORTIC INTIMAS *in vitro*[a,b,c]

| | *Cholesterol content (mg)* | $^{3}H$-*data* | | | | $^{14}C$-*data* | | |
|---|---|---|---|---|---|---|---|---|
| | | *specific activity (disint./min/mg)* | *influx (μg/day)* | *influx (μg/day per mg/ml)* | *relative influx* | *specific activity (disint./min/mg)* | *influx (μg/day)* | *influx (μg/day per mg/ml)* |
| Free cholesterol | 6.96 ± 4.02 | 1258 ± 436 | 144.4 ± 21.2 | 67.1 ± 9.9 | 1 | 1316 ± 430 | 181.8 ± 34.4 | 84.5 ± 16.0 |
| Cholesterol ester | 14.71 ± 6.80 | 2559 ± 605 | 315.4 ± 82.1 | 36.5 ± 9.5 | 0.53 ± 0.09[d] | — | — | — |
| Cholesterol ester | | | | | | | | |
| saturated | 4.32 ± 1.86 | 2198 ± 532 | 68.6 ± 11.1 | 34.8 ± 5.6 | 0.82 ± 0.12 | — | — | — |
| monounsaturated | 6.19 ± 3.09 | 2691 ± 524 | 204.0 ± 58.4 | 45.8 ± 13.1 | 1 | — | — | — |
| polyunsaturated | 4.21 ± 1.85 | 3050 ± 463 | 98.2 ± 26.4 | 44.0 ± 11.8 | 0.97 ± 0.03 | — | — | — |

[a] Means and standard errors of means of 3 experiments.
[b] Data for serum used given in Table 5.
[c] Whole aortas not halves were used in these experiments.
[d] Significant at $< 5$ % level.

present as cholesterol ester distributed between the individual cholesterol esters. Practically all of the $^{14}C$ was present as free cholesterol. The $^{3}H/^{14}C$ ratio of the free cholesterol was 1.26 and that of the cholesterol ester 804. The $^{3}H/^{14}C$ ratio of the free cholesterol was obtained by replicate determinations with a standard deviation of 0.03 ($n = 8$). This is of some importance since a rise in this ratio in the intima is interpreted as hydrolysis of cholesterol ester following entry of the $^{3}H/^{14}C$-labelled serum into the intima.

The influx of cholesterol and of the various cholesterol esters into the intima in the *in vitro* experiments is shown in Table 6. The influx was calculated on the same basis as that for the *in vivo* experiments, except that in this case the specific activity of the serum bathing the artery was constant throughout the experiment. In addition, as the free cholesterol was labelled with both $^{3}H$ and $^{14}C$, it was possible to calculate the influx of free cholesterol from both the [$^{3}H$]- and the [$^{14}C$]cholesterol present. The amount of free cholesterol and of cholesterol ester entering the intima ($\mu g$/day) in these experiments is similar to the figures obtained for the *in vivo* experiments, both with respect to the free cholesterol and cholesterol ester and with respect to the individual groups of cholesterol esters. It needs to be borne in mind that in these experiments influx into whole aortas and not into aortic halves is being calculated. The relative influx of free cholesterol as compared with cholesterol ester also closely resembles that shown in the *in vivo* experiments. There is no significant difference, however, between the relative entry of the individual groups of cholesterol esters. It will be observed from Table 6, that the influx of free cholesterol as calculated from the $^{14}C$ data is about 20 % higher than that calculated from the $^{3}H$ data. This difference suggested that the lipoprotein labelling produced by the $^{14}C$ differed somewhat from that produced by the $^{3}H$, one being labelled *in vivo* and the other *in vitro*. In order to determine whether the [$^{3}H$]- and [$^{14}C$] cholesterol was distributed in the same proportion in the various lipoprotein fractions of the serum, the serum lipoproteins were separated by ultracentrifugation, extracted, and the $^{3}H$ and $^{14}C$ present in the free cholesterol of the various fractions determined. This data is shown in Table 7. It can be seen that while the bulk of both the [$^{3}H$]- and the [$^{14}C$]cholesterol is present in the fraction with a density less than 1.006, there is some difference in the relative distribution of the label; slightly more $^{14}C$ and less $^{3}H$ labelled free cholesterol is present in the

TABLE 7

LIPOPROTEIN ANALYSIS OF $^{3}H/^{14}C$ CHOLESTEROL-LABELLED HYPERCHOLESTEROLAEMIC SERUM BY ULTRACENTRIFUGATION

| *Density* | *Free cholesterol* | | |
|---|---|---|---|
| | *$^{3}H$ (%)* | *$^{14}C$ (%)* | *$^{3}H/^{14}C$ ratio* |
| < 1.006 | 59.1 | 49.6 | 2.29 |
| 1.006–1.019 | 25.3 | 29.5 | 1.47 |
| 1.019–1.063 | 13.2 | 17.3 | 1.46 |
| 1.063–1.21 | 2.5 | 3.6 | 1.32 |

TABLE 8

INFLUX OF LIPOPROTEIN $^{3}H/^{14}C$-LABELLED CHOLESTEROL AND CHOLESTEROL ESTERS INTO CHOLESTEROL-FED RABBIT AORTIC MEDIA *in vitro*[a,b,c]

| | *Cholesterol content (mg)* | *$^{3}H$-data* | | | | *$^{14}C$-data* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *specific activity (disint./min/mg)* | *influx (μg/day)* | *influx (μg/day per mg/ml)* | *relative influx* | *specific activity (disint./min/mg)* | *influx (μg/day)* | *influx (μg/day per mg/ml)* | *relative influx* |
| Free cholesterol | 2.14 ± 0.40 | 10152 ± 1472 | 558 ± 81 | 259 ± 38 | 1 | 10872 ± 2222 | 752 ± 127 | 350 ± 59 | — |
| Cholesterol ester | 5.75 ± 1.30 | 20832 ± 4671 | 1198 ± 212 | 139 ± 24 | 0.53 ± 0.04 | — | — | — | — |
| Cholesterol ester | | | | | | | | | |
| saturated | 1.49 ± 0.50 | 29210 ± 7120 | 279 ± 51 | 142 ± 26 | 0.87 ± 0.03 | | | | |
| monounsaturated | 2.81 ± 0.47 | 22070 ± 6050 | 732 ± 153 | 164 ± 34 | 1 | | | | |
| polyunsaturated | 1.45 ± 0.34 | 35350 ± 10030 | 334 ± 71 | 150 ± 32 | 0.91 ± 0.04 | | | | |

[a] Means and standard errors of means of 3 experiments.
[b] Data for serum used given in Table 5.
[c] Whole aortas not halves were used in these experiments.