

higher density lipoproteins. This skewing of distribution of ^{14}C compared with ^3H may explain the slight difference in the influx data calculated from the two labels, presumably by increased entry of the smaller lipoprotein particles labelled with ^{14}C .

Influx of cholesterol, cholesterol ester and the individual groups of cholesterol ester into the media in the *in vitro* experiments is shown in Table 8. It will be noted that the influx into the media in these experiments is considerably higher than that into the media in the *in vivo* experiments, and exceeds that into the intima in the corresponding *in vitro* experiments. The influx of free cholesterol in relation to ester cholesterol, however, bears the same relationship as in the intima. More free cholesterol enters the media than would be expected on the basis of its concentration in the serum of the incubation medium. The entry of individual groups of cholesterol esters is also shown in Table 8. The relative influx of monounsaturated exceeds that of both saturated and polyunsaturated but differences are not significant. Thus, although the influx into the media in the *in vitro* experiments is greatly in excess of that in the *in vivo* experiments, the relative amounts of ester and free and of individual cholesterol esters is essentially that shown for the previously described experiments.

The contribution of hydrolysis of cholesterol ester in the artery to the apparent influx of free cholesterol is shown in the data given in Table 9. Hydrolysis of the pre-

TABLE 9

ESTERIFICATION AND HYDROLYSIS OF LIPOPROTEIN $^3\text{H}/^{14}\text{C}$ -LABELLED CHOLESTEROL BY ATHEROSCLEROTIC RABBIT AORTAS *in vitro*

	Time (h)	Experiment	% Cholesterol ester		$^3\text{H}/^{14}\text{C}$ free cholesterol
			^3H	^{14}C	
Serum	0		90.9	1.6	1.26
	4		91.1 ± 0.2^a	1.4 ± 0.3^a	1.21 ± 0.03^a
Intima	4	1	82.8	2.1	0.97
	4	2	87.6	15.3	0.86
	4	3	83.1	1.5	0.97
Media	4	1	85.8	1.4	0.86
	4	2	82.9	1.4	1.00
	4	3	83.3	1.1	1.00

^a Mean of 8 determinations with standard deviations.

dominantly ^3H -labelled cholesterol ester in the intima would give rise to an elevation of the $^3\text{H}/^{14}\text{C}$ ratio of the free cholesterol present and so provides a sensitive indication of the presence of hydrolysis in the artery. No hydrolysis of the serum occurred during incubation for 4 h, the $^3\text{H}/^{14}\text{C}$ ratio altering from 1.26–1.21 over a 4 h incubation period as shown in Table 9. The $^3\text{H}/^{14}\text{C}$ ratio of the free cholesterol in the aortic intima and media in the three experiments carried out is also shown in Table 9. Under these circumstances the $^3\text{H}/^{14}\text{C}$ ratio fell slightly in both intima and media. No evidence of hydrolysis of the lipoprotein cholesterol ester was therefore obtained.

DISCUSSION

The entry of cholesterol and cholesterol ester into the normal and atherosclerotic arterial intima has been the subject of a number of studies^{4,6-8,30}. The results reported in the present paper confirm those of other workers for the atherosclerotic intima^{4,6} in that the entry both *in vivo* and *in vitro* of radioactively labelled free cholesterol in relation to cholesterol ester is twice that expected on the basis of their respective plasma concentrations. The reason for the greater relative influx of free cholesterol, however, is open to question, is as the mechanism of influx. Entry of cholesterol into the intima may be by an active or a passive mechanism, or both. The work of JENSEN^{30,31} on normal rabbit aorta suggests that there is active transport of plasma cholesterol into the intima accompanied by hydrolysis of the cholesterol ester. Experiments by NEWMAN AND ZIVERSMIT⁶ on atherosclerotic rabbit aorta, and HASHIMOTO AND DAYTON^{7,8} on normal rat aorta, however, suggest that entry of cholesterol does not require cellular or enzymic activity.

The possibility that hydrolysis of cholesterol ester in the arterial wall contributes to the apparent influx of cholesterol, however, has not been excluded in the work reported by other investigators. Hydrolysis of cholesterol ester has been reported for both the normal and atherosclerotic wall^{9,10} and it seemed possible that this mechanism might affect the calculation of cholesterol influx. In the present work this possibility has been excluded by using double-labelled lipoprotein cholesterol and cholesterol ester. It can be seen from a consideration of the properties of the double-labelled lipoprotein used in these experiments that it is possible to detect very small amounts of hydrolysis of the cholesterol ester entering the arterial intima. 5% hydrolysis of the [³H]cholesterol ester would produce an increase in the ³H/¹⁴C ratio of the free cholesterol of about 50%. No such shift occurred, in fact a slight fall in the ratio was observed. Under the circumstances of the present experiments, therefore, none of the calculated cholesterol influx can be accounted for by hydrolysis of the labelled cholesterol ester entering the wall. The high relative influx of free cholesterol must therefore be accounted for either by selective entry of free cholesterol or by exchange with lipoprotein in the intima. Radioactively labelled free cholesterol exchanges readily with plasma lipoprotein³², whereas cholesterol ester does not. The exchange of free cholesterol between the plasma and intima may therefore account for the entry of radioactive free cholesterol into the intima but exchange of cholesterol ester is unlikely to account for the entry of radioactive cholesterol ester. Two alternative possibilities present themselves, namely, esterification of free cholesterol in the intima or direct entry of radioactive cholesterol ester as lipoprotein. In our *in vitro* experiments (Table 9) and those of other workers¹⁵, esterification of radioactive cholesterol is difficult to demonstrate in the intima. Entry of cholesterol ester directly as plasma lipoprotein would therefore seem more likely.

The finding, from the *in vivo* studies, that most of the aortic labelled cholesterol, both free and ester, is in the intima, is in accord with the autoradiographic findings of ADAMS *et al.*³³, although, in a later paper, using a multiple layering technique,

ADAMS *et al.*³⁴ report differences in distribution of labelled cholesterol across the aortic wall according to the degree of atherosclerosis. It should be noted that, in the present paper, calculation of influx of cholesterol and cholesterol ester into the arterial media, is based on the assumption that the cholesterol entering the media has the same specific activity as plasma cholesterol. If radioactive cholesterol in the arterial media is derived from the vasa vasorum, the calculation is valid. If the radioactive cholesterol is derived from the intima, however, the calculated influx could be an underestimate because of the lower intimal specific activities. The influx of radioactive cholesterol into the intima, is similar *in vivo* and *in vitro*. However, the influx of cholesterol into the media is much greater *in vitro* than *in vivo*, probably due to the more direct exposure of the arterial media/adventitia to radioactive cholesterol from the incubation medium.

The efflux of the labelled cholesterol from the aorta in $\mu\text{g}/\text{day}$, exceeds influx by some 50 times. Since more than 80 % of the radioactivity in the aorta is present in the intima, it is reasonable to assume that efflux of labelled cholesterol *in vitro* was mainly from the intima. The high apparent efflux may be explained if a cholesterol pool of high specific activity were present in the intima, as suggested by NEWMAN AND ZILVERSMIT⁶. To investigate this possibility the efflux of [³H]cholesterol from the artery was studied in relation to time. Data from only one experiment is available, but with this limitation in mind it is possible, using a semilogarithmic plot, to resolve the efflux into two components. In this experiment efflux of the more rapidly removed component of labelled cholesterol did not appear to depend on the availability of lipoprotein in the incubation medium. The observation that efflux of labelled cholesterol occurs into incubation medium containing Hank's solution alone contrasts with the observations of DAYTON AND HASHIMOTO⁸ for normal rat aorta, in which relatively little efflux occurred into buffer solution but considerable efflux took place into a solution containing serum lipoprotein. This contrast may be due to a difference between the normal and atherosclerotic intima: greater amounts of plasma lipoprotein being present in the atherosclerotic intima because of surface disorganization or increased permeability. This lipoprotein would then be removed into an aqueous medium over a subsequent incubation period. Another possibility is a contribution from cell breakdown or from cells denuded from the incubated tissue. Efflux of the more slowly removed component is appreciably greater, as indicated by the $T_{\frac{1}{2}}$, when Hank's-serum is used rather than Hank's solution alone. This difference is likely to represent exchange of radioactive intimal cholesterol with that in the serum lipoprotein of the medium.

In this experiment, as in all the efflux experiments, more than 80 % of the labelled cholesterol was present in the intima at the end of the 4 h *in vitro* incubation period and this amount contained both free and ester labelled cholesterol. However, since the $T_{\frac{1}{2}}$ for the efflux of [³H]cholesterol from the larger less active pool into the Hank's-serum incubation medium was 25 h, considerable recycling of [³H]cholesterol possibly occurred during the 96 h study of influx *in vivo*. The influx figures as calculated from the counts/min that accumulated in the intima over the 96 h period may

therefore be rather low. However, the validity of the comparisons, on a relative basis, of cholesterol and cholesterol ester is not necessarily influenced by such considerations. The relative efflux of free cholesterol is twice that of cholesterol ester as was the case for the relative influx. The cholesterol of the intima, therefore, is turning over more rapidly than the cholesterol ester. This may simply reflect a process of physicochemical exchange, however, and need not necessarily reflect the mechanism of the overall accumulation of cholesterol ester in the atherosclerotic lesion.

The other aspect of the present paper was to obtain some information regarding the relative entry of different groups of cholesterol esters into the atherosclerotic arterial intima. There is now considerable evidence that the atherosclerotic intima differs in composition, both in man and experimental animals, from that of the serum, in that it contains a higher proportion of cholesterol oleate and a lower proportion of cholesterol linoleate³⁵. In the present paper, this shift of cholesterol ester pattern has been confirmed in that the aortic intima contains more cholesterol oleate and less cholesterol linoleate than the cholesterol esters of the terminal plasma. In the acute experiments reported in which the entry of [³H]cholesterol esters into the already atherosclerotic intima was observed *in vivo*, it was also shown that the influx in $\mu\text{g}/\text{day}$ of monounsaturated cholesterol ester exceeded that of polyunsaturated and saturated cholesterol ester. This agrees with the findings of SWELL *et al.*²². Even when serum concentrations of individual cholesterol esters were taken into account, the relative influx of ³H-labelled monounsaturated cholesterol esters was still significantly in excess of that of the polyunsaturated cholesterol esters. There are four possibilities to account for these differences, *viz.* (i) differential entry, (ii) differential removal, (iii) differential hydrolysis or differential esterification in the arterial wall, and (iv) differential exchange of cholesterol ester fatty acids. With regard to the last two possibilities, it has been observed in a series of experiments that fatty acid is incorporated into cholesterol ester in both experimental and in human atherosclerotic lesions^{14,36}. NEWMAN *et al.*¹⁵ have shown that of fatty acids synthesised *in vivo* from [¹⁴C]acetate in the rabbit atherosclerotic aorta, saturated and monounsaturated sorts are incorporated into cholesterol ester to a greater extent than polyunsaturated. In experiments currently being carried out in our laboratory, the relative incorporation of different fatty acids into cholesterol ester in the atherosclerotic lesion has been studied³⁷ and it has been shown that oleic acid is incorporated preferentially into cholesterol ester in the lesion. However, since little esterification at all of the [¹⁴C]-cholesterol present could be demonstrated in our experiments and in those of others^{6,15} the possibility of differential esterification of free cholesterol is in doubt.

As regards differential hydrolysis, it has been shown that cholesterol esters are hydrolysed in the atherosclerotic lesion^{9,10}, cholesterol oleate being hydrolysed relatively slowly³⁸. In the present experiments, however, the absence of significant hydrolysis of cholesterol ester in the intima indicates that the greater relative influx of monounsaturated over polyunsaturated cholesterol ester cannot be accounted for by preferential hydrolysis of the polyunsaturated cholesterol ester.

With respect to the removal of cholesterol esters from the arterial wall, it has been suggested that the polyunsaturated and saturated cholesterol esters are removed more readily than are the monounsaturated³⁸. It was suggested by these workers, however, that such removal is dictated by the relative hydrolysis of the different esters. ABDULLA *et al.*³⁹ have shown that the cholesterol esters containing polyunsaturated fatty acids are removed from granulomatous lesions more rapidly than are the more saturated cholesterol esters, and suggest that this mechanism is responsible for the differential removal of cholesterol esters from the atherosclerotic lesion. There is, however, little information about the relative removal of cholesterol esters as such from the atherosclerotic intima. SWELL *et al.*²² deduce from calculated influxes per day and from net accumulation of cholesterol ester classes that saturated cholesterol esters are retained to the greatest extent, followed by monounsaturated and linoleate esters. It is possible that the data in the present paper may reflect the removal from the atherosclerotic intima of polyunsaturated and saturated cholesterol esters more rapidly than of monounsaturated.

It would seem, therefore, that the processes of entry and removal of cholesterol esters influence the composition of the lesion in such a way as to favour the accumulation of cholesterol oleate in the atherosclerotic intima.

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