The Uptake of Lipids by Rat Liver Cells

By JOAN A. HIGGINS and C. GREEN
Department of Biochemistry, University of Liverpool

(*Received* 31 *August* 1965)

1. Unesterified cholesterol, cholesterol esters and triglycerides of chylomicrons were taken up at the same rate by isolated hepatic parenchymal cells. 2. On incubation of hepatic cells, isolated 2min. after the injection of chylomicrons *in vivo*, the chylomicron triglyceride associated with the cells underwent hydrolysis. 3. In cells isolated 5min. after the injection of chylomicrons, the chylomicron triglyceride bound to the hepatic cells was accessible to added clearing factor lipase. 4. 'Ghost' hepatic cells had the same binding capacity and lipolytic activity per cell as intact cells. 5. Of all subcellular fractions studied, the 'plasma membrane' fraction showed the greatest capacity per unit weight for non-esterified fatty acid and chylomicron triglyceride binding and for triglyceride hydrolysis. 6. Once non-esterified fatty acids entered the hepatic cell, they were apparently metabolized in the same manner, whether taken up from the circulation as such or derived from chylomicron triglyceride.

Isolated rat liver cells in suspension take up both chylomicrons and non-esterified fatty acids. They also bring about hydrolysis of the chylomicron triglyceride and evidence has been obtained suggesting that the enzyme involved is in the plasma membrane (Green & Webb, 1964). This paper describes further studies on the uptake of lipids by liver cells *in vivo* and *in vitro* and on the site of action of the lipase. A preliminary report of some of the findings has been published (Webb & Green, 1964).

MATERIALS AND METHODS

The suspensions of isolated rat hepatic parenchymal cells and all materials, preparations and analytical methods, other than those listed below, were as described by Green & Webb (1964). The cell suspensions were always examined by phase-contrast microscopy and shown to consist almost entirely of single parenchymal cells. As described by Jacob & Bhargava (1962), other cells made up $< 5\%$ of the total.

[4-^{14}C]Cholesterol, [9,10-$^{3}H_2$]palmitic acid and glycerol tri[1-^{14}C]palmitate were obtained from The Radiochemical Centre, Amersham, Bucks. Human plasma albumin was obtained as described by Ashworth & Green (1963) or purchased from L. Light and Co. Ltd., Colnbrook, Bucks., and freed of fatty acids by the method of Goodman (1957). Palmitic acid–albumin complexes were prepared according to the method of Ashworth & Green (1963).

Hepatic cell 'ghosts'. A slight modification of the method of Levin & Thomas (1961) was used. Isolated cells were extracted three times with each of the following solutions: (1) 0·16M-KCl containing 5g. of sodium citrate ($Na_3C_6H_5O_7$, $2H_2O$) per 1.; (2) 0·4M-KCl containing 7·5g. of sodium citrate per 1.; (3) M-KCl containing 10g. of sodium citrate per 1. In addition, all solutions contained 186mg. of iodoacetic acid per 1. Extractions were carried out over 10min. at 0° with occasional inversion of the tubes. Final extracts were free of protein, DNA and RNA.

Fractionation of liver cells. In the earlier experiments, the method used by Wallach & Ullrey (1962) to obtain surface membranes of Ehrlich ascites cells was adapted for liver tissue. The liver was homogenized in about 10vol. of 0·25M-sucrose in a Potter–Elvehjem homogenizer and centrifuged at 16000*g* for 15min. The precipitate (P) was washed twice and the combined supernatants were centrifuged at 105000*g* for 30min. to give a crude microsomal preparation. This was washed once, suspended in sucrose solution (ρ1·17) and layered on top of a continuous density gradient (ρ1·15–1·17) of sucrose. After centrifugation at 105000*g* for 16hr. three suspended subfractions (numbered I, II and III in order of increasing density) and a precipitate were obtained. Nuclear and mitochondrial fractions were obtained from precipitate (P) by resuspending it in 0·25M-sucrose to the volume of the original homogenate and centrifuging as described by Glover & Green (1957).

In later experiments the modifications proposed by Wallach & Ullrey (1964) were adopted. Liver cell plasma membranes were also obtained by the method of Emmelot, Bos, Benedetti & Rümke (1964).

Washing procedures. After incubation with either chylomicrons or albumin-bound palmitic acid, isolated liver cells were sedimented by centrifuging at 500*g* for 2min. They were washed twice by resuspending in Hanks solution (15–30 vol.) and centrifuging at 500*g* for 2min. After only one such washing only 3% of the chylomicron lipid associated with the cells was removed by centrifuging in Hanks solution at 20000*g* for 60min. (Green & Webb, 1964). Contamination of the washed cells by the palmitic acid–albumin complex was checked by using ^{131}I-labelled albumin. Over

the range of times and concentrations of cells and albumin used in this work, where 6–40% of the palmitic acid of a palmitic acid–albumin complex was taken up by the cells, < 0·001% of the albumin remained associated with them.

After the incubation of chylomicrons or albumin-bound palmitic acid with subcellular particles, the particles were always isolated by sedimentation from the Hanks solution at 105000*g* for 30 min. The particles formed a firm pellet and the unbound chylomicrons migrated centripetally to form a layer at the surface of the clear supernatant. The pellets were twice resuspended in Hanks solution and centrifuged at 105000*g* for 30 min.

Palmitic acid–albumin complexes were tested before use by centrifuging at 105000*g* for 60 min. followed by measurement of the palmitic acid content of the supernatant. No loss of palmitic acid could be detected. In addition, in one experiment the albumin contents of the incubation media were checked by means of the Folin–Ciocalteu reagent after centrifugation of the various subcellular particles. No loss of albumin could be detected, although 8–42% of the palmitic acid had been taken up by the particles.

Rat intestinal lymph chylomicrons. These were obtained as described by Green & Webb (1964) after the administration of 5μc of glyceryl tri[^{14}C]palmitate or 12μc of [^{3}H]-palmitic acid and 10μc of [^{14}C]cholesterol.

Post-heparin plasma. This was used as the source of clearing factor lipase and was obtained from rats by the method of Tidwell, McPherson, Moore, Pope & Freeman (1964).

Injections. All injections were made under Nembutal anaesthesia into the exposed femoral vein over a period of 30–60 sec. Not more than 2 mg. of lipid was given on any occasion.

Incubations. All incubations were carried out in Hanks solution (Hanks & Wallace, 1949) containing sodium succinate (10 mM) at 37° under air.

Extraction and separation of lipids. Lipids were extracted and washed by the method of Folch, Lees & Sloane-Stanley (1957) and chromatographed on silicic acid (Barron & Hanahan, 1958). Fatty acids were separated from triglycerides by the method of Borgström (1952).

Measurement of radioactivity. Portions of the lipid solutions were evaporated to dryness under nitrogen in glass counting vessels and dissolved in 5 ml. of scintillation fluid. This contained 5 g. of 2,5-diphenyloxazole and 0·3 g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 l. of toluene. Samples were counted to a statistical error of < 4% in a Packard Tri-Carb Scintillation Spectrometer. For simultaneous measurement of ^{14}C and ^{3}H, both simultaneous equation and screening methods (Okita, Kabara, Richardson & LeRoy, 1957) were used originally but, as no significant difference was found between them, only the latter was used in the later experiments.

RESULTS

Doubly-labelled chylomicrons were used to follow the uptake of unesterified cholesterol, esterified cholesterol and triglyceride + NEFA* by isolated hepatic cells. The results (Fig. 1) show that all three are taken up together.

On incubation, the ^{14}C content of the unesterified

* Abbreviation: NEFA, non-esterified fatty acids.

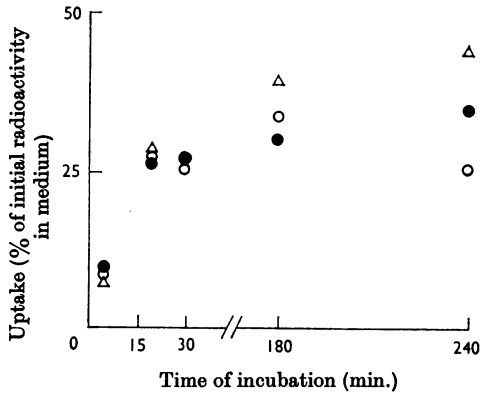

Fig. 1. Uptake of chylomicron lipid by isolated hepatic cells. Liver cells (22·1 mg. of nitrogen) were incubated with 12·5 mg. of chylomicrons labelled with [^{14}C]cholesterol and [^{3}H]palmitic acid. At suitable time-intervals, samples were removed and the cells washed before extraction and chromatography of the lipid. Uptake was determined from the ^{14}C and ^{3}H contents of the lipid fractions. ●, Triglyceride + NEFA: ○, cholesterol esters; △, unesterified cholesterol.

cholesterol of the cells rises while that of the cholesterol esters falls. It is known that liver contains cholesterol esterases (Deykin & Goodman, 1962; Brot, Lossow & Chaikoff, 1964), and hydrolysis of the esters could have occurred under the influence of these enzymes. Another factor which would increase the radioactivity in the unesterified sterol fraction is a slow exchange of inert sterol in the cells with radioactive sterol in the unbound chylomicrons. That cellular cholesterol can take part in such a process has been shown in experiments with isolated rat liver cells and human β-lipoproteins (Basford, 1965).

Hydrolysis in vitro *of chylomicron triglyceride bound* in vivo. Previous work (Green & Webb, 1964) demonstrated the ability of isolated hepatic cells to bind chylomicrons and hydrolyse the constituent triglycerides. To relate this to the process normally occurring in the intact animal, a rat was injected with [^{14}C]palmitic acid-labelled chylomicrons (2 mg. of lipid). After 2 min. the liver was removed and the hepatic cells were isolated from it. These were incubated in Hanks solution for 2 hr. and the radioactivity of the cell triglycerides and NEFA was followed during this period by analyses carried out on portions of the cell suspension.

It can be seen from Fig. 2 that chylomicron triglycerides taken up *in vivo* are hydrolysed *in vitro*. Although results could not be obtained at time-intervals shorter than 40 min. after injection (the time taken to isolate the cells), the degree of

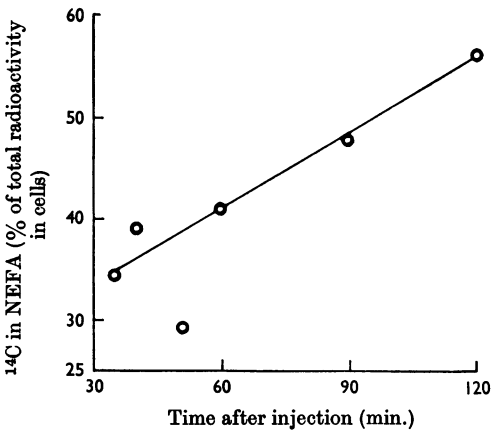

Fig. 2. Hydrolysis *in vitro* of chylomicron triglyceride taken up by hepatic cells *in vivo*. Details given in the text.

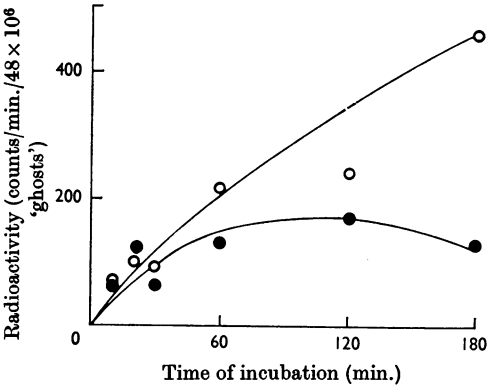

Fig. 3. Uptake and hydrolysis of chylomicron triglyceride by isolated hepatic cell 'ghosts'. The system contained 287×10^6 cell 'ghosts' and 3·5mg. of [^{14}C]palmitic acid-labelled chylomicrons. At suitable intervals the radioactivity present in the triglyceride (●) and NEFA (○) of the 'ghosts' was determined.

Table 1. *Effects of extraction with salt solutions on the composition of isolated hepatic cells*

The procedure is described in the Materials and Methods section.

	Unextracted cells	'Ghost' cells
Total lipid (% dry wt.)	21·2	37·3
Lipid phosphorus (% lipid)	2·8	2·7
Total phosphorus (mg./mg. of total lipid)	0·065	0·027
Total nitrogen (mg./mg. of total lipid)	0·62	0·38
(mg./10^6 cells)	0·25	—

lipolysis at this and subsequent times is roughly that expected on the basis of the earlier experiments carried out completely *in vitro*.

Uptake and hydrolysis of chylomicron triglyceride by 'ghost' hepatic cells. 'Ghost' hepatic cells were prepared as described in the Materials and Methods section. The effects of the extraction procedure can be seen in Table 1. As very little lipid is removed and as the phospholipid/total lipid ratio does not alter, the lipid content was taken as the basis for making comparisons. A good deal of protein and nucleic acid is extracted and this is reflected in the nitrogen/lipid and total phosphorus/lipid ratios. Microscopically, the cells were pale but otherwise appeared normal.

Suspensions of the 'ghost' cells were incubated with [^{14}C]palmitic acid-labelled chylomicrons. Three experiments were performed, one of which is shown in Fig. 3. The ghost cells took up chylomicron lipid to the same extent as non-extracted cells (about 10μg. of triglyceride per 10^6 cells) but in two experiments the process was slower. Thus the shapes of the curves shown in Fig. 3 differ from those seen with control cells (Green & Webb, 1964). With control cells, the triglyceride content rises rapidly to a maximum at 10–30min. and then falls as hydrolysis proceeds. In 3hr. the mean percentage of the total ^{14}C in the NEFA associated with the 'ghost' cells rose to 49·8%, whereas with control cells 48·8% was in the NEFA at 3hr. (Green & Webb, 1964).

Location of chylomicrons after uptake. If chylomicrons are first bound to the plasma membrane and the triglycerides hydrolysed before entering the hepatic cells, then all intact chylomicron triglyceride associated with the cells will be on the outside and should be accessible to enzymes in the medium. If, on the other hand, triglyceride enters the cell intact it should not be readily accessible to large molecules in the medium. To investigate these possibilities, isolated cells were exposed to the action of clearing factor lipase (glycerol-ester hydrolase, EC 3.1.1.3) in the form of post-heparin rat plasma.

The effect of the enzyme on the hydrolysis of the endogenous triglyceride of isolated cells was first investigated. Hepatic cells were labelled by injecting [^3H]palmitic acid intraperitoneally into a rat. Two hours later the cells were isolated and incubated with either normal or post-heparin plasma for 30min. Because of the possibility of migration of radioactive NEFA into the medium during the incubation, the cells were not separated from the medium after the incubation but the lipids of the cells and medium combined were extracted and triglycerides and NEFA separated.

The results (Table 2) show that the low initial NEFA/triglyceride ratio of the cells increases when they are incubated in normal plasma. As these cells cannot carry out resynthesis of compound lipids, the normal equilibrium maintained in the intact animal between their breakdown and synthesis no longer exists and this could be the cause of the increase in NEFA radioactivity. On the other hand it could be the result of the destruction of a small number of the cells with the liberation of their contents into the medium where they become exposed to the enzymes of other cells. The absolute amount of NEFA, in any case, remains very small and, most important, the addition of clearing factor lipase does not cause additional lipolysis.

When cells obtained from livers removed 5min. after the injection of [^{14}C]palmitic acid-labelled chylomicrons were incubated in normal or post-heparin plasma the results shown in Table 3 were obtained. As in the previous experiment, cells and plasma were not separated after the incubation. The weight of triglyceride in the cells was determined before the incubation and the total triglyceride hydrolysis has been calculated on this basis. The weight of NEFA present in the plasma was determined before incubation, and as this did not change during a 30min. incubation without cells, this quantity was subtracted from the weight of NEFA found in the system after incubation to obtain the cell NEFA content. As expected, the specific activity of the NEFA associated with the liver cells was always much lower than that of the total fatty acids of the original chylomicrons, due to dilution by NEFA derived from unlabelled chylomicrons in the blood as well as from the liver itself. The cells when isolated contained 558μg. of triglyceride/mg. of nitrogen, of which only 3·3μg. was contributed by the adsorbed radioactive chylomicrons. Hence, the hydrolysis of a very small proportion of the intracellular triglyceride may explain why the NEFA specific activity falls during the incubation. Although it is only about 20% of that of the injected chylomicron fatty acids, it is still 10–100 times that of the total triglyceride in the system.

At the start of the incubation *in vitro*, 23·7% of the ^{14}C associated with the cells is in the NEFA. This is the proportion expected after the 40min. needed for isolation of the cells (see Fig. 2). After incubation with normal plasma, this is increased by half, but after incubation with post-heparin plasma, it is almost trebled. The measurements of actual quantities of triglyceride and NEFA indicate that there is an increase in the hydrolysis of endogenous lipid in the presence of post-heparin plasma which was not shown in the experiments recorded in Table 2. However, although the absolute amount of NEFA formed in this way is

Table 2. *Action of clearing factor lipase on endogenous lipids of isolated hepatic cells*

The cells (7·5mg. of nitrogen), isolated from a rat liver 2hr. after the injection of 10μC of [^{3}H]palmitic acid, were incubated for 30min. in 2ml. of plasma. Lipid was then extracted from the whole system and NEFA and triglyceride were separated. Hydrolysis is expressed as the ratio ^{3}H in NEFA: ^{3}H in triglycerides.

	Triglyceride hydrolysis (%)	
Treatment of cells	Expt. 1	Expt. 2
Extracted immediately	1·1	1·9
Incubated in normal plasma	4·9	5·3
Incubated in post-heparin plasma	3·4	2·5

Table 3. *Hydrolysis of bound chylomicrons by clearing factor lipase*

Cells were isolated from the liver of a rat 5min. after injection of [^{14}C]palmitic acid-labelled chylomicrons (1mg. of lipid) and the suspension was divided into three parts. One was extracted immediately and the others (containing 5–12mg. of nitrogen) were incubated for 30min. with 2ml. of normal or post-heparin plasma before extraction. Total hydrolysis is calculated as wt. of NEFA after incubation/wt. of triglyceride in the cells before incubation. Correction was applied for the NEFA present in the plasma as described in the text. Hydrolysis of chylomicron triglyceride is calculated as ^{14}C in NEFA/^{14}C in triglycerides. The relative specific activity of NEFA is the specific activity expressed as a percentage of that of the original chylomicron total fatty acids. Results are expressed as means ± S.E.M., with the numbers of experiments in parentheses except for the total triglyceride hydrolysis figures which are the means of three results with the ranges in parentheses.

Treatment of cells	Cell NEFA (μg./mg. of N)	Total triglyceride hydrolysis (%)	Chylomicron triglyceride hydrolysis (%)	Relative sp. activity of NEFA
None	12·2 ± 0·4 (7)	2·2 (1·4–4·2)	23·7 ± 7·2 (5)	26·1 ± 3·9 (5)
Incubated with normal plasma	20·2 ± 5·5 (6)	3·6 (1·1–7·2	36·3 ± 5·9 (5)	16·8 ± 5·8 (5)
Incubated with post-heparin plasma	36·4 ± 6·5 (6)	6·6 (4·7–7·5)	67·9 ± 3·2 (5)	14·7 ± 4·3 (5)

high in relation to that produced by hydrolysis of the chylomicron triglycerides, the % hydrolysis of the endogenous triglyceride is still very low.

Site of lipid binding and triglyceride hydrolysis. In experiments to find the initial site of NEFA binding, [^{3}H]palmitic acid (64μg.) bound to albumin (7moles/mole) was injected into a rat. Two minutes later, the liver was perfused with ice-cold Hanks solution and homogenized and fractionated as described in the Materials and Methods section. In control experiments, a similar amount of albumin-bound [^{3}H]palmitic acid was mixed into the homogenate of a normal rat liver before fractionation. Nuclear and mitochondrial pellets were washed three times with 0·25M-sucrose. The crude microsomal pellet was washed twice and then fractionated by density gradient centrifugation. The results are shown in Table 4. Although the supernatant fraction and the precipitate obtained during the microsomal sub-fractionation were not themselves analysed, it was possible in two experiments, where portions of the original homogenate were taken, to determine the [^{3}H]-palmitic acid in these fractions by difference. Together they contained 9% of the total ^{3}H in one experiment and 11% in the other.

There is no clear-cut localization of ^{3}H in any one cell fraction whether the NEFA were injected or added to the homogenate. However, in terms of binding affinity, there are big differences between individual fractions. The microsomal sub-fraction I (corresponding to the plasma membrane fraction of ascites cells) has a much greater capacity (weight for weight) for binding lipid than any other, although it makes up only a very small part of the cell mass. The possibility that redistribution of NEFA occurred during the cell fractionation in the experiments where they were injected *in vivo* cannot be excluded.

When similar experiments were carried out with ^{14}C-labelled chylomicrons (1·1mg. of lipid) comparable results were obtained(Table 5). There was

Table 4. *Subcellular distribution of* [^{3}H]*palmitic acid in rat liver*

Fractionations of rat liver homogenates were carried out as described in the text either after the injection of [^{3}H]palmitic acid *in vivo* or after the addition of [^{3}H]palmitic acid to homogenates *in vitro*. Values for the experiments *in vivo* and *in vitro* are expressed as the means of four results ±S.E.M. and as the means of two results respectively. To allow comparison of experiments *in vivo* and *in vitro*, the microsomal sub-fraction I has been given a value of 1000 for the ^{3}H present/mg. of lipid-free dry wt. and the values for all other fractions have been calculated on this basis.

		^{3}H content (% of total)		^{3}H content/mg. of lipid-free dry wt. (arbitrary units)	
Cell fraction		Injection *in vivo*	Addition *in vitro*	Injection *in vivo*	Addition *in vitro*
Nuclear		23·6±3·6	16·6	13±2·6	12
Mitochondrial		16·8±3·0	33·5	25±7·4	35
Microsomal sub-fractions	I	19·2±4·2	5·2	1000	1000
	II	27·9±3·1	28·9	483±73	430
	III	12·5±1·9	16·2	148±53	323

Table 5. *Subcellular distribution of* [^{14}C]*palmitic acid-labelled chylomicrons in rat liver after injection* in vivo *and after addition to homogenates* in vitro

All figures are the means of two experiments. For comparison of experiments *in vivo* and *in vitro*, microsomal sub-fraction I was given a value of 1000 for the ^{14}C present/mg. of lipid-free dry wt. and the values for all other fractions were calculated on this basis. Details are given in the text.

		^{14}C content (% of total)		^{14}C content/mg. of lipid-free dry wt. (arbitrary units)	
Cell fraction		Injection *in vivo*	Addition *in vitro*	Injection *in vivo*	Addition *in vitro*
Nuclear		30	36	25	38
Mitochondrial		32	22	39	29
Microsomal sub-fractions	I	9	9	1000	1000
	II	17	3	224	26
	III	8	30	124	293

no localization of the isotope in any one fraction but again the microsomal sub-fraction I had by far the greatest binding capacity.

In addition to the site of binding, the site of triglyceride hydrolysis was investigated in experiments *in vitro*. Rat livers were homogenized and fractionated and each of the microsomal sub-fractions and the precipitate obtained during the fractionation were incubated separately in Hanks solution with [^{14}C]palmitic acid-labelled chylomicrons for 3hr. The total lipid bound and the extent of hydrolysis was then determined (Table 6). There were big differences in binding by the more dense fractions in the two experiments performed. In particular, the precipitate, which was very variable in quantity, showed wide variation. In each case, sub-fraction I produced much more ^{14}C-labelled NEFA per unit weight than any other, and, even when the others bound relatively large amounts of lipid, very little of the triglyceride was hydrolysed. It may be noted that some fractions bound almost all of the lipid presented to them.

The results given in Table 5 showed a relatively high content of chylomicron lipid in microsomal sub-fraction I, whether the chylomicrons were injected *in vivo* or added to the liver homogenate. It was possible that combination of chylomicron lipid with microsomal vesicles of sub-fractions II and III had lowered the density of the vesicles sufficiently for them to appear with sub-fraction I on gradient centrifugation, thus giving this sub-fraction an artificially high lipid content. The finding that isolated sub-fraction I binds much more lipid, per unit weight, than isolated sub-fractions II and III shows that the differences found in the earlier experiments were not due to such effects.

After these experiments were completed,

Table 6. *Binding of chylomicrons and hydrolysis of triglyceride by microsomal sub-fractions of rat liver*

In Expt. 1 the various fractions were prepared from four pooled rat livers. The whole of each fraction was incubated with [^{14}C]palmitic acid-labelled chylomicrons (0·85mg. of lipid) in 5ml. of Hanks solution at 37° for 3hr. In Expt. 2, a similar fractionation was carried out but only portions of each fraction were incubated as in Expt. 1 with chylomicrons (1·1mg. of lipid). The sub-fractions were sedimented by centrifugation after the incubation and washed once before extraction. The amount of triglyceride bound has been calculated from the total ^{14}C in the NEFA+triglyceride fraction. In calculating the NEFA produced, correction has been made for the small amount of NEFA present initially in the chylomicrons.

Microsomal sub-fraction	Lipid-free dry wt. of sub-fraction (mg.)		Triglyceride bound (μg./mg. of lipid-free dry wt.)		NEFA produced (μg./mg. of lipid-free dry wt.)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
I	9·1	10·0	93	101	56	32
II	52·0	28·1	6	29	3	3
III	12·9	30·0	14	30	9	3
Precipitate	113·2	6·5	3	164	1	6

Table 7 *Binding of NEFA and chylomicrons and hydrolysis of chylomicron triglyceride by liver membrane preparations*

The modified method of Wallach & Ullrey (1964) and the method of Emmelot *et al.* (1964) were used to prepare the membrane fractions for these determinations. NEFA binding was determined by incubating 68·4μg. of [^{3}H]palmitic acid bound to albumin (7moles/mole) with 3·4–3·9mg. (lipid-free dry wt.) of the membrane preparation in 5ml. of Hanks solution for 3hr. Binding was calculated from the ^{3}H present in the membrane NEFA. Binding and hydrolysis of chylomicron triglyceride by the membrane preparation of Emmelot *et al.* (1964) was determined by incubating about 2mg. (lipid-free dry wt.) of the preparation with chylomicrons (0·45mg. of lipid) for 3hr. in 5ml. of Hanks solution. The binding of triglyceride was calculated from the total ^{14}C in the NEFA+triglyceride of the fractions. The figures in parentheses for chylomicron binding and NEFA production by the membranes prepared according to Wallach & Ullrey are taken from the results of the experiments in Table 6, where the original method of these authors (Wallach & Ullrey, 1962) was used. All results are expressed in μg./mg. of lipid-free dry wt. and are the means of two experiments.

Method of membrane preparation	NEFA binding	Chylomicron triglyceride binding	NEFA production from triglyceride
Emmelot *et al.* (1964)	8·5	132	52
Wallach & Ullrey (1964)	7·8	(97)	(44)

Emmelot *et al.* (1964) described modifications of the method of Neville (1960) for isolating liver cell plasma membranes. Lipid-binding and triglyceride hydrolysis by such preparations were therefore investigated and compared with those by microsomal sub-fraction I isolated by the technique of Wallach & Ullrey (1962, 1964) (Table 7). It is apparent that the two types of membrane preparation are remarkably similar in both lipid-binding ability and lipolytic activity. These similarities, and the differences from other membrane subfractions (Tables 4–6), strongly suggest that they are derived from the same source.

Metabolism of albumin-bound and chylomicron-derived NEFA. A series of experiments was carried out to compare the metabolism by the rat liver of albumin-bound and chylomicron-derived NEFA. Rats were injected in one femoral vein with 1mg. of protamine sulphate to inhibit plasma clearing factor lipase. One minute later, a mixture of [^{14}C]palmitic acid-labelled chylomicrons and albumin-bound [^{3}H]palmitic acid was injected into the other femoral vein. After given intervals of time, the rats were killed and their livers were perfused with ice-cold Hanks solution. Lipid was extracted from the whole tissue and the ^{14}C and ^{3}H contents of each lipid class were determined. The results are given in Fig. 4.

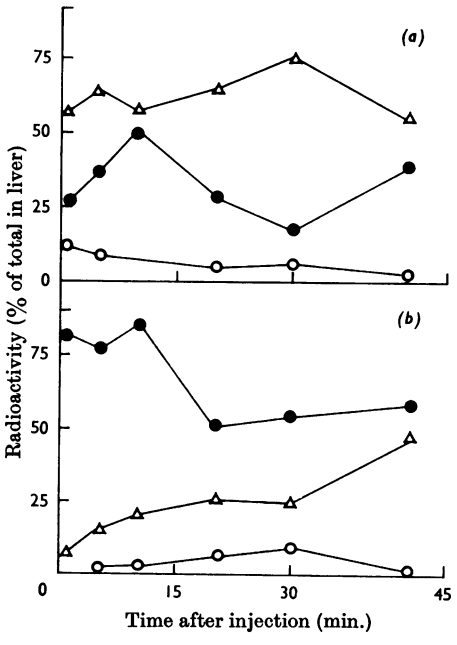

Fig. 4. Metabolism in rat liver of (*a*) albumin-bound [^{3}H]-palmitic acid and (*b*) [^{14}C]palmitic acid-labelled chylomicrons injected simultaneously into the femoral vein. Each point is a mean of four results obtained from separate experiments on four rats. ○, NEFA; ●, triglyceride; △, phospholipid.

Apart from the ^{3}H present at the very earliest times after injection there is never more than a low percentage of either isotope in the NEFA fraction. The curves of triglyceride radioactivity both show a maximum at about 10min. and a minimum at about 30min. The major differences are in the phospholipids. Their ^{3}H content rises very rapidly to a high value and remains higher than that of the triglycerides, whereas their ^{14}C content rises slowly throughout the experiment and does not exceed that of the triglycerides at any stage. In other experiments, it was established that there was no significant incorporation of labelled ^{3}H or ^{14}C into the sterol esters over the relevant time-interval.

DISCUSSION

The initial experiments were designed to test the validity of using isolated hepatic cells in the study of lipid uptake and triglyceride hydrolysis. Three findings indicate that they do provide a suitable test system. Firstly, three different chylomicron components are taken up at the same rate by isolated cells (Fig. 1), confirming the earlier conclusion that the whole chylomicron is bound (Green & Webb, 1964). It is well established that, *in vivo*, chylomicrons are removed intact from the circulation by liver (Dustin, Fredrickson, Laudat & Ono, 1961; Goodman, 1962; Nestel, Havel & Bezman, 1963). Secondly, hydrolysis of chylomicron triglyceride occurs *in vitro* whether the initial uptake takes place *in vivo* or *in vitro*. Thirdly, on the assumption that binding of chylomicrons *in vitro* does not involve destruction of their structure, the process is not one of random adsorption but affects only a small area of the cell surface. Thus, when isolated cells are incubated for 5min. in the presence of excess of chylomicrons, the maximum binding is about 40μg. of chylomicron lipid/mg. of cell nitrogen (Green & Webb, 1964), which is equivalent to 4×10^{6} cells (Table 1). Assuming that the average diameter of the chylomicrons is 500mμ, it can be calculated that roughly 200 chylomicrons are taken up per cell. This corresponds to a coverage of only about 40μ^2 of the approx. 2000μ^2 of the surface of a spherical cell 25μ in diameter.

An earlier finding with isolated hepatic cells that NEFA liberated from bound chylomicron triglycerides was removed when the cells were incubated in a solution of albumin (Green & Webb, 1964) suggested that the cell plasma membrane was the site of triglyceride hydrolysis. This view is strongly reinforced by the results of the present study. Triglyceride associated with hepatic cells

5min. after the injection of labelled chylomicrons is hydrolysed in the presence of clearing factor lipase. Since clearing factor lipase does not catalyse the hydrolysis of any significant proportion of the endogenous cell triglyceride, the triglyceride which is split has not been re-formed inside the cell after lipolysis. The chylomicron lipid associated with the liver cells at short time-intervals after its injection into the circulation thus appears to be outside the plasma membrane and in a state very similar to its original form.

Uptake and metabolism of NEFA and triglycerides by cell membrane fractions. Uptake of lipid by liver cell membrane fractions has been shown previously. Studies both *in vivo* and *in vitro* have shown the appearance of chylomicron lipids and NEFA in mitochondrial and microsomal fractions of rat liver (Fritz, 1964; Stein & Shapiro, 1959, 1960; Havel, Felts & Van Duyne, 1962; Reshef & Shapiro ,1965). As the plasma membrane is the first part of the cell with which exogenous material comes into contact, the present study concentrated on this fraction.

The preparations from rat liver obtained by the method of Emmelot *et al.* (1964) have been shown by these authors by thorough biochemical and morphological examination to be of plasma membranes. The preparations used in the present work were examined by phase-contrast microscopy and shown to consist of planar sheets as described by Emmelot *et al.* (1964). The method of Wallach & Ullrey (1962, 1964) was designed for the isolation of plasma membrane preparations of ascites cells and has not previously been applied to liver. It has been shown, however (Ashworth & Green, 1966), that the lipid compositions of liver membrane preparations made by the two methods are very similar and are quite different from any other liver cell membrane fraction. It has now been established (Table 7) that the preparations of Emmelot *et al.* (1964) and of Wallach & Ullrey (1962, 1964) are very similar with respect to NEFA binding, chylomicron binding and chylomicron triglyceride hydrolysis and that in these respects also they differ from other cell fractions (Table 6). This provides strong evidence that the microsomal sub-fraction I of the Wallach & Ullrey (1962, 1964) procedure is derived from the plasma membrane of the liver cell.

All the subcellular fractions of the liver cell that were studied in this investigation had the capacity to take up chylomicron lipid and albumin-bound NEFA (Tables 5 and 6). However, the plasma membrane had the greatest capacity per unit weight for binding lipid and for catalysing triglyceride hydrolysis. Although the plasma membrane represents only a very small proportion of the total membranes of the cell, this high activity supports the view that initial binding of chylomicrons and subsequent lipolysis of their triglyceride occur at this site. Since the amount of the plasma membrane fraction isolated by the procedures of either Emmelot *et al.* (1964) or Wallach & Ullrey (1962, 1964) represented an unknown proportion of the total plasma membrane of the cell, no estimate can be made of the total lipid binding capacity and lipolytic activity of the plasma membrane *vis à vis* other cell membranes.

Metabolism of albumin-bound and chylomicron-derived NEFA. Although previous experiments have been carried out in which the appearance of injected chylomicron lipid or albumin-bound NEFA in liver lipids has been followed, no study has been made in which the two have been injected simultaneously. When this is done under conditions in which the plasma clearing factor lipase is inhibited, neither affects the metabolism of the other (Fig. 4). Thus the results shown in Fig. 4(*b*) resemble those of Belfrage, Borgström & Olivecrona (1963) and Olivecrona & Belfrage (1965), who injected small doses of homologous chylomicrons into fed rats, and those shown in Fig. 4(*a*) resemble those of Göransson & Olivecrona (1964), who injected albumin-bound palmitic acid.

Injected NEFA do not accumulate as such in the liver but are immediately incorporated into compound lipids, principally phospholipid (Fig. 4*a*). After injection of [^{14}C]palmitic acid-labelled chylomicrons, radioactive NEFA also do not accumulate although the triglyceride is hydrolysed. A comparison of the rate of incorporation of the isotope into phospholipid (Fig. 4*b*) with the rate of hydrolysis of the chylomicron triglyceride by the isolated hepatic cells (Green & Webb, 1964) suggests that, once inside the cell, NEFA are treated similarly whatever their origins and that hydrolysis of chylomicron triglycerides at the cell surface is the rate-limiting step in their metabolism.

Sites other than the plasma membrane have been proposed for the hydrolysis of chylomicron triglyceride. Felts & Mayes (1965) proposed that lipolysis occurred before uptake by the liver. They found in the isolated, perfused rat liver that, unless clearing factor lipase was induced in or added to the perfusate, chylomicron triglyceride was not taken up by the tissue. They criticized earlier contrary findings (Morris, 1963; Rodbell, Scow & Chernick, 1964) on the grounds that heparin was used in the experiments as an anticoagulant. Ashworth, DiLuzio & Riggi (1963) also suggested that lipolysis might occur in the space of Disse. These proposals are not consistent with much of the work in this and the earlier study (Green & Webb, 1964).

Another proposal is that hydrolysis occurs inside the cell within pinocytic vesicles (Ashworth,

Stembridge & Sanders, 1960; Ashworth *et al.* 1963) or within the lumen of the endoplasmic reticulum (Robinson, 1964). The present studies *in vitro* with isolated cells do not necessarily conflict with this as it could be argued that the enzyme is normally present in the plasma membrane which *in vivo* pinches off into pinocytic vesicles on contact with chylomicrons and that it is within these that the enzyme acts: since the isolated cells are incapable of pinocytosis lipolysis occurs outside the cell. This possibility has been discussed elsewhere (Green & Webb, 1964) but the results of the experiments using post-heparin plasma after chylomicron uptake *in vivo* (Tables 2 and 3) are strongly against it.

We are grateful to the U.S. Public Health Services for financial support (award AM-05282-03). J. A. H. thanks the Department of Scientific and Industrial Research for a research studentship.

REFERENCES

Ashworth, C. T., DiLuzio, N. R. & Riggi, S. J. (1963). *Exp. molec. Path.* Suppl. **1**, 83.

Ashworth, L. A. E. & Green, C. (1963). *Biochim. biophys. Acta*, **70**, 68.

Ashworth, L. A. E. & Green, C. (1966). *Science*, **151**, 210.

Ashworth, C. T., Stembridge, V. A. & Sanders, E. (1960). *Amer. J. Physiol.* **198**, 1326.

Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.

Basford, J. M. (1965). Ph.D. Thesis: University of Liverpool.

Belfrage, P., Borgström, B. & Olivecrona, T. (1963). *Acta physiol. Scand.* **58**, 111.

Borgström, B. (1952). *Acta physiol. scand.* **25**, 111.

Brot, N., Lossow, W. J. & Chaikoff, I. L. (1964). *J. Lipid Res.* **5**, 63.

Deykin, D. & Goodman, D. S. (1962). *J. biol. Chem.* **237**, 3649.

Dustin, J.-P., Fredrickson, D. S., Laudat, Ph. & Ono, K. (1961). *Fed. Proc.* **20**, 270.

Emmelot, P., Bos, C. J., Benedetti, E. L., & Rümke, P. H. (1964). *Biochim. biophys. Acta*, **90**, 126.

Felts, J. M. & Mayes, P. A. (1965). *Nature, Lond.*, **206**, 195.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.

Fritz, I. B. (1964). *Amer. J. Physiol.* **206**, 1217.

Glover, J. & Green, C. (1957). *Biochem. J.* **67**, 308.

Goodman, D. S. (1957). *Science*, **125**, 1296.

Goodman, D. S. (1962). *J. clin. Invest.* **41**, 1886.

Göransson, G. & Olivecrona, T. (1964). *Acta physiol. scand.* **62**, 224.

Green, C. & Webb, J. A. (1964). *Biochim. biophys. Acta*, **84**, 404.

Hanks, J. H. & Wallace, R. E. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 196.

Havel, R. J., Felts, J. M. & Van Duyne, C. M. (1962). *J. Lipid Res.* **3**, 297.

Jacob, S. T. & Bhargava, P. M. (1962). *Exp. Cell. Res* **27**, 453.

Levin, E. & Thomas, L. E. (1961). *Exp. Cell. Res.* **22**, 363.

Morris, B. (1963). *J. Physiol.* **168**, 564.

Nestel, P. J., Havel, R. J. & Bezman, A. (1963). *J. clin. Invest.* **42**, 1313.

Neville, D. M. (1960). *J. biophys. biochem. Cytol.* **8**, 413.

Okita, G. T., Kabara, J. J., Richardson, F. & LeRoy, G. V. (1957). *Nucleonics*, **15**, 111.

Olivecrona, T. & Belfrage, P. (1965). *Biochim. biophys. Acta*, **98**, 81.

Reshef, L. & Shapiro, B. (1965). *Biochim. biophys. Acta*, **98**, 73.

Robinson, D. S. (1964). In *Lipid Transport*, p. 194. Ed. by Meng, H. C., Coniglio, J. G., Lequire, V. S., Mann, G. V. & Merrill, J. M. Springfield, Ill.: Charles C. Thomas.

Rodbell, M., Scow, R. O. & Chernick, S. S. (1964). *J. biol. Chem.* **239**, 385.

Stein, Y. & Shapiro, B. (1959). *Amer. J. Physiol.* **206**, 1217.

Stein, Y. & Shapiro, B. (1960). *J. Lipid Res.* **1**, 326.

Tidwell, H. C., McPherson, J. C., Moore, W. T., Pope, J. L. & Freeman, A. (1964). *Proc. Soc. exp. Biol., N.Y.*, **116**, 720.

Wallach, D. F. H. & Ullrey, D. (1962). *Biochim. biophys. Acta*, **64**, 526.

Wallach, D. F. H. & Ullrey, D. (1964). *Biochim. biophys. Acta*, **88**, 620.

Webb, J. A. & Green, C. (1964). *Biochem. J.* **91**, 24P.