SYNTHESIS AND SECRETION OF VERY LOW DENSITY LIPOPROTEIN (VLDL) BY PRIMARY CULTURES OF RAT HEPATOCYTES

As discussed in the introduction, very low density lipoprotein (VLDL) secretion is a highly complex process which requires the synthesis of specific lipids and apolipoproteins and their association into a particle transported into the sinusoidal space.\textsuperscript{50,70,71,217} The liver is the major site of synthesis, assembly and secretion of VLDL.\textsuperscript{67} The apoproteins are apparently synthesised by bound ribosomes and lipid components are synthesised by enzymes located in the cytoplasmic side of the endoplasmic reticulum.\textsuperscript{71,218} Although the events involved in the formation and secretion of plasma lipoproteins are understood in outline, many questions remain concerning details of the process. The mechanism of sequestration of lipids in the cisternal space and the sequence of events involved in the packaging and assembly of the lipoprotein components remain to be elucidated. Davis et al.\textsuperscript{219} in a recent report, suggested that the majority of both molecular weight forms of apoB are localized in the endoplasmic reticulum, the initial site of VLDL assembly. Their data suggested that if proteolysis occurs
in vivo, the degradation of apoB in the endoplasmic reticulum may in part determine the portion of apoB entering VLDL assembly/secretion pathway. Higgins and Huston\textsuperscript{220} have observed that although triglycerides appear to be sequestered in the endoplasmic reticulum, much of the phospholipids and cholesterol found in the secreted lipoprotein is not packaged with the triglyceride until this reaches the Golgi membrane.

There is no definite information about the origin of LDL whether it is synthesised as such and secreted by the liver or whether it arises as a result of catabolism of the secreted VLDL. Fidge \& Poulis\textsuperscript{221} observed that LDL can be secreted directly by the liver in rats. On the other hand, Davis\textsuperscript{130} et al could find only the synthesis of VLDL and not that of LDL by the liver. In view of this lack of definite information on the synthesis of LDL, an attempt was made to gain information on this aspect using hepatocytes isolated from the liver of rats. This was considered particularly important in view of the fact that LDL is the most atherogenic of all the lipoproteins. Further, detailed information on the synthesis and secretion of VLDL by hepatocytes is required to study the role of various factors on the metabolism of these lipoproteins. Therefore experiments were carried out to study the synthesis and secretion of VLDL by hepatocytes and to examine whether LDL is produced by these cells in vitro.
culture dish. These cells later flattend out. After about 4 hours, unattached cells were removed and then the cell layer was washed. By this time cells were spread and appeared as monolayers under light microscope (Plate IA). In 24 hours they aggregated into trabecular structures. The hepatocytes were not contaminated with Kupfer cells as evidenced by the lack of phagocytosis of fluorescein isothiocyanate conjugated IgG.

2. Synthesis of VLDL and LDL by primary cultures of rat hepatocytes: The synthesis and secretion of VLDL and LDL by rat hepatocytes in culture were studied by metabolic labelling with $^{3}$H-leucine. Results are given in table (Table.3).

There was significant incorporation of radioactivity into trichloro acetic acid (TCA) precipitable material in the medium and the cell layer even at 3 hours. The radioactivity associated with the VLDL fraction in the medium (d<1.006) increased with time reaching a maximum at 12 hours (Fig.7). The radioactivity associated with the lipoprotein fraction (d-1.006 - 1.06) secreted into the medium was very low during the earlier stages of incubation but showed significant amounts at later stages (Fig.8). At 12 hours nearly 70-75% of the total $^{3}$H-leucine radioactivity associated with the secreted lipoproteins was in VLDL and about 20-25% in the fraction of the density range 1.006 - 1.06 (Table.3). With increase
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Plate I A: Hepatocytes - 20 hours after plating seen under a phase contrast microscope.
Plate I B: VLDL - SDS PAGE.

Rat VLDL isolated by sequential ultracentrifugation was dialyzed in 0.15M NaCl and concentrated by liophilization. The protein was then dissolved in Laemli's buffer containing 3% SDS and electrophoresed on 4.5% to 15% gradient polyacrylamide gel under reducing conditions.
Fig. 7  The synthesis and secretion of VLDL by primary cultures of rat hepatocytes:

Hepatocytes were labelled with $^{3}H$-leucine (50pCi) for different time intervals. The medium was collected and VLDL was isolated by sequential ultracentrifugation using carrier rat serum. The values given are the mean of four experiments.
The synthesis and secretion of LDL by primary cultures of rat hepatocytes:

Hepatocytes were labelled with 50μCi of ($^3$H)-leucine for different time intervals. The medium was collected and the lipoproteins in the LDL density range was isolated by sequential ultracentrifugation as described in the text. The values given are the mean of four experiments.
Table 3

Incorporation of (\(^3\)H)-leucine into lipoproteins

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>VLDL [CPM/mg cell protein]</th>
<th>LDL [CPM/mg cell protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8476±1590</td>
<td>2643±512</td>
</tr>
<tr>
<td>6</td>
<td>18209±2003</td>
<td>4586±489</td>
</tr>
<tr>
<td>12</td>
<td>34500±4180</td>
<td>9800±882</td>
</tr>
<tr>
<td>18</td>
<td>28734±3048</td>
<td>12408±2600</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated with 50\(\mu\)Ci of (\(^3\)H)-leucine for different time intervals and secreted lipoproteins were separated. The values given are the mean of four different experiments ± SD.
in the labelling period, there was decrease in the fraction of the radioactivity associated with the secreted VLDL.

It was found that there was steady increase in the incorporation of (³H)-leucine into immunoprecipitable cell associated apoB with increase in time indicating continuous production of apoB by hepatocytes in culture.

3. Distribution of apoB in VLDL and LDL secreted by hepatocytes in culture: The (³H)-leucine labelled lipoproteins secreted by the hepatocytes were isolated by sequential ultracentrifugation. The apoproteins of the lipoproteins were separated by SDS-PAGE and it was found that nearly 50-55% of the radioactivity was associated with apoB in the VLDL fraction. In the lipoprotein fraction of the density range 1.006 - 1.06 nearly 60% of the radioactivity was associated with apoB (Table 4).

Antiserum raised against rat apoB was found to react with both apoB₁₀₀ and B₄₈ (Plate II B). Ouchterlony's double immunodiffusion test showed that the antiserum was specific for rat LDL and VLDL, as it did not show any cross reaction with rat serum HDL, albumin or human serum VLDL and LDL (Plate II A). Using this antiserum it was again found that about 60% of (³H)-leucine radioactivity in VLDL secreted by rat hepatocytes in culture was apoB.
Plate II A: Immunodiffusion: The antibodies were raised against rat apoB in rabbits as described in the text. Ouchterlony's immunodiffusion was carried out on 1% agarose in a humidified atmosphere against (a) rat VLDL (b) rat LDL and (c) rat HDL.

Plate II B: Autoradiogram of immunoprecipitated apoB: Radioactive apoB was immunoprecipitated and electrophoresed on 4.5% to 15% gradient gel under reducing conditions. The gel was prepared for autoradiogram and exposed to preflashed X-ray film for 10 days at -70°C.
Table 4

Incorporation of (\(^3\)H)-leucine into apoB

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Total counts in lipoproteins [CPM/mg cell protein (^{\times}10^{-3})]</th>
<th>SDS-PAGE</th>
<th>Immunoprecipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>34.50±4.11</td>
<td>18.53±2.79</td>
<td>20.9±2.81</td>
</tr>
<tr>
<td>LDL</td>
<td>9.80±0.88</td>
<td>5.93±0.76</td>
<td>6.26±1.02</td>
</tr>
</tbody>
</table>

The cells were incubated with 50\(\mu\)Ci of (\(^3\)H)-leucine for 12 hours. The lipoproteins secreted into medium were separated. The total radioactivity in each lipoprotein fraction as well as that in apoB analyzed by SDS-PAGE (7.5%)/immunoprecipitation and electrophoresis were determined. The results given are the mean of four different experiments ± SD.
and about 65% in the lipoprotein fraction d-1.006 - 1.06 (Table 4). Since this antibody was found to react with both apoB\textsubscript{100} and B\textsubscript{48} the immunoprecipitable radioactivity from medium of the culture was taken as the total apoB. It appeared that during earlier stages of incorporation, nearly 70% of the immunoprecipitable apoB radioactivity was associated with VLDL.

4. Incubation of prelabelled total lipoproteins with hepatocytes in culture: The results discussed above indicated that at earlier time intervals there was only very little incorporation of \(^3\text{H}\)-radioactivity into apoB associated with the lipoprotein fraction of the density range 1.006 - 1.06, which slowly increased with time. This fraction may contain LDL and probably IDL also. It can be due to a lower rate of synthesis and secretion of LDL by hepatocytes when compared to that of VLDL or due to a partial conversion of VLDL to LDL via IDL or due to both. The conversion of VLDL to LDL is possible in the light of the fact that hepatocyte plasma membrane contains hepatic triglyceride lipase. This possibility was examined by incubating hepatocytes maintained in culture in presence of prelabelled total lipoproteins. There was significant decrease in radioactivity in the VLDL fraction with a proportionate increase in the radioactivity associated with the lipoprotein fraction of the density range 1.006 - 1.06 with time (Fig. 9). In control experiments, where the medium containing prelabelled lipoproteins
Fig. 9 Conversion of prelabelled lipoproteins:

(\(^3\)H)-leucine labelled total lipoproteins synthesised and secreted by rat hepatocytes were isolated and incubated with hepatocytes maintained in primary culture. Medium was collected at different time intervals, VLDL (\(\bullet\)) and LDL (\(\Delta\)) were isolated by ultracentrifugation and quantitated as described in the text. Test -\((0,\Delta)\), Control (no cells) - \((\bullet,\Delta)\).
alone was incubated at 37°C there was no change in radioactivity indicating that cells were required for the conversion to take place. This also indicated that the conversion of VLDL to LDL was not due to the action of any enzyme present in association with the prelabelled lipoproteins.

5. Effect of deoxyglucose on the synthesis and secretion of (\(^3\)H)-apoB: Results discussed above indicated that apoB synthesised by hepatocytes is distributed both in VLDL and LDL that are secreted into the medium. During 6-12 hours of labelling nearly 70% of the total apoB was found to be present in VLDL while the remaining was present in the lipoprotein fraction in the density range 1.006 - 1.06 in the medium. The apoB is known to be a glycoprotein and the possibility, that glycosylation is essential for the secretion of apoB containing lipoproteins was examined, using 2-deoxyglucose an inhibitor of protein glycosylation.

The results are given in table (5) and 't' values are given in table (5a).  

2-deoxyglucose did not affect the secretion of apoB into the medium when compared to the control. Cell associated apoB also showed no significant alteration in the presence of deoxyglucose. These results indicated that dolichol linked glycosylation is not critical in the secretion of apoB containing lipoproteins.
Table 5

Incorporation of $({}^3\text{H})$-leucine into ApoB - Effect of deoxyglucose

<table>
<thead>
<tr>
<th></th>
<th>Media [CPM/mg cell protein]</th>
<th>Cell layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9768±829</td>
<td>1953±228</td>
</tr>
<tr>
<td>Deoxyglucose</td>
<td>10092±1096</td>
<td>2012±280</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated with 20μCi of $({}^3\text{H})$-leucine for 12 hours in presence of 2-deoxyglucose ($10^{-3}$ M) and the incorporation of label into total apoB was determined. The results given are the mean of four different experiments ± SD.

Table 5a

't' values

<table>
<thead>
<tr>
<th>'t' between control &amp; test</th>
<th>Media</th>
<th>Cell layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyglucose</td>
<td>0.47</td>
<td>0.33</td>
</tr>
</tbody>
</table>
6. Effect of colchicine on the synthesis and secretion of apoB: 
Colchicine is an antitubulin drug which disturbs the tubular assembly. 
The effect of colchicine on the secretion of apoB containing lipoproteins 
into the medium was studied. The results are given in table (6) and 
't' values are given in table (6a).

Colchicine (10μM) significantly decreased the incorporation of 
radioactivity into VLDL secreted into the medium. There was about 40% 
inhibition at the concentration tested. The incorporation of 
radioactivity into apoB secreted into the medium was also significantly 
reduced at this concentration of the drug. These results indicated that 
disruption of intracellular assembly impaired the secretion of apoB 
containing lipoproteins.

Discussion

The results presented above indicate that primary cultures of 
rat hepatocytes synthesise and secrete lipoprotein in the density range 
of serum VLDL. The synthesis of VLDL was observed as early as 3 
hours and increased steadily with time and reached a maximum at 12 
hours as measured by incorporation of (3H)-leucine. Bell Quint and 
Forte reported maximum VLDL secretion by rat hepatocytes in culture 
at 6.5 hours. But under conditions used by us the incorporation of 
radioactivity into VLDL was only about 50% of the maximum at this
Table 6

Incorporation of (\(^3\)H)-leucine into VLDL & apoB - Effect of colchicine

<table>
<thead>
<tr>
<th></th>
<th>ApoB</th>
<th>VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM/mg cell protein</td>
<td>CPM/mg cell protein</td>
</tr>
<tr>
<td>Control</td>
<td>9768±829</td>
<td>16842±212</td>
</tr>
<tr>
<td>Colchicine</td>
<td>3687±744</td>
<td>9876±1003</td>
</tr>
</tbody>
</table>

Hepatocytes were incubated with 20\(\mu\)Ci of (\(^3\)H)-leucine for 12 hours in presence of colchicine (10\(\mu\)M) and the incorporation of label into secreted VLDL and apoB was determined. The results given are the mean of four experiments ± SD.

\(p < 0.01\)

Table 6a

't' values

<table>
<thead>
<tr>
<th>'t' between control &amp; test</th>
<th>ApoB</th>
<th>VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>10.92</td>
<td>8.86</td>
</tr>
</tbody>
</table>
time. Davis et al\textsuperscript{130} reported that more than 95\% of the triglyceride secreted into the medium by rat hepatocytes was present in VLDL. They studied the secretion of VLDL by cells after maintaining them in culture for 24 hours. The VLDL secreted by rat hepatocytes in culture has been shown to contain apoB and apoE as the major apolipoproteins. They reported that newly synthesised VLDL did not contain detectable amounts of apoC. But other reports showed that VLDL isolated from liver perfusates\textsuperscript{30,222} and from freshly isolated hepatocytes\textsuperscript{223} contained low amounts of apoC indicating similarity to serum VLDL. It has also been reported\textsuperscript{30,224} that the lipid composition of newly secreted VLDL by hepatocytes was generally similar to that of VLDL secreted in liver perfusions, with a reduced amount of cholesteryl ester\textsuperscript{130}.

Electrophoretic analysis and immunoprecipitation studies showed that about 50-60\% of the \((^3\text{H})\)-radioactivity associated with the VLDL is apoB. Martin-Sanz et al\textsuperscript{164} also observed a similar rate of incorporation. This represents the total apoB \((\text{apoB}_{100}+\text{apoB}_{48})\) as our antiserum was found to cross react with both forms of the apoB.

Primary cultures of rat hepatocytes also secreted lipoprotein in the density range of LDL as evidenced by floatation of \((^3\text{H})\)-labelled lipoproteins from culture medium by sequential centrifugation at a density range of 1.006 to 1.06 g/ml. Electrophoretic analysis and immunoprecipitation studies showed that about 65\% of the \((^3\text{H})\)-
The lipoprotein fraction isolated from the medium in the LDL density range can be formed by direct synthesis by the cells or by partial conversion of the VLDL or due to both. Support for direct secretion of LDL was obtained by Illingworth in vivo studies in squirrel monkeys by blocking VLDL catabolism by injecting Triton W.R. 1339 and monitoring the incorporation of radioactive leucine into LDL apoB. Direct hepatic secretion of lipoprotein particles within the LDL density range has also been described in liver perfusion studies in pigs and in non human primates.
The other possibility that the VLDL secreted into the medium is converted into lipoproteins with higher density was examined in experiments with prelabelled lipoproteins. The results showed that there was significant decrease in the radioactivity associated with VLDL fraction with a corresponding increase in the fraction floating in the density range of 1.006 - 1.06, indicating that hepatocytes might have converted part of the VLDL into lipoprotein particles having density in the range of LDL.

Nascent hepatic VLDL synthesised by the hepatic cells with apoB as the major apoprotein and small amounts of apoE, acquires small amounts of both apoC-II (which activates lipoprotein lipase)\textsuperscript{51,52} and apoC-III (which inhibits lipoprotein lipase and also block receptor interaction)\textsuperscript{81,82} probably from the HDL secreted into the medium. The VLDL particles are probably then hydrolysed by lipoprotein lipase present in the plasma membrane or secreted into the medium. Progressive loss of apoC along with hydrolysis of tryglycerides results in the formation of LDL. Another possibility is that hepatic lipase bound to plasma membrane is the enzyme involved in the conversion of the secreted VLDL to LDL\textsuperscript{56,59}. It is active in the absence of C-apoproteins, although apoA-II enhances its activity\textsuperscript{58}. This enzyme can therefore hydrolyse nascent VLDL which contain very little apoC.

Presence of small amounts of (\textsuperscript{3}H)-leucine labelled lipoproteins which floats in the LDL density region in the medium of hepatocytes
even during earlier time intervals may suggest that hepatocytes may also produce lipoprotein particles in the LDL density range. However, the possibility that reuptake of the secreted lipoproteins by the hepatocytes cannot be ruled out. A faster rate of uptake of the secreted VLDL than of LDL may also explain the observed results. Clear cut experiments which are designed to inhibit reuptake of the secreted lipoproteins by the hepatocytes and inhibit action of lipoprotein lipase and triglyceride lipase are required to arrive at definite conclusions regarding the origin of LDL.

The synthesis and secretion of VLDL by primary cultures of hepatocytes, were also sensitive to a number of drugs which were known to affect lipoprotein metabolism. Colchicine, an antitubulin drug which is known to be a potent inhibitor of VLDL secretion \(^{229}\) and other processes dependent on microtubular function, also caused inhibition of secretion of VLDL by about 40% at 10\(\mu\)M concentration in our experiments. Since apoB was the major protein in both VLDL and LDL and at earlier time intervals the predominant lipoprotein produced was VLDL, the measurement of apoB secretion in response to colchicine was studied and found to be reduced by about 50%. Cycloheximide also has been shown to inhibit VLDL production (data not given).

It has been reported that secretion of VLDL from hepatocytes is not affected in the presence of tunicamycin, which inhibits
glycosylation\textsuperscript{230}. This indicated that N-linked glycosylation of apoB is not necessary for VLDL synthesis, assembly and secretion. But tunicamycin appeared to have a potential inhibitory effect on protein synthesis as well\textsuperscript{231}. Therefore these results have been derived after correcting for this. Deoxyglucose is another substance which is known to inhibit glycosylation and does not have any significant inhibitory effect on protein synthesis\textsuperscript{232}. Incubation of cultured animal cells with 2-deoxyglucose leads to the formation of both UDP-2-deoxyglucose and GDP-2-deoxyglucose as well as dolichyl phosphate-2-deoxyglucose\textsuperscript{232}. The major compound involved in the inhibition of glycosylation is GDP-2-deoxyglucose. GDP-2-deoxyglucose serves as a sugar donor for the formation of dolichyl-PP-(Glc-NAc)$_2$-2-deoxyglucose which cannot be further elongated, and is not transferred to protein. The results obtained with 2-deoxyglucose confirms the finding obtained with tunicamycin that N-glycosylation of apoB is not necessary for VLDL synthesis, assembly and secretion\textsuperscript{229}.

All these results indicate that the hepatocyte culture system that we have been working with, is a suitable system for the study of the regulation of very low density lipoproteins. Since, during earlier time intervals most of the lipoproteins produced was in the VLDL density range, and since the apolipoprotein B (apoB) content per VLDL particle appears to be constant\textsuperscript{223}, and an integral component unlike other apoproteins such as apoE, apoC etc. which are exchangable, the secretion of apoB can be taken as a measure of the VLDL production.