Role of Lipoprotein-X in the Pathogenesis of Cholestatic Hypercholesterolemia

Uptake of Lipoprotein-X and its Effect on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase and Chylomicron Remnant Removal in Human Fibroblasts, Lymphocytes, and in the Rat

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cract. Cholestasis is accompanied by the appearance of lipoprotein-X (LP-X) in plasma. This lipoprotein has a high content of unesterified cholesterol and phospholipids and appears to be ineffective in suppressing the enhanced hepatic cholesterogenesis of cholestasis. Its role as a possible causative factor for cholestatic hypercholesterolemia was investigated. When $^{125}$I-LP-X was injected into rats, it disappeared rapidly from the circulation. Calculated on the basis of gram wet weight, spleen took up more LP-X than liver. Prior ligation of the bile duct reduced the uptake in spleen. Experiments with isolated perfused rat liver showed that nonparenchymal cells (NPC) took up over eightfold more $^{125}$I-LP-X than hepatic parenchymal cells (PC). Incubation of PC, NPC, human lymphocyte suspensions, or fibroblast cultures with LP-X showed that NPC bound more LP-X than PC or fibroblasts. Lymphocytes took up 20-fold more LP-X than PC and the activity of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase was depressed by LP-X. Lymphocytes isolated from cholestatic patients showed low activity of this enzyme. The activity was increased by LP-X in isolated perfused livers, but suppressed in isolated microsomes.

LP-X competitively inhibited the uptake of chylomicron remnants in isolated perfused livers and hepatocytes. In contrast, degradation of LDL by perfused livers, which were isolated from ethinyl estradiol-treated rats or human fibroblast cultures, remained unchanged in the presence of LP-X.

The results indicate that cholesterol transported by LP-X is mainly taken up by the cells of the reticuloendothelial system. It increases the activity of hepatic HMG-CoA reductase and suppresses remnant uptake, thus emphasizing a major role of LP-X in cholestatic hypercholesterolemia.

Introduction

Hypercholesterolemia and hyperphospholipidemia that occur secondary to cholestasis are accompanied by the appearance of an abnormal lipoprotein, lipoprotein-X (LP-X), in plasma. It exists as a bilayer vesicle of equimolar phospholipids and unesterified cholesterol, which contains small amounts of albumin in its internal aqueous compartment with apoproteins (apo) C and D absorbed on its surface. Apo B, the major apoprotein of normal low density lipoprotein (LDL), is not present in LP-X (1-4). The appearance of LP-X in plasma has been noted after bile duct ligation or insertion of the common bile duct into the venous system in experimental animals (5). In addition, incubation of bile or biliary lipoproteins with serum leads to their conversion into LP-X. The biliary lipoproteins that are synthesized in liver, together with bile salts, are necessary for LP-X formation (6). Data from in vitro

A part of this work was presented as a preliminary report at the 6th International Symposium on Atherosclerosis, Berlin, June 1982 (Abstr. 697).

This work is dedicated to Professor Werner Creutzfeldt, Department of Internal Medicine, University of Göttingen, on his 60th birthday.

Received for publication 24 June 1983 and in revised form 24 April 1984.

1. Abbreviations used in this paper: apo, apoprotein; apo A, B, C, and E, apoprotein A, B, C, and E; DMSO, dimethylsulfoxide; HMG-CoA, 3-hydroxy-3-methylglutaryl Coenzyme A; LDL, lipoprotein-deficient serum; LP-X, lipoprotein-X; NPC, nonparenchymal cells; PC, parenchymal cells; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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0021-9738/84/09/0867/13 $1.00
Volume 74, September 1984, 867-879
and in vivo experiments suggest that during cholestasis regulation of bile components into plasma leads to the formation of LP-X (7). Despite high levels of unesterified cholesterol in plasma, in the form of LP-X during cholestasis, hepatic cholesterogenesis is enhanced (8, 9). Bile acids, which are increased both in plasma and liver, may not be implicated in hepatic cholesterogenesis (9–11). LP-X has no significant effect on hepatic cholesterol synthesis in normal rats that have a bile fistula (12).

Conflicting explanations have been offered to explain increased hepatic cholesterogenesis during cholestasis. It has been suggested that cholestasis impairs the negative feedback inhibition of cholesterol synthesis at the cellular level (13). In contrast, it has been shown that impairment in the supply of lymphatic lipoproteins, rather than a defect in this feedback inhibition, causes cholestatic hypercholesterolemia (11). However, infusion of lymphatic lipoproteins did not completely suppress enhanced cholesterogenesis in selective or complete biliary-obstructed rats (14).

Bile contains a lipid-protein complex that inhibits 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase activity in isolated liver microsomes (15, 16). Studies with isolated perfused rat livers showed that lipoprotein fractions (d < 1.063 g/ml) from the plasma of cholestatic rats increased the activity of the reductase, which was in contrast to the lipoprotein fractions from plasma of normal rats (17). LP-X, which is the main biliary lipoprotein and the major lipoprotein in cholestatic serum that is found in this density range, was not directly tested in these studies. Although the appearance of LP-X is a characteristic feature of cholestasis and biliary obstruction in man and in experimental animals (4–7), its possible role in bringing about cholestatic hypercholesterolemia has not been examined.

Accordingly, this study was designed to determine the site of LP-X uptake and its effect on HMG-CoA reductase and on the uptake of LDL and chylomicron remnants by the liver. The data presented here are consistent with the concept that LP-X plays a major role in bringing about the hypercholesterolemia that is observed during cholestasis and biliary obstruction.

Methods

Materials. All chemicals and biochemicals were obtained from Sigma Chemie, GmbH (München, Federal Republic of Germany), E. Merck, GmbH (Darmstadt, Federal Republic of Germany) or Boehringer (Mannheim, Federal Republic of Germany). Bovine serum albumin, fraction V, fatty acid poor was obtained from Miles Biochemicals (Kankakee) cell culture medium and fetal calf serum were from NUNC GmbH (Wiesbaden, Federal Republic of Germany), and Lymphoprep was from Nyegaard & Co. (Oslo, Norway). 32P-sodium iodide, 3-hydroxy-[3-14C]-methylglutaryl CoA, [2-3H]-mevalonic acid lactone, [26(27)-3H]-cholesterol, and cholesteryl-[1-14C]-oleate were purchased from Amersham Buchler GmbH & Co. KG (Braunschweig, Federal Republic of Germany).

Animals. Male Wistar SPF rats (MüIns Rattus, Brunthal, Federal Republic of Germany) weighing between 200 and 300 g were used. They had 12-h dark and light periods and were fed ad libitum with standard rat food (diet 1324, Altromin International Lager, Federal Republic of Germany).

Liver perfusion. Livers were perfused by a technique described previously (18), using Krebs-Ringer bicarbonate medium that contained 2.5% albumin and 10% hemoglobin in the form of human erythrocytes. Perfusions were started with 120 ml of medium. The first 20 ml were allowed to pass through the liver and be discarded. Recirculating perfusion was then commenced. Bile was collected throughout the perfusion. The viability of the preparation was checked by measuring the rate of release of glucose, the lactate/pyruvate ratio, and the adenosine nucleotide content of the liver.

Isolated liver cells. Hepatocytes were isolated by the standard method (19) with some minor modifications. Hyaluronidase was omitted from the medium. Nonparenchymal cells (NPC) were isolated by differential centrifugation (20). The biochemical viability of cell preparations was tested as described previously (18, 20).

Cultured human fibroblasts. Fibroblasts that were derived from skin biopsies of normal subjects were maintained in Dulbecco’s minimal essential medium, which contained 25 mM NaHCO3, 20 mM Hepes buffer, pH 7.4, and 10% fetal calf serum. Culture medium was also supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml. For experiments, cells between 8 and 10 passages were seeded into 60mm plastic dishes that contained 3 ml medium. The medium was changed after 3 d, and on the sixth day 2 ml medium that contained 10% lipoprotein-deficient serum (LDS) (2.5 mg protein/ml) was used in place of fetal calf serum. Experiments were performed 24 h later.

Mononuclear cells. Venous blood was drawn into heparin-containing syringes (100 U heparin/10 ml blood) under sterile conditions. Mononuclear cells were isolated over Lymphoprep gradient (21, 22). The cell suspension was incubated in RPMI-1640 medium that contained either 10% fetal calf serum or 10% LDS that was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). After 2 h at 37°C, nonadherent cells were transferred to 60-mm plastic dishes. Experiments were performed 48 h later. The purity of the cells was assessed after staining with the May-Grunwald-Giemsa stain, which revealed that >90% of mononuclear cells were lymphocytes. This was confirmed in some experiments by histochemical staining for peroxidase and non-specific esterase reactions (23) (negative for lymphocytes). The term lymphocytes is used for these cells throughout this report.

HMG-CoA reductase. The enzyme activity in the isolated perfused liver was measured by taking biopsies at various time intervals during the course of the perfusion. Microsomes were isolated from liver homogenates in 0.05 M Tris buffer, pH 7.4, which contained 0.25 M sucrose, 0.01 M EDTA, and 0.01 M dithiothreitol (24). Microsomal protein (50–100 μg) in 100 μl buffer was used for the measurement of HMG-CoA reductase activity in a final volume of 0.2 ml reaction mixture that contained 100 mM Tris buffer, pH 7.4, 5 mM dithiothreitol, 3 mM NADP, 22 mM glucose 6-phosphate, 3.5 U/ml glucose 6-phosphate dehydrogenase, and 43 μM [3-14C]-HMG-CoA (8.3 μCi/μmol, sp act). The reaction was linear for 2 h and assays were carried out routinely for 1 h. The reaction was stopped by addition of 0.06 ml 2.5 N HCl that contained 3.6 μmol 1H-mevalonic acid lactone as a carrier and internal standard (25). After further incubation at 37°C for 30 min the reaction vials were centrifuged and the supernates (50 μl) were assayed by thin-layer chromatography on silica gel plates, which were developed in benzene-acetone (1:1 vol/vol) for separation of HMG-CoA from mevalonolactone. The areas that corresponded to mevalonolactone (Rf 0.5–0.7) were scraped into scintillation vials that
contained Instagel (Packard Instrument International, Zurich, Switzerland) and counted for \(^{3}H\) and \(^{14}C\)-radioactivity in a Berthold liquid scintillation spectrometer (Berthold Laboratory, Wildbad, Federal Republic of Germany). The recovery of mevalonolactone was 80-85%.

The enzyme activity in lymphocytes was measured by centrifuging the cell suspensions, and after discarding the supernatates the remaining cell pellets were solubilized in 0.2 ml 50 mM potassium phosphate buffer, pH 7.4, which contained 5 mM dithiothreitol, 5 mM EDTA, 0.2 M KCl, and 0.25% pyro-EOB (25). The enzyme activity was assayed as in the procedure for liver microsomes, except that the protein content in the reaction mixture was 200-300 \(\mu g\) and an incubation time of 2 h was employed for the reaction.

Lipoproteins. Human LDL (solvent density 1.019-1.063 \(g/ml\)) and human LDI (solvent density > 1.215 \(g/ml\)) were prepared from plasma of normal subjects by differential ultracentrifugation using standard techniques (26). LDL was labeled with \(^{125}I\) by the iodine monochloride method for iodination of lipoproteins (27), which resulted in a radiolabeled preparation of LDL with specific activity 200-300 cpm/ng protein.

Chylomicron remnants were prepared from chylomicrons that were obtained from 2-h postprandial plasma of normal human subjects and from rat lymph. Chylomicrons were incubated with postheparin rat plasma (2 \(ng\) chylomicron cholesterol and 4.5 \(ml\) postheparin plasma that contained 5% albumin) for 30-45 min at 37°C (28, 29). The remnants were then isolated by centrifugation at 100,000 \(g\) for 100 min at 10°C at a solvent density of 1.019 \(g/ml\). After dialysis against 0.154 M NaCl-0.25 mM EDTA, pH 7.4, for 24 h, the remnants were labeled with cholesteryl-[\(^{14}C\)]-olate that was dissolved in dimethyl sulfoxide (DMSO) (28) and also with \(^{125}I\). The labeling efficiency of radiiodination was 16% on average and the TCA insoluble radioactivity that was measured was 95%, which corresponded to 300-400 cpm/ng protein. About 17% of the radioactivity was found in the lipid moiety. The chemical composition of the remnants from human plasma was 63% triglycerides, 14% cholesterol, of which 3% was unesterified, 16% phospholipids, and 7% protein. Chylomicrons that were obtained from rat lymph had a similar lipid composition, but the protein content was only 2%. Analysis by electrophoresis (SDS-PAGE) on polyacrylamide gel in the presence of sodium dodecyl sulfate showed the presence of apo A-I, apo B-48, apo B-100, apo C-I, and apo E in both chylomicrons and chylomicron remnants.

Isolation, labeling, and characterization of lipoprotein-X and its precursor, the bile lipoprotein. In order to attain a protein labeling of LP-X that assures high stability, specificity, and prevents exchange of protein label with other plasma proteins or apoproteins, it was necessary to label the core protein of LP-X, its albumin portion. This can only be achieved by iodination of the precursor lipoprotein of LP-X, which is the bile lipoprotein that carries only albumin, as earlier demonstrated (6). We confirmed this finding by analysis of this lipoprotein by SDS-PAGE and double immunodiffusion with specific antibodies to apo A-I, apo A-II, apo E, apo B, and apo C.

The bile lipoprotein was labeled with \(^{125}I\)-sodium iodide. In order to make the protein core accessible for radiiodination, the isolated lipoprotein fraction was first treated with 0.1% bile salt mixture, which consisted of equal parts of cholic acid, deoxycholic acid, and taurocholic acid (30). After radiiodination, the lipoprotein fraction was dialyzed against 0.005 M Tris buffer, pH 7.4, which contained 0.25 mM EDTA and 0.15 M NaCl, for >24 h. Fatty acid free unlabeled albumin was then added to give a final concentration of 2%, and the solvent density was adjusted with NaCl to 1.065 \(g/ml\) for a second ultracentrifugation at 150,000 \(g\) for 20 h at 10°C. The floating \(^{125}I\)-lipoprotein particles were collected from the supernatant. The labeling efficiency of radiiodination was 10±2\% (\(n=8\)). The TCA precipitable protein radioactivity was 91±2\% corresponding to 100-200 cpm/ng protein, and 19±2\% of the radioactivity was found in the lipid moiety. Such a lipoprotein fraction has previously been designated as LP-X\(_{\text{nor}}\) because it shares the physical and chemical characteristics of LP-X but is devoid of apo C and D (6).

If this material comes in contact with plasma or plasma lipoprotein fractions, it immediately acquires apo C and D and converts thereby into LP-X with its characteristic composition. This LP-X does not contain apo E or B as earlier demonstrated (6), and also confirmed by immunoelectrophoresis in this study (Fig. 1). This does not necessarily contradict other reports (31, 32) in which apo E was demonstrated in the density region in which LP-X is also present. In these reports it was not shown whether apo E was associated with cathodic-migrating lipoprotein. If radiolabeled LP-X is brought into contact with serum, 91±0.5\% of the radioactivity is recovered in the LP-X band on lipoprotein electrophoresis in agar gel (Rapidophor). Isolation of LP-X from serum was achieved using the previously described technique which consisted of a combination of ultracentrifugation and cold ethanol fractionation (3, 6).

The chemical composition of LP-X after radiiodination did not differ from that of unlabeled LP-X and was found to be 32±1.6\% unesterified cholesterol, 1.7±0.3\% esterified cholesterol, 2.1±0.1\% triglycerides, 56.5±4\% lecithin, 5.3±0.4\% phosphatidylethanolamine, and 2.4±0.4\% protein.

Analytical techniques. The protein content of cell extracts and lipoproteins was measured by the method of Lowry et al. (33). The concentration of cholesterol, phospholipids, and triglycerides was determined by standard enzyme kit methods (18). Lipoproteins were identified by lipoprotein electrophoresis (Lipidophor technique, Immuno A.G., Vienna, Austria) and LP-X by electrophoresis on agar gel by Rapidophor technique (Immuno A.G., Vienna, Austria), and also by chemical and immunochemical analysis (6). \(^{125}I\)-radioactivity in blood, serum, lipoproteins, cells, and tissues was measured directly using a Beckman Gamma 4000 (Beckman Instruments Inc., Irvine, CA).

\(^{14}C\)-radioactivity in the tissues or blood was measured after solu-

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**Figure 1.** Lipoprotein electrophoresis in 1% agar. (A) LP-X positive and negative sera. The lipoprotein bands were visualized by polyanionic precipitation. LP-X shows typical cathodic migration (6). (B and C) Immunoelectrophoresis of LP-X positive serum in 1% agar against anti-apo C and anti-apo B (B) and against anti-apo C and anti-apo E (C). Immunoelectrophoresis demonstrates absence of apo B and apo E in LP-X.

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bilitation in NCS (Amersham Searle Corp., Arlington Heights, IL). Mathematical analysis of data for decay curves and Lineweaver-Burk plots were done with the help of a Wang MUP type 2200 computer (Wang Laboratories, Lowell, MA).

The apolipoprotein composition of the remnants and LP-X was determined by SDS-PAGE on 10% polyacrylamide gel (34) after the lipoproteins were delipidated with ethanol-diethyl ether (3:1). For differentiation of apo B-100 and apo B-48, 3% gels were used (35). Immunoelectrophoresis was done on 1% agar gels (36, 37) using antibodies specific for apo A-I, apo B, apo C (polyspecific for C-I, C-II, and C-III), and apo E.

Noniodide TCA soluble radioactivity served as a measure of lipoprotein degradation. TCA was added to 2 ml of culture medium or perfusate to give a final concentration of 5%. After centrifugation, 1 ml of TCA supernate was treated with 50 μl 30% H₂O₂ and 20 μl of 40% KI. The molecular iodine was extracted with 2 ml chloroform. The two phases were separated by centrifugation at 4°C. An aliquot of the aqueous phase (500 μl) was assayed for radioactivity.

Results

Removal of LP-X in vivo

In order to establish the site of LP-X removal, ¹²³¹-I-LP-X that was reconstituted from bile lipids was injected intravenously into normal rats. ¹²³¹-I-LP-X disappeared rapidly from the circulation, as shown by the plasma decay curve (Fig. 2). A rapid initial decline in ¹²³¹-I-radioactivity was followed by a slow decline. Analysis of the plasma decay curve (Fig. 2) yielded a mean biological half-life of 15.5 min, as calculated from the fast component of the curve, and a mean biological half-life of 9.5 h corresponding to a mean fractional catabolic rate of 1.782 day⁻¹, as calculated from the slow component of the curve. A similar plasma decay curve was obtained for LP-X that was isolated from the serum of cholestatic rats (5). Since labeling of LP-X was achieved by iodination of the core protein of bile lipoprotein, it was necessary to establish that the plasma decay curve and the distribution of ¹²³¹-I reflected that of LP-X and not that of albumin. Rats were therefore injected with ¹²³¹-I albumin, which was labeled in the same manner as LP-X, in an amount equivalent to the amount of protein in LP-X. The decline in ¹²³¹-I-radioactivity in the plasma differed significantly from the results that were obtained with ¹²³¹-I-LP-X. Moreover, in contrast to the results for ¹²³¹-I-LP-X, the liver and spleen took up equal but small amounts of ¹²³¹-I-radioactivity. The plasma decay curve and the distribution of radioactivity in the organs did not change significantly over a longer time period (data not shown).

Measurement of ¹²³¹-I-radioactivity in various organs at various time intervals after administration of ¹²³¹-I-LP-X revealed that most of the radioactivity was recovered in the spleen and liver, while only negligible amounts were found in the heart, skeletal muscle, kidney, and lung (Table I). The amount of radioactivity in the spleen was fourfold and 11-fold higher than in the liver after 1 and 24 h, respectively, when a comparison was made on the basis of radioactivity per gram wet weight. On the basis of total weight, the percentage of ¹²³¹-I-radioactivity in the liver was 16, 15, 13, 12, and 9% at 1, 2, 4, 6, 8, and 24 h after administration of LP-X. Spleen had accumulated between 6 and 7% of the radioactivity at all of these time intervals.

The mean biological half-life of ¹²³¹-I-LP-X in these tissues of the rat was calculated so that it could be compared with the slow component of the plasma decay curve. The values obtained were 51.6 h for spleen, 18.3 h for liver, 16.2 h for heart, 15.9 h for lung, 16.3 h for kidney, and 11.3 h for skeletal muscle. A similar rate of disappearance and accumulation in the tissues of LP-X was also observed when LP-X, labeled in its unesterified cholesterol moiety with ³⁴C cholesterol that was solubilized in DMSO, was administered intravenously to normal rats (data not shown).

Cholestaticosis caused by bile duct ligation affects the rate of removal of LP-X by the spleen but not by the liver. Thus, 2 d after bile duct ligation, 81% of the radioactivity of noncholestatic control rats was still found in the spleen and after 8 d this decreased further to 68% (data not shown).

Uptake of LP-X in vitro

Isolated perfused liver. Livers were perfused with ¹²³¹-I-LP-X, prepared from bile lipids, which was added to the perfusion medium at 30 min after the start of the perfusion (Fig. 3). During the first 30 min, ~25–30% of the added radioactivity disappeared from the medium, but thereafter the radioactivity
in the medium remained unchanged. Likewise, no further accumulation of radioactivity in the liver was noted after the first 30 min. Such a pattern of removal kinetics is not indicative of active uptake by the liver. Only ~0.8–1.0% of $^{125}$I-LP-X added to the medium was degraded during 3 h of perfusion. When 1,435 μg $^{125}$I-LP-X was added to the medium, 10.28 μg were degraded, and upon addition of 766 μg, 7.66 μg were degraded. LP-X that was reisolated from perfusion medium showed similar organ distribution, as was noted with $^{125}$I-LP-X when injected into rats. Also, LP-X that was reisolated from perfusion medium exhibited similar kinetics of removal from the medium as compared with bile $^{125}$I-LP-X.

It was of interest to investigate whether parenchymal or nonparenchymal cells preferentially remove $^{125}$I-LP-X from the circulation. Rat livers were first perfused with a medium that contained $^{125}$I-LP-X, and the cells were then separated. 55 min after the start of the perfusion the radioactivity in the liver was found to be $3.0 \times 10^4$ cpm/g (Table II). The livers were then perfused with a medium that contained collagenase for a further 20 min in order to disperse the tissue for isolation of the hepatocytes. These blanched livers had a radioactivity of $2.1 \times 10^4$ cpm/g, while the amount of radioactivity in the hepatocytes that were isolated from the dispersed livers was

**Table II. $^{125}$I-Activity in Isolated Hepatocytes from Livers Perfused with $^{125}$I-LP-X**

<table>
<thead>
<tr>
<th>Total</th>
<th>Tissue (cpm/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-LP-X added at 15 min</td>
<td>$9.6 \times 10^4$</td>
</tr>
<tr>
<td>Counts in perfusion medium at 55 min</td>
<td>$6.5 \times 10^4$</td>
</tr>
<tr>
<td>Counts in liver tissue at 55 min</td>
<td>$3.0 \times 10^4$</td>
</tr>
<tr>
<td>Counts in medium at 75 min (new medium)</td>
<td>$1.35 \times 10^4$</td>
</tr>
<tr>
<td>Counts in liver tissue at 75 min</td>
<td>$2.100 \times 10^4$</td>
</tr>
<tr>
<td>Counts in medium with collagenase</td>
<td>$0.148 \times 10^4$</td>
</tr>
<tr>
<td>Counts in liver dispersion (filtered)</td>
<td>$0.670 \times 10^4$</td>
</tr>
<tr>
<td>Counts in isolated hepatocytes (0 min)</td>
<td>$1.712 \times 10^4$</td>
</tr>
<tr>
<td>Counts in isolated hepatocytes incubated 60 min</td>
<td>$0.481 \times 10^4$</td>
</tr>
<tr>
<td>Counts in NPC (0 min)</td>
<td>$4.354 \times 10^4$</td>
</tr>
</tbody>
</table>

Livers were perfused with erythrocyte-free medium for 15 min, and 1 ml of $^{125}$I-LP-X was then added to the medium (see Fig. 3) and perfusion continued until 55 min. At the end of this time, livers were perfused with new medium that contained collagenase. NPC were obtained by differential centrifugation of PC-free supernate.
0.519 \times 10^6 \text{ cpm/g}, and in NPC was 4.354 \times 10^4 \text{ cpm/g}. This radioactivity was retained by the cells even after incubation in the medium at 37°C. These results show that hepatocytes that were isolated from livers perfused with \(^{125}\text{I}-\text{LP-X}\) contained only 17% of the radioactivity found in the whole organ.

In order to determine if such a marked difference in the uptake of radioactivity between PC and NPC existed in vivo, rats were first injected with \(^{125}\text{I}-\text{LP-X}\). The livers were then isolated for preparation of the cells. Table III shows the distribution of \(^{125}\text{I}\)-radioactivity expressed as cpm/g. Again it was seen that NPC have taken up about 10-fold more \(^{125}\text{I}-\text{LP-X}\) than PC. This difference in the uptake was also evident when the results were expressed on the basis of protein content. Thus, the radioactivity found in the PC and NPC was 88 cpm/mg cell protein and 972 cpm/mg cell protein, respectively.

**Cells in suspension or in culture.** The uptake of \(^{125}\text{I}-\text{LP-X}\) by isolated human lymphocyte suspensions, isolated PC, and NPC from rat liver and monolayer cultures of human skin fibroblasts is shown in Fig. 4. \(^{125}\text{I}-\text{LP-X}\) was removed from the incubation medium by all these cell types in a concentration-dependent manner. The uptake of LP-X by PC and fibroblasts was only a fraction of that observed in NPC or lymphocytes. NPC took up 4–6-fold more LP-X than PC, and lymphocytes 20-fold more than PC. No saturation kinetics of uptake was noted in any of these cell types. Even raising the concentration of LP-X to 110 \(\mu\text{g/ml}\) did not result in any saturation kinetics. When \(^{125}\text{I}-\text{LP-X}\) was employed in a concentration range of 2.3–110 \(\mu\text{g}\), 0.846±0.05% of the added dose was degraded.

In order to establish if LP-X was taken up due to specific binding by a receptor, cells were incubated with \(^{125}\text{I}-\text{LP-X}\) in a concentration of 0.5 \(\mu\text{g}\) LP-X protein/ml medium, together with increasing amounts of unlabeled LP-X. No displacement of the uptake of labeled LP-X was noted, even in the presence of 60-fold excess of unlabeled LP-X. This suggests that LP-X is taken up by the cells in a nonspecific manner.

**Table III. Distribution of \(^{125}\text{I}\)-Radioactivity in PC and NPC in Rats after Intravenous Injection of \(^{125}\text{I}-\text{LP-X}\)**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Per gram wet weight</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (0 min)</td>
<td>59.7</td>
<td>452</td>
</tr>
<tr>
<td>Liver after 20 min preperfusion</td>
<td>25.3</td>
<td>174</td>
</tr>
<tr>
<td>Perfusion medium (20 min)</td>
<td>—</td>
<td>112</td>
</tr>
<tr>
<td>Dispersed liver after collagenase per</td>
<td>—</td>
<td>111.8</td>
</tr>
<tr>
<td>Medium after collagenase perfusion</td>
<td>—</td>
<td>50.7</td>
</tr>
<tr>
<td>PC</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>NPC</td>
<td>66.1</td>
<td></td>
</tr>
</tbody>
</table>

Rats weighing 160–170 g were injected with \(^{125}\text{I}-\text{LP-X}\) (32.5 \(\mu\text{g}\) LP-X protein) 30 min before perfusion of livers for isolation of PC and NPC. Values are the average of two experiments.

**Figure 4.** Concentration-dependent uptake of \(^{125}\text{I}-\text{LP-X}\) by liver PC and NPC, suspensions of human lymphocytes, and monolayer cultures of human skin fibroblasts. Incubations were carried out at 37°C for 2 h. At the end of the incubation time, monolayer cultures or cell pellets were washed three times with medium containing 0.2% albumin and then twice with albumin-free medium. Cells were dissolved in 1.0 ml of 0.1 N NaOH for measurement of radioactivity and protein. Values are mean±SD of six experiments.

**Effect of LP-X on the activity of HMG-CoA reductase**

**Lymphocytes.** Since the uptake of LP-X by lymphocytes was much higher than that observed in hepatocytes or monolayer cultures of fibroblasts, it might be expected that cholesterol that is transported by LP-X is able to suppress the activity of HMG-CoA reductase in these cells. As shown in Fig. 5, LP-X suppressed the enzyme activity when the lymphocytes were incubated in fetal calf serum or LDS. The degree of inhibition was similar for LDL and LP-X.

The data obtained from these incubation experiments are in agreement with the results of measurements performed on lymphocytes that were isolated from the blood of cholestatic patients who were LP-X positive. The enzyme activity was significantly lower, as compared with that measured in control subjects, being 0–11.5 compared with 55 pmol/mg protein per h (Table IV).

**Isolated perfused liver.** In order to determine whether there is a direct effect of LP-X on the activity of HMG-CoA reductase, which is increased in cholestasis, isolated livers were perfused with a medium that contained 30–40 mg LP-X cholesterol per dl, which corresponded to the levels of choles-
terol that were found in rat plasma. As shown in Fig. 6, a twofold increase in the activity of the enzyme was noted at the end of the perfusion period in livers, which had been perfused in the absence of lipoproteins in the medium. This may be due to the loss of cholesterol by the liver during perfusion (38). Addition of LDL to the medium did not suppress this increase in enzyme activity. However, addition of LP-X to the medium resulted in a marked, more than fivefold increase in hepatic HMG-CoA reductase activity.

Isolated microsomes. Incubation of liver microsomes with LP-X or LDL showed that LP-X, in contrast to LDL, suppressed the activity of the reductase in a concentration-dependent manner (Fig. 7). In order to exclude the possibility that LP-X interferes with the assay of the enzyme activity, microsomes that had been incubated with LP-X for 2 h were reisolated and the enzyme activity was measured. However, even after this incubation, the activity of HMG-CoA reductase remained suppressed in the LP-X free system (data not shown).

Since LP-X contains bile salts (3, 31), the effect of a mixture of bile salts (cholic, deoxycholic, chenodeoxycholic, and lithocholic acids) on the enzyme activity in microsomes was examined. Even at concentrations 5–10-fold higher than those present in LP-X (100–300 μM), no significant effect on the activity of the enzyme was observed. Thus it appears unlikely that the direct effect of LP-X on the activity of HMG-CoA reductase in isolated microsomes is due to the bile salts in LP-X particles (data not shown).

Table IV. Clinical Chemical Parameters in Serum and Activity of HMG-CoA Reductase in Lymphocytes from Blood of Cholestatic Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>LP-X</th>
<th>γ-GT (U/l)</th>
<th>AP (U/l)</th>
<th>GOT (U/l)</th>
<th>GPT (U/l)</th>
<th>Lymphocytes HMG-CoA reductase (pmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W.L.</td>
<td>215</td>
<td>259</td>
<td>++</td>
<td>372</td>
<td>369</td>
<td>78</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>B.M.</td>
<td>75</td>
<td>54</td>
<td>+</td>
<td>106</td>
<td>258</td>
<td>35</td>
<td>74</td>
<td>11.5</td>
</tr>
<tr>
<td>B.E.</td>
<td>469</td>
<td>139</td>
<td>+++</td>
<td>918</td>
<td>1,500</td>
<td>44</td>
<td>72</td>
<td>1.3</td>
</tr>
<tr>
<td>K.S.</td>
<td>300</td>
<td>265</td>
<td>+++</td>
<td>400</td>
<td>500</td>
<td>90</td>
<td>130</td>
<td>4.5</td>
</tr>
<tr>
<td>W.M.</td>
<td>200</td>
<td>245</td>
<td>+++</td>
<td>250</td>
<td>400</td>
<td>85</td>
<td>150</td>
<td>3.5</td>
</tr>
<tr>
<td>Controls</td>
<td>150–260</td>
<td>50–170</td>
<td>—</td>
<td>≤18</td>
<td>≤190</td>
<td>≤18</td>
<td>≤22</td>
<td>54.8±6.7 (n = 10)</td>
</tr>
</tbody>
</table>

Lymphocytes were obtained from the blood of patients with cholestasis. After isolation they were incubated in RPMI medium that contained 10% fetal calf serum for 2 h. The reductase activity was measured in nonadherent cells.


Figure 7. Effect of LP-X and LDL on the activity of HMG-CoA reductase in isolated liver microsomes. Microsomes (60 µg protein) were incubated in triplicate with varying concentrations of either LP-X or LDL at 37°C for 20 min. At the end of this incubation period, the incubate was assayed for the reductase activity. Values are the average of two separate microsomal preparations (100% activity = 296.2 pmol mevalonate/mg protein per min).

Influence of LP-X on uptake of chylomicron remnants

In view of the conflicting reports about the ability of lymph lipoproteins to suppress the enhanced hepatic cholesterogenesis after biliary ligation (11, 14), the effect of LP-X on the uptake of chylomicron remnants and LDL by the liver was examined. Preliminary experiments with chylomicron remnants, which were obtained by the treatment of human chylomicrons with postheparin plasma of rats, and with rat lymph chylomicrons that were treated in a similar manner revealed no significant difference in their uptake by isolated hepatocytes. Therefore, only human chylomicron remnants were employed in the following experiments.

Cholesteryl-[1-14C]-oleate labeled remnants were added to the perfusion medium and were rapidly removed by the isolated perfused liver. As shown in Fig. 8, after 90 min perfusion only 10% of the 14C-radioactivity remained in the medium. In the presence of LP-X, however, the rate of remnant removal by the liver from the medium was significantly decreased. The lower rate of LP-X clearance was paralleled by the diminished amount of 14C-radioactivity that was found in the liver tissue (Fig. 8). Likewise, the inhibitory effect of LP-X on the removal of chylomicron remnants was exhibited by isolated hepatocytes at all concentrations of remnants used (Fig. 9). In order to establish the specificity of remnant uptake by hepatocytes, the isolated cells were incubated with 125I-labeled remnants in the presence of varying amounts of unlabeled remnants. As indicated in Fig. 10, 50-fold excess of unlabeled remnants reduced the uptake of 125I-labeled remnants to 25%. In the presence of 50 mM EDTA and in the absence of calcium ions (see Fig. 10) from the incubation medium, a similar degree of inhibition of remnant uptake was observed. Thus ~75% of the uptake is specific.

Under our experimental conditions and with the concentration used, LP-X inhibited the specific uptake of remnants by hepatocytes by ~70% (Fig. 10). As in the case of remnants

Figure 8. Effect of LP-X on uptake of cholesteryl-[1-14C]-oleate-labeled chylomicron remnants in isolated perfused rat livers. Remnants were added to the perfusion medium after 30 min of equilibration (140 µg protein and 453 µg cholesterol/dl medium). LP-X was present in the medium from the start of the perfusion. Values are the average of two perfusions.

Figure 9. Inhibition of uptake of cholesteryl-[1-14C]-oleate-labeled chylomicron remnants by LP-X in isolated hepatocytes. Hepatocytes (100–150 mg wet weight) were incubated in 5 ml of incubation medium at 37°C for 1 h. Incubations were carried out in triplicate and values are the average of two cell preparations.
that were labeled with cholesteryl-[1-14C]-oleate, LP-X inhibited the uptake of 125I-labeled remnants at all the concentrations of remnants tested (Fig. 11). The Lineweaver-Burk plot, as used in enzyme kinetics, was obtained from the data on the uptake of 125I-labeled remnants. The intercepts of the best fit linear regression curves yielded the following constants for remnants: $K_m = 4.85 \mu g$ of protein · ml$^{-1}$, and $V_{max} = 108.81$ ng of protein · mg$^{-1}$ cell protein. In the presence of LP-X the values were $K_m = 9.69 \mu g$ of protein · ml$^{-1}$, and $V_{max} = 96.71$ ng of protein · mg$^{-1}$ cell protein. Thus, LP-X increased the $K_m$ but did not significantly alter the $V_{max}$. This increase in the $K_m$ in the presence of LP-X was also observed for remnants labeled with cholesteryl-[1-14C]-oleate.

**Influence of LP-X on cellular uptake of LDL**

Plasma LDL is catabolized by a variety of cells in culture, after specific binding to high affinity surface receptors (39). However, in normal rats only a small amount of circulating LDL is reported to be taken up by the liver (40), which suggests that this organ does not usually possess a large number of LDL receptors. Administration of ethinyl estradiol results in the expression of specific binding sites for LDL on the hepatic plasma membranes (41, 42). Similarly, livers of estrogen-treated rats bind and degrade human LDL as well as rat LDL in amounts that are several fold higher than those found in normal livers (42). We measured the production of nonprotein-bound 125I from 125I-LDL, which served as a measure of LDL degradation, by the perfused livers that were isolated from rats treated with ethinyl estradiol, in the presence and absence of LP-X. In contrast to the experiments performed with chylomicron remnants, the presence of LP-X in the medium had no significant effect on hepatic LDL degradation, as judged by the appearance of 125I nonprotein-bound radioactivity in the perfusion medium during a perfusion period of 3 h (Fig. 12). Monolayer cultures of human fibroblasts, whose LDL receptor activity was increased by incubation in LDS, were also incubated with 125I-LDL in the presence or absence of LP-X. No appreciable effect of LP-X on the degradation of LDL was found (see Fig. 13). Thus, LP-X does not influence the receptor-dependent high affinity binding and degradation of LDL in fibroblasts. The effect of LP-X on the uptake of chylomicron remnants by the liver appears therefore to be specific.

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**Figure 10.** Displacement of 125I-labeled chylomicron remnants by excess of unlabeled remnants in hepatocytes. Hepatocytes were preincubated for 30 min in the absence or presence of LP-X (0.4 mg LP-X cholesterol/ml medium). After this preincubation, 125I-remnants and increasing concentrations of unlabeled remnants were added to the incubation medium (see methods for chemical composition and specific activity). Incubations were carried out in triplicate at 37°C for 1 h. Values are the mean±SD of six cell preparations.

**Figure 11.** Concentration-dependent uptake of 125I-labeled chylomicron remnants by isolated hepatocytes in the presence and absence of LP-X. Each point represents the average of triplicate incubations for each concentration. Values are the mean±SD of three cell preparations.

**Figure 12.** Degradation of 125I-LDL by isolated perfused livers from ethinyl estradiol-treated rats in the presence and absence of LP-X. Rats were injected subcutaneously with 17α-ethinyl estradiol in a daily dose of 5 mg/kg body weight. On the sixth day, livers were perfused with or without LP-X for 30 min. Human 125I-LDL was added to the medium (900 μg LDL protein/dl medium) at 30 min. Values are the average of three perfusions.
Discussion

Induction of cholestasis by bile duct ligation in experimental animals such as rats, dogs, and pigs leads to the appearance of an abnormal lipoprotein, LP-X, in plasma. This lipoprotein also appears in humans with biliary stasis and it disappears after correction of biliary obstruction (3, 4, 7).

Since bile acids have been shown not to be directly implicated in hepatic cholesterogenesis, their increase in plasma and liver during cholestasis seems to be of minor importance in the development of cholestatic hypercholesterolemia. This study was initiated to examine whether LP-X, which is a consistent feature of cholestasis, may be the cause of the hypercholesterolemia of cholestasis, possibly because of its unusual chemical composition (3, 4). On the basis of data on its uptake, its effect on the activity of HMG-CoA reductase, and on the uptake of normal lipoproteins, an attempt will be made to explain at least in part the pathogenesis of the increased hepatic cholesterol synthesis in cholestasis and of the cholestatic hypercholesterolemia.

Intravenous administration of $^{125}$I-LP-X into rats results in a biphasic plasma decay curve. The reason for the initial fast disappearance of LP-X remains unclear and may reflect its distribution. LP-X differs from other lipoproteins, in that it is readily removed by the spleen rather than by the liver, when results are compared on a gram weight basis.

Liver tissue consists of PC and NPC, which may have different relative rates of uptake of LP-X. Study of the distribution of $^{125}$I-radioactivity in isolated livers that were perfused with $^{125}$I-LP-X, or in perfused livers that were isolated from rats that were injected with $^{125}$I-LP-X before cell isolation suggests that cells of the reticuloendothelial system are responsible for removal of LP-X from the circulation. Experiments with isolated cell suspensions also indicate that only lymphocytes exhibit a significant uptake of LP-X. Even though it is concentration dependent, it is not saturable and is nonspecific.

Thus, during cholestasis, even though the levels of plasma-unesterified cholesterol are high, this fraction of cholesterol that is transported in the form of LP-X is not taken up by the liver, and therefore cannot exert a feedback control on hepatic cholesterol biosynthesis. This view is strengthened by the observations that LP-X failed to suppress cholesterogenesis in perfused livers that were isolated from normal rats with biliary diversion (12).

In cholestasis, enhanced cholesterogenesis is accompanied by increased activity of HMG-CoA reductase despite elevated levels of unesterified cholesterol (43, 44), the reasons for which were unclear and the subject of our study. Since bile acids do not appear to directly affect hepatic cholesterol synthesis, biliary lipids which reflux into plasma during cholestasis need to be considered. It has been reported that bile contains a protein-lipid complex that is capable of inhibiting HMG-CoA reductase in isolated microsomes (15, 16). On the other hand, the total lipoprotein fraction of cholestatic rat serum has been shown to increase the activity of HMG-CoA reductase several fold in perfused liver (17). The activating factor was found to be in the $d < 1.063$ g/ml fraction, but was not identified. The density fraction $d < 1.063$ g/ml in both bile and cholestatic plasma contains LP-X or its precursors. We found that the reductase activity in perfused liver was enhanced over twofold in the presence of LP-X. This contrasts with the data obtained in lymphocytes, where, concomitant with the uptake of LP-X, the activity of the reductase is diminished to an extent comparable with that of LDL. Contrary to the situation in perfused livers, LP-X is capable of inhibiting the activity of the reductase in isolated microsomes, whereas LDL is ineffective. Thus, when no barriers for the uptake of LP-X exist, its cholesterol moiety is capable of inhibiting the reductase activity. This agrees well with other reports, which suggests that the delivery of unesterified cholesterol to liver microsomes suppresses the reductase activity (45). The failure of LDL to suppress the activity of HMG-CoA reductase may be a reflection of the inability of this lipoprotein to enrich the liver microsomes with unesterified cholesterol because of its physical configuration.

The activation of the enzyme activity in liver by LP-X may be explained on the basis of its high phospholipid content for the following reasons. Infusion of phospholipids into rats or of Intralipid into human neonates results in a rise in the content of unesterified cholesterol and LP-X (46) in plasma. Furthermore, phospholipid liposomes can leach cholesterol from cell membranes (47) and increase the activity of HMG-CoA reductase in hepatocytes (48). Phospholipid-apoprotein complexes can also promote an efflux of steroids from cells in tissue culture (49, 50). Taken together with our data, these observations suggest that LP-X in the circulation causes an efflux of cholesterol from cells that do not readily take up this lipoprotein, such as hepatocytes and fibroblasts. In contrast,
LP-X suppresses the activity of HMG-CoA reductase in cell types such as lymphocytes in vitro or lymphocytes that were isolated from cholestatic patients, where LP-X is taken up and delivers its cholesterol to the cells, thereby suppressing the activity of HMG-CoA reductase.

During a series of current experiments (manuscript in preparation), we prelabeled the cholesterol pool of fibroblasts with LDL that was labeled with $^{3}$H-cholesterol and incubated them in the presence and absence of LP-X that was labeled with $^{14}$C-cholesterol. The influx of $^{14}$C-cholesterol from LP-X into fibroblasts was negligible, which confirmed the data that was obtained for the uptake of $^{125}$I-LP-X by these cells. However, the efflux of $^{3}$H-radioactivity from the cells was considerably increased in the presence of $^{14}$C-LP-X. This provides an experimental support for the capability of LP-X to promote the efflux of cellular cholesterol. Additional experiments are required and are in progress to elucidate the mechanisms and to quantitatively evaluate this efflux.

Chylomicron remnants are rapidly removed from the circulation by the liver and are most effective in suppressing hepatic cholesterogenesis (51, 52). The supply of these chylomicrons of intestinal origin was shown by Weis and Dietschy (11) to be responsible for regulating cholesterogenesis in biliary diversion, enterolymphatic diversion, and biliary ligation. They suggested that during cholestatic the liver was still sensitive to feedback regulation of cholesterogenesis by lymph lipoproteins. This is in contrast to the suggestion that cholesterol feedback control is impaired in cholestatic livers (13). Cooper et al. (14), while confirming the ability of lymph lipoproteins to suppress the elevated hepatic cholesterol synthesis in rats with biliary diversion, failed to obtain complete suppression of cholesterogenesis in bilirubin-oxidized rats. Should chylomicrons, or specifically, their remnants, be able to suppress the enhanced cholesterogenesis of cholestasis, it would then be expected that their uptake is unimpaired. However, a marked hypertriglyceridemia, possibly due to accumulation of chylomicron remnant-like particles, is noted in patients with liver disease who have LP-X present in their serum (53). In bile duct ligated rats, a delayed clearance of chylomicron remnants from plasma has been reported (54), which is in agreement with the results reported here. However, they are at variance with those of Weis and Dietschy (11). The experimental design of these studies was such that lymph lipoproteins were infused immediately after bile duct ligation. LP-X in serum is not noted until 5–10 h after bile duct ligation in rats, and reaches a peak at 40 h (5). This may explain the unimpaired uptake of lymph lipoproteins and correction of enhanced hepatic cholesterogenesis that was noted by these authors.

In our experiments, the presence of LP-X caused the inhibition in hepatocytes of the uptake of chylomicron remnants that were labeled with either cholesteryl-$[1-^{14}$C]-oleate or $^{125}$I at all concentration of remnants tested. Analysis of the data from the Lineweaver-Burk plot suggest competitive inhibition in the presence of LP-X. In contrast to this, the uptake of LDL by perfused livers from rats treated with ethinyl estradiol, a treatment known to induce hepatic receptors for LDL (40–42), normally not expressed by liver, is unchanged in the presence of LP-X. Similarly, no effect of LP-X was noted on high affinity binding and degradation of LDL in fibroblasts, which possess apo B-E receptors for LDL binding (39). Hepatic membranes from various animal species and from humans have been shown to possess a specific receptor for lipoproteins that contain apo E, in addition to B-E receptor for uptake of LDL (55, 56). Apo-B-E receptor activity, which is not easily detectable in adult liver membranes, is subject to acute down-regulation in response to infusion of intestinal lymph lipoproteins or taurocholate. However, the apo E receptor activity remains unchanged by such treatment (56).

From our results, it appears that LP-X, although devoid of apo E, specifically affects the hepatic apo E receptors, and causes an inhibition of remnant uptake. Further data are required to elucidate the nature of this LP-X mediated, apo E receptor inhibition in hepatocytes and other cells. Furthermore, data on the underlying mechanisms that are responsible for this alteration may prove useful in gaining a better understanding of the mode of action of this receptor under physiological conditions, as it is believed to be primarily responsible for the clearance of dietary cholesterol.

Considering the data presented in this report, we suggest that cholestasis causes the appearance of a cholesterol rich, abnormal lipoprotein (LP-X) in plasma, which cannot deliver its cholesterol to the liver. Because of its high phospholipid content, it leaches cholesterol from cells such as hepatocytes, which do not take it up, and thereby increases the activity of HMG-CoA reductase in these cells. Its presence in plasma causes, by a still unknown mechanism, an inhibition of the uptake of chylomicron remnants by the liver, which thereby renders this class of lipoproteins unable to exert their regulatory role on hepatic HMG-CoA reductase and cholesterogenesis.

Acknowledgments
We thank Miss Christina Wiese and Miss Sylvia Kumm for excellent technical assistance and Mr. Bernd Kruse for mathematical analysis of the data. We are grateful to Miss Erika Bauer for typing the manuscript. These studies were supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 89, Cardiology-D 3400 Göttingen, Federal Republic of Germany).

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