Cholestasis: Lamellar Structure of the Abnormal Human Serum Lipoprotein

Abstract. *An abnormal lipoprotein was visualized directly in serum by electron microscopy of preparations negatively stained with potassium phosphotungstate. It appears as a unique disk-shaped particle with major axis measuring 400 to 600 angstroms and minor axis measuring about 100 angstroms. Chemical analysis, viscosity measurements, and x-ray diffraction analysis of purified preparations indicate that the particle, consisting of a one-to-one molar mixture of cholesterol and choline phosphatides associated with a small amount of protein, is a flattened vesicle, the wall of which is a continuous lipid bilayer.*

The major lipid classes of human serum normally occur in defined proportions in particles containing specific apoproteins. These serum lipoproteins are generally divided into four major groups by physical techniques of ultracentrifugal flotation or electrophoresis: (i) chylomicrons, (ii) very low density (VLDL) or pre-beta lipoproteins, (iii) low density (LDL) or beta lipoproteins, and (iv) high density (HDL) or alpha lipoproteins. Whereas most human hyperlipoproteinemias are associated with altered concentrations of apparently normal serum lipoproteins, the hyperlipidemia of obstructive liver disease represents a remarkable exception.

Obstruction to bile flow in humans selectively increases free cholesterol and phospholipids in the serum in approximately equimolar amounts (*1*). It was suggested from chemical studies of fractionated serum lipoproteins that these changes resulted from accumulation of one or more abnormal serum lipoproteins in response to biliary obstruction (*2*). Although the density and electrophoretic mobility of the bulk of the abnormal lipoproteins are similar to that of LDL, isolated fractions have abnormal chemical composition (*2, 3*). Recently, a relatively homogenous lipoprotein which does not react with antiserums to normal LDL has been isolated from the LDL fraction from patients with cholestasis (*4*).

The observations reported here are based on studies stimulated by reports on the properties of the abnormal lipoprotein: (i) it is separated from LDL by column chromatography on agarose gel (*5*); (ii) it contains antigenic determinants to serum albumin and to a polypeptide with carboxyl-terminal alanine (*6*) normally present in VLDL and HDL (*7*); (iii) like certain polypeptides of VLDL and HDL, it promotes the hydrolysis of triglycerides by lipoprotein lipase (*8*).

Serums from three patients with clinical evidence of cholestasis were compared by electron microscopy to those of three normolipidemic subjects and a patient with hyperbetalipoproteinemia. Lipoproteins were separated by preparative ultracentrifugation (*3*) except that D_2O in 0.15*M* sodium chloride solution containing 0.04 percent disodium ethylenediaminetetraacetate was used in most cases to raise the density of serum to 1.040 or 1.063 g/ml for flotation of LDL and the abnormal lipoprotein. Lipoprotein fractions were separated on agarose gel columns (*9*). Lipids of serum and lipoprotein fractions were measured by standard methods (*8*). Electrophoresis was performed on agarose gel (*10*). Protein was estimated by the method of Lowry (*11*).

Samples were prepared for electron microscopy by a standard procedure. Copper grids (300 mesh; Fullam) were covered with a thin parlodion film prior to carbon coating in a Siemens vacuum evaporator. A drop of fresh whole serum or lipoprotein fraction was applied with a Pasteur pipette to the carbon surface. After 5 to 15 seconds, excess fluid was removed by absorption onto filter paper and a drop of 2 percent potassium phosphotungstate (*p*H 6.35 to 6.40) was quickly added to the moist surface for 5 to 15 seconds. Excess stain was removed with filter paper and the grid was dried in air. All grids were examined promptly in a Hitachi HS-8 electron microscope at 50 kv and photographed at initial magnifications of 22,000 and 50,000 diameters.

With the electron microscope, we and others have examined negatively stained preparations of ultracentrifugal fractions of normal serum lipoproteins. Individual classes of lipoproteins can be identified on the basis of size, shape, substructure, and staining properties (*12*). In the present study, we found that these normal lipoproteins can also be visualized and identified in whole serum. In normal and in hyperbetalipoproteinemic serums, LDL of about 200 to 250 Å diameter predominate (Fig. 1a). The VLDL are larger and more polydisperse with respect to diameter (250 to 600 Å) and often have an electron-translucent core surrounded by a characteristic dark halo. Small chylomicrons and larger VLDL of about 1000 Å diameter are indistinguishable. The HDL are much smaller (about 100 Å) and take up more of the aqueous phosphotungstate stain. The electron microscopic image of whole serums from patients with cholestasis is strikingly different (Fig. 1b). A new structure, heretofore undescribed, occurs in these serums in numbers proportionate to the severity of the associated hyperlipidemia. The abnormal particles appear, by negative staining with potassium phosphotungstate, to be coin or disklike with a remarkable tendency to form rouleaux (Fig. 1, b and c). Larger structures resembling myelin figures are also observed in these serums and often appear continuous with single disks and rouleaux (Fig. 1b), which suggests a structural transformation. Although VLDL and LDL are readily seen, HDL are not (Fig. 1b), in agreement with previous data showing low amounts of alpha lipoproteins in cholestasis (*2, 3*).

After ultracentrifugation (10^8 $g \cdot$min, density 1.006 g/ml) of serums from patients with cholestasis, a wide yellow band is seen overlying the narrow band of LDL. The fluid above the wide band is removed with the aid of a tube slicer, and the bands are then collected with a Pasteur pipette. The amounts of the narrow and wide bands correspond roughly to the proportions of the normal and abnormal LDL particles visualized by electron microscopy of the whole serum. The wide band contains a mixture of three different particles (see cover). Its upper portion contains the greatest concentrations of disks relative to the spherical particles of about 200 to 250 Å characteristic of LDL. The major axis of the disks is 400 to 600 Å, and they tend to adhere to one another in rouleaux, producing overlapping images. This band also contains a few small VLDL distinguishable by their electron translucent cores (see cover). The lower portion of the wide band contains a larger proportion of normal appearing LDL particles. The narrow band contains mainly LDL together with some disks. The density of the wide band is raised to 1.040 or 1.063

with D_2O and the material is recentrifuged for 10^8 $g \cdot$ min. The yellow layer floating at the surface is applied to the agarose gel columns in a volume of 5 to 10 ml.

In material from D.C., a girl with a hepatoma and clinical and laboratory evidence of obstructive jaundice, gel column fractions of 10 ml showed three protein peaks. The first peak, at a K_D (distribution coefficient) of 0.23 contained the abnormal particles, which typically appear in rouleaux after concentration by diafiltration (Fig. 1c). The rare appearance, judged by electron microscopy, of contaminating normal LDL is evidently correlated with the very low cholesteryl ester content of this fraction: 0.3 percent cholesteryl esters, 1.9 percent triglycerides, 33 percent cholesterol, 61 percent phospholipids, and 3.4 percent protein. On agarose gel electrophoresis, the material separated in a broad band from the γ to the α_2 region. It was not stained by lipid crimson and stained faintly with oil red O. Beginning at K_D 0.33 (on the downslope of the first peak), a few normal LDL particles were seen by electron microscopy (Fig. 1d). The second peak, at K_D 0.58, contained chiefly normal LDL particles with a scattering of the abnormal disks, as determined by negative staining (Fig. 1e). The chemical composition of this material also indicates mainly beta lipoprotein with a small amount of the abnormal lipoprotein: 26 percent cholesteryl esters, 7 percent triglycerides,

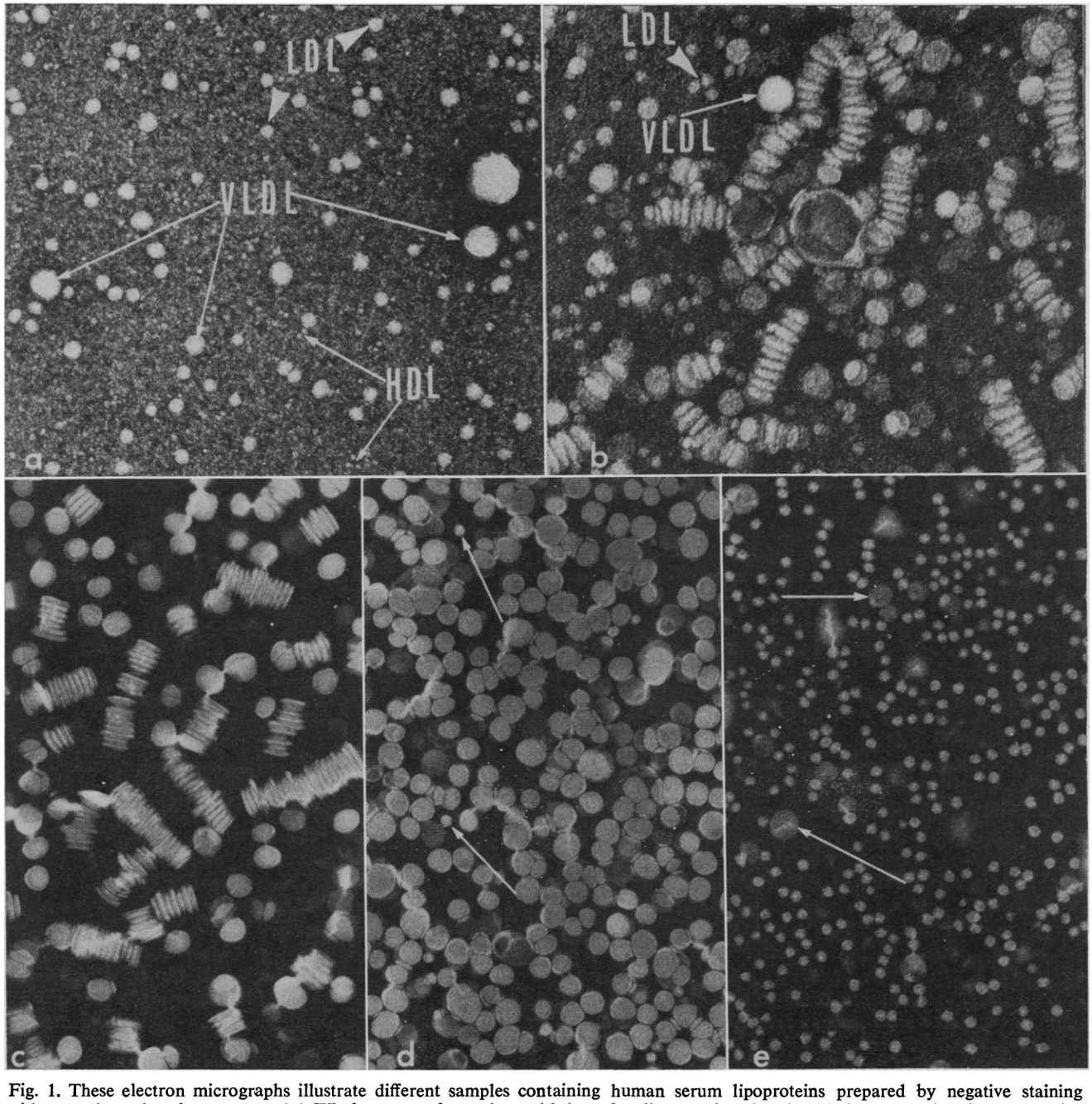

Fig. 1. These electron micrographs illustrate different samples containing human serum lipoproteins prepared by negative staining with potassium phosphotungstate. (a) Whole serum of a patient with hyperbetalipoproteinemia; (b) whole serum of patient D.C. with cholestasis; (c–e) fractions of density = 1.006 to 1.040 g/ml lipoproteins from patient D.C. separated by chromatography on agarose gel; (c) virtually uncontaminated disks, often in rouleaux, are seen at a K_D of 0.23; (d) at a K_D of 0.33, a few LDL particles are also seen (arrows); (e) LDL particles predominate at K_D 0.58, but a few disks are seen (arrows) ($\times$ 100,000).

15 percent cholesterol, 35 percent phospholipids, and 17 percent protein. The K_D of the third protein peak was 0.84. It contained other serum proteins but was not investigated further.

The same pattern was obtained with serums from the other two patients. In L.K., a man with obstructing carcinoma of the ampulla of Vater, the isolated abnormal lipoprotein was pooled, concentrated, and rechromatographed. This highly purified fraction contained 0.3 percent cholesteryl esters, 0.9 percent triglycerides, 31 percent cholesterol, 65 percent phospholipids, and 2.5 percent protein. In D.E., a woman with primary biliary cirrhosis, the fraction contained more LDL as determined by electron microscopy and had the following composition: 4.0 percent cholesteryl esters, 3.3 percent triglycerides, 27 percent cholesterol, 59 percent phospholipids, and 6.3 percent protein. From these data we suggest that the small amount of cholesteryl esters and some of the triglycerides are contained in contaminating LDL rather than in the abnormal lipoprotein. The abnormal particles are composed primarily, if not exclusively, of phospholipid (mainly phosphatidylcholine) and cholesterol in a molar ratio close to 1 : 1 together with small amounts of serum albumin and the several small glycoproteins normally found in VLDL and HDL (*13*).

The protein content of rechromatographed fractions of the abnormal serum lipoprotein is substantially lower than usually reported (*4*) and is of interest not only because of the improved purification possible with gel filtration (*5*) but because these particles retain their property of promoting the hydrolysis of phospholipid-stabilized emulsions of triglycerides by lipoprotein lipase (*13*). Certain polypeptides of VLDL and HDL have this property (*8*), which suggests that they have an affinity for interfacially oriented phospholipids and might be transferred to the surface of the abnormal lipoprotein from circulating VLDL or HDL.

Dispersions of phosphatidylcholine can incorporate up to an equimolar quantity of cholesterol in lamellar liquid crystals (*14, 15*). These structures can be modified and more readily dispersed into vesicles less than 1000 Å in diameter by addition of certain proteins (*16*). As visualized by negative staining with potassium phosphotungstate, adding lysolecithin to equimolar dispersions of cholesterol and lecithin produces particles which have structure and dimensions remarkably similar to the abnormal lipoprotein of cholestasis (*14*). It was suggested by Bangham and Horne that the disks they produced consist of two sheets of bimolecular leaflet, annealed at the periphery by the lysolecithin.

The phospholipids of our abnormal lipoprotein consist almost entirely of choline phosphatides, mainly lecithin, but also contain 1 to 3 percent lysolecithin (*13*). In electron micrographs of the abnormal lipoprotein negatively stained with phosphotungstate, the disklike structures in rouleaux occasionally exhibit a dark line along, but not usually extending to the ends of, the major axis. Negative staining with ammonium molybdate or fixation with osmium tetroxide prior to negative staining produces images consistent with flattened vesicles. These observations indicate that the negative stain can penetrate into an apparently narrow aqueous compartment and suggest the presence of a continuous lipid bilayer enclosing a potential water space.

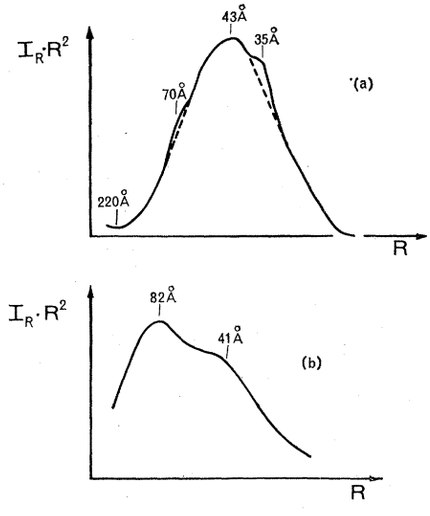

Fig. 2. The corrected (*19*) radial densitometer traces of the low-angle x-ray patterns of abnormal whole serum and of the purified abnormal lipoprotein. The abscissas are proportional to the distance, R, from the center of the pattern and the ordinates, to the photographic film density, I_R, multipled by the square of the distance, R^2. (a) For the isolated disks the continuous, corrected curve peaks at 43 Å; closely similar curves have been found for hydrated lecithin-cholesterol mixtures (*18*); traces of orders 1 (at 70 Å) and 2 (at 35 Å) of a lamellar, stacked structure are also seen superimposed on the broad peak. These may be due to disks in rouleaux or to the myelin figures which readily form in the concentrated material. (b) For the whole serum the continuous, corrected curve shows a peak at 82 Å and a shoulder at about 41 Å. In Fourier syntheses computed from these data and phased as for a lipid bilayer, the cross-sectional profile in both cases shows two peaks, identified with the lecithin head groups, separated by a trough identified with the lecithin fatty acid chains and the cholesterol. For the whole serum data, there is a peak adjacent to the lipid head group peak, that is, centered 48 Å from the center of the lipid bilayer.

The low-angle x-ray diffraction pattern of a concentrated dispersion of the abnormal lipoprotein in 0.20*M* aqueous NaCl was recorded using a Franks low-angle point-focusing camera (*17*) and compared to the pattern reported for mixtures of lecithin and cholesterol (*15, 18*). After the continuous curve of diffracted intensity was corrected for disorientation of the disks (*19*), it showed the form (Fig. 2a) predicted for a lipid bilayer (*18, 19*) and found for mixtures of lecithin and cholesterol with water (*18*). Together with the chemical analysis, this result indicates that the predominant lecithin and cholesterol form a bilayer, that is, the lecithin head groups are arranged in two layers 40 to 45 Å apart, center to center, with the fatty acid chains and cholesterol at the center of the membrane. Thus the x-ray results show that the disks visualized by electron microscopy (Fig. 1c) consist of a bilayer sheet which is doubled back as though to form a flattened vesicle; the overall thickness of the two sheets, each 50 to 60 Å, accounts for the value of the disk thickness, about 100 Å, measured in electron micrographs. The protein contained in the disks has not been located by x-ray diffraction analysis.

Only possible traces have been seen of the regularly spaced diffuse bands predicted for the low-angle diffraction pattern of two sheets lying parallel to one another at constant spacing. Thus, there appears to be a swelling of the space within each disk. Under these circumstances, one cannot decide from the x-ray pattern whether the rouleaux seen in Fig. 1c, or similar chains, exist in 0.20*M* saline. In considering this point, some viscosity measurements are given below.

The low-angle x-ray pattern of whole serum containing the abnormal particles (Fig. 2b) differs considerably from that of the isolated particles. The differences are of the form predicted for a lipid bilayer with a superficial layer of protein molecules and observed for cytochrome c added to phospholipid vesicles (*20*). Serum albumin was, therefore, added to the isolated particles. The resulting pattern closely re-

sembled that of the abnormal whole serum.

Although the viscosity of the purified material varies considerably from preparation to preparation, it is usually very high and tends to increase with time. Intrinsic viscosity as high as 1.0 dl g^{-1} (25°C) has been observed under conditions of both high and low shear, which indicates the formation of highly asymmetrical aggregates. The relative viscosity of fresh whole serum from affected patients is above the normal range (up to 2.2 at a concentration of 1.9 g of the abnormal lipoprotein per deciliter). Since the expected intrinsic viscosity of disks with an axial ratio of five as calculated by the Simha equation (*21*) is 0.045 dl g^{-1}, the increased viscosity observed in whole serum can be accounted for either by a monomeric dispersion of the disks or by linear aggregates of axial ratio not exceeding four. The marked increase observed in the viscosity of the material in the purified state suggests that other elements of serum interfere with the formation of highly asymmetrical aggregates. That serum albumin and perhaps other proteins exert such an effect is suggested by the finding that incubation of a viscous purified preparation for 1 hour at 25°C with an amount of serum albumin equal to the protein content of the abnormal lipoprotein resulted in a marked decrease in viscosity. The hypothesis that protein molecules in the serum coat the surfaces of the abnormal lipoprotein will account for both the x-ray pattern of the whole serum and the effect of albumin upon viscosity of the purified material.

The viscometric behavior indicates that only moderately asymmetrical particles exist in the native serum of patients with cholestasis. The electron microscopic images and x-ray diffraction patterns are consistent with particles in the form of partially flattened vesicles, the walls of which are a continuous lipid bilayer of the width expected for an equimolar mixture of choline phosphatides and cholesterol. The unique disklike appearance of the abnormal lipoprotein can be expected to facilitate investigation of its origin and metabolic fate.

ROBERT L. HAMILTON
RICHARD J. HAVEL, JOHN P. KANE
ALLEN E. BLAUROCK*, TEIZO SATA†
Cardiovascular Research Institute and
Departments of Anatomy and
Medicine, University of California School of Medicine, San Francisco

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* Present address: Biophysics Department, King's College, London, England.
† Present address: Research Institute of Angiocardiology, Kyushu University School of Medicine, Fukuoka, Japan.

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Deuterium Effects on Binding of Reduced Coenzyme Alcohol Dehydrogenase Isoenzyme EE

Abstract. *Determination of dissociation constants by two different methods yield the following mean values in 20 millimolar phosphate, pH 7.0, 25°C: 0.27 micromolar for reduced nicotinamide adenine dinucleotide (NADH); 0.29 micromolar for NADH with deuterium in the nicotinamide 4-B position (B-NADD); and 0.46 micromolar for NADH with deuterium in the nicotinamide 4-A position (A-NADD). These results indicate that dehydrogenases are capable of recognizing and distinguishing the appropriate hydrogen in the coenzyme already in the initial binding reaction.*

Various class A dehydrogenases—enzymes responsible for the stereospecific proton transfer to and from the A side of reduced nicotinamide adenine dinucleotide (NADH)—exhibit significant kinetic isotope effects in the hydrogen transfer step. These include yeast alcohol dehydrogenase (*1, 2*), malate dehydrogenase (*3*), lactate dehydrogenase (*4, 5*), and liver alcohol dehydrogenase (*1, 6–9*). Some of the results obtained suggested direct interaction between the enzyme and the coenzyme involving the proton to be transferred.

However, these interpretations are not without ambiguity; it therefore seemed desirable to make a direct comparison of the dissociation constants of the enzyme-NADH complexes with those of stereospecifically labeled enzyme-NADD complexes (*10*). Any significant interaction between the enzyme and the transferable proton should produce an observable isotope effect on this dissociation constant. Although such effects have been reported for dissociation constants calculated from rate constants (*1, 3, 4, 7*), no direct equilibrium measurements have been reported. To remedy this situation we have studied the LADH-NADD complexes; this enzyme is perhaps the most widely investigated example among the class A dehydrogenases.

The necessity for a purified LADH sample is quite evident in equilibrium studies which require relatively large amounts of enzyme. We observed that the commercial preparations of LADH, used in many laboratories for kinetic studies, slowly destroyed NADH in binary complexes under equilibrium conditions, a fact also reported by Theorell *et al.* (*11*). In order to remove this contaminant we adopted a purification procedure that resulted in the isolation of LADH isoenzyme EE (*12*). Thus, in contrast to all but the most recent results (*13*), the equilibrium constants reported here refer to homogeneous reactions involving only one isoenzyme.

We purified LADH (Boehringer) in lots of 8 to 10 mg by chromatography