treatment of the inhibitors with an excess of dithiothreitol should render them ineffective. The results in Table 1 show that neither the tumor inhibitors nor the standard sulfhydryl reagents inhibit phosphofructokinase if they have first been allowed to react with dithiothreitol (except for iodoacetamide which inhibited 32 percent when treated with dithiothreitol compared with 75 percent when not treated). When dithiothreitol was added after the tumor inhibitor was incubated with enzyme, activity was increased only slightly over that with inhibitor alone. As was expected, the inhibition of phosphofructokinase by *N*-ethylmaleimide or iodoacetamide, each of which forms a carbon-sulfur bond with the enzyme, was not reversed by dithiothreitol; but inhibition by Ellman's reagent, which forms a disulfide bond with the enzyme, was reversible.

Direct evidence for a reaction of taxodione with the sulfhydryls of phosphofructokinase is shown in Table 2. When phosphofructokinase was denatured with sodium dodecyl sulfate, ten molecules of taxodione per enzyme protomer resulted in the loss of about 12 sulfhydryl groups, indicating about a 1:1 reaction. When taxodione (10 moles per mole of enzyme) was incubated with the enzyme for 5 minutes, 5 m*M* ATP plus 2 m*M* fructose-6-phosphate protected six to seven of the sulfhydryl groups of the protomer. This incubation time and the substrate concentration are the same as those used in the kinetics experiment of Fig. 1; thus the prevention of inhibition by substrates is correlated with the protection of sulfhydryl groups. In this experiment, a concentration of phosphofructokinase about 800 times that used in the kinetics experiments was required in order to have sufficient protein for accurate determination of the enzyme's sulfhydryl content. Because of the larger relative concentrations of the other tumor inhibitors required for inhibition and their limited water solubility, they could not be tested in this system (*16*).

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16. Dr. H. I. Hadler (personal communication) has found that vernolepin acts as a thiol reagent in a mitochondrial system.
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Identity of Very Low Density Lipoprotein Apoproteins of Plasma and Liver Golgi Apparatus

Abstract. *In the rat, very low density lipoproteins isolated from hepatocyte Golgi apparatus, liver perfusates, and whole plasma appear identical in many respects. With specific immunochemical techniques and polyacrylamide-gel electrophoresis it can be demonstrated that the very low density lipoproteins from all three sources contain the same major lipoprotein apoproteins.*

The liver is the site of biosynthesis of at least one of the families of plasma lipoproteins, namely the triglyceride-rich, very low density lipoproteins (VLDL) (*1*, *2*). An additional site of origin for VLDL is the intestine (see *2*).

Evidence has been presented from electron microscopy suggesting that the 300- to 1000-Å particles within the hepatocyte Golgi apparatus are the precursors of plasma VLDL (*3*). A cell fraction rich in Golgi apparatus, isolated from rat liver, contains lipoprotein particles similar to plasma VLDL in morphology, immunochemical reactivity, and chemical composition (*4*). Delipidated plasma VLDL in man contains a number of apoproteins whose functional importance and site of origin remain unclear (*5*). It is not known whether all these apoproteins are assembled into VLDL in the liver, reflect VLDL from several tissues, or are partially adsorbed onto the VLDL after its release into the circulation. The first of these alternatives seems likely in that the major apoproteins of plasma VLDL have now been found in the VLDL recovered from liver Golgi apparatus and in the VLDL released from liver during perfusion of the isolated organ.

The Golgi apparatus were isolated from rat livers with the use of a barbital-buffered homogenizing medium and an unbuffered sucrose gradient (*4*). Lipoprotein particles were obtained by sonication of the Golgi apparatus cell fraction in barbital buffer (37.5 m*M* sodium diethyl barbiturate; 7.3 m*M* diethyl barbituric acid at *p*H 8.4) for 20 to 25 seconds (at tap No. 2 with a Branson Sonifier, model S110, equipped with a 13 by 0.3 cm microtip and operated at 3 amperes). The Golgi VLDL were floated free from the fragmented Golgi membranes at a density of 1.006 by ultracentrifugation at 100,000*g* for 16 hours (No. 50 Spinco rotor). They were removed in a small volume, mixed with 0.15*M* sodium chloride and recentrifuged in a similar manner. The low density lipoproteins (LDL) were isolated at densities of 1.006 to 1.035 (*6*).

Livers of 290-g rats were perfused in situ for 6 hours at 37°C at a flow rate of 20 ml/min with a plasma-free medium (*6*). The perfusate consisted of Krebs-Ringer bicarbonate buffer plus 5 percent (weight to volume) crystalline bovine serum albumin, 0.1 percent glucose, a sufficient number of washed rat erythrocytes to give a hematocrit of 25 percent, and a mixture of 11 amino acids (*7*). Perfusate VLDL were isolated and washed twice in 0.15*M* sodium chloride by ultracentrifugation at 100,000*g* for 16 hours. The VLDL isolated from the Golgi apparatus, perfusate, and plasma were dialyzed against 0.01 percent ethylenediaminetetraacetate in distilled water (*p*H 7) for 24 to 36 hours. These native lipo-

proteins were subjected to agarose electrophoresis, immunoelectrophoresis, and Ouchterlony immunodiffusion. Other lipoprotein preparations were lyophilized, totally delipidated, and then solubilized in 0.2*M* tris-HCl buffer (*p*H 8.2) containing 0.1*M* sodium decyl sulfate and 6*M* urea (*5*). These are referred to as Golgi apo-VLDL, perfusate apo-VLDL, and plasma apo-VLDL. Immunochemical analyses were carried out as described (*8*). Antiserums to the apoproteins (*9*) reacted with no plasma proteins except the lipoproteins and their apoproteins. Polyacrylamide-gel electrophoresis was performed with 15 percent acrylamide. The gels were prepared in *8M* urea at *p*H 8.9 with tris-HCl buffers (*5*).

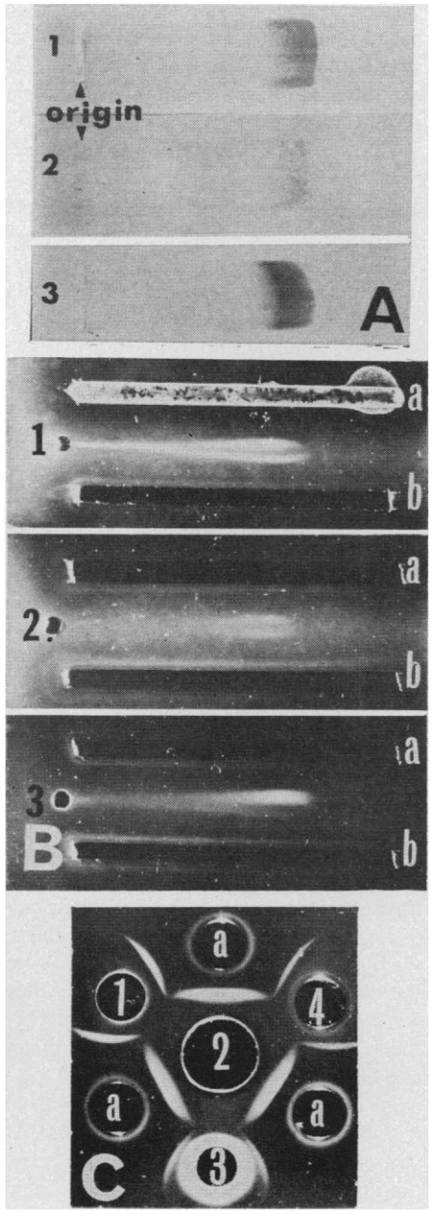

Fig. 1. Native lipoproteins. (A) Agarose electrophoresis. (B) Immunoelectrophoresis. (C) Ouchterlony immunodiffusion. Key: 1, Plasma very low density liproproteins; 2, Golgi very low density lipoproteins; 3, perfusate very low density lipoproteins; 4, plasma low density lipoproteins; a, antiserum to apoprotein VLDL; b, antiserum to apoprotein of plasma high density lipoprotein.

Native Golgi VLDL and perfusate VLDL migrated, on agarose-gel electrophoresis, with the same mobility as plasma VLDL (Fig. 1A). By immunoelectrophoresis, native Golgi VLDL, perfusate VLDL, and plasma VLDL appeared to be identical (Fig. 1B). By Ouchterlony immunodiffusion, a precipitin line of identity between Golgi VLDL, perfusate VLDL, plasma VLDL, and plasma LDL was obtained with antiserums prepared specifically to the apoproteins of plasma VLDL (Fig. 1C). The immunodiffusion studies indicate that Golgi VLDL, perfusate VLDL, and plasma VLDL share a major protein determinant with the plasma LDL. This LDL protein is often referred to as the B apoprotein (*10*).

After delipidated plasma VLDL was solubilized in decyl sulfate, several apoproteins could be isolated and characterized by gel filtration, polyacrylamide-gel electrophoresis, and immunochemical techniques. With similar procedures four major apoproteins have been identified in human plasma VLDL (*5*). In the rat, polyacrylamide-gel electrophoresis of plasma apo-VLDL revealed six bands, numbered 1 to 6, plus additional protein which remained in the stacking gel (Fig. 2). Most of the proteins responsible for the multiple bands have been isolated and characterized (*9*). The material remaining in the stacking gel was immunochemically identical to apoprotein B; furthermore, bands 4, 5, and 6 were immunochemically identical but different from the protein responsible for band 2 (*9*).

In Golgi apo-VLDL (Fig. 2), protein corresponding to the B apoprotein remained in the stacking gel, and there were four bands (Nos. 2, 4, 5, and 6) which appeared to correspond to bands in plasma apo-VLDL. When the isolated apoproteins responsible for bands 2, 4, and 5 from the plasma apo-VLDL were added to Golgi apo-VLDL, the acrylamide-gel pattern showed coelectrophoresis. This provided additional evidence of the identity of these proteins from plasma and Golgi VLDL.

Perfusate apo-VLDL contained the same bands (Nos. 2, 4, 5, and 6) as Golgi and plasma apo-VLDL, plus bands 1 and 3 found only in plasma apo-VLDL. Golgi apo-VLDL also contained three other bands (a, b, and c) which were absent from plasma VLDL but present in perfusate apo-VLDL. We could not demonstrate that the faintly

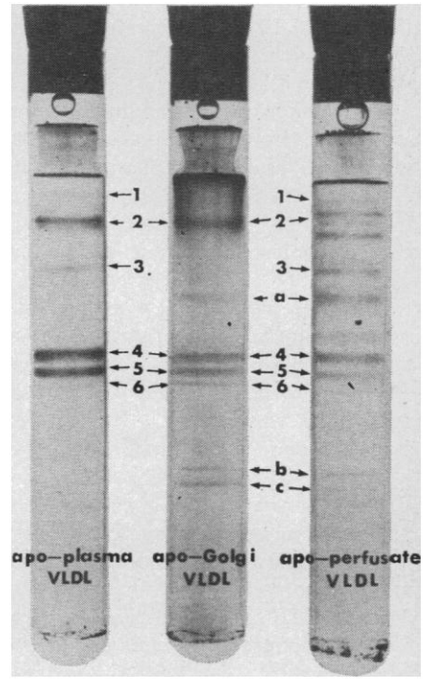

Fig. 2. Apo-lipoproteins: Polyacrylamide-gel electrophoresis of the apoproteins of plasma, perfusate, and Golgi VLDL.

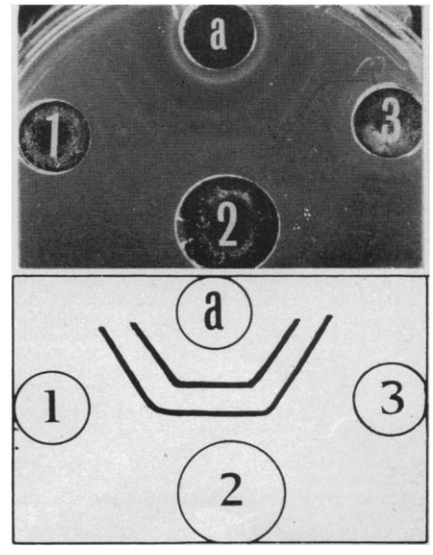

Fig. 3. Apo-lipoproteins: Ouchterlony immunodiffusion of the apoproteins of plasma, perfusate, and Golgi VLDL. (1, plasma apo-VLDL; 2, Golgi apo-VLDL; 3, perfusate apo-VLDL; a, antiserum to apo-VLDL.)

stained bands (1 and 3) of plasma and perfusate apo-VLDL were present in the Golgi apo-VLDL. The amounts present in the small quantity of Golgi material may have been too small to detect, or these bands may represent proteins added in small quantity to VLDL after it leaves the Golgi apparatus. On the other hand, there were three minor bands (a, b, and c) in both Golgi apo-VLDL and perfusate apo-VLDL which were not detected in plasma apo-VLDL.

With Ouchterlony immunodiffusion, apo-VLDL from the Golgi apparatus, hepatic perfusate, and plasma formed two precipitin lines of identity, neither due to B apoprotein, when they were reacted against antiserum to plasma apo-VLDL (Fig. 3). These two precipitin lines were not evident when the antiserum to apo-VLDL was first absorbed with the plasma VLDL apoproteins equivalent to bands 2 and 4. One precipitin line was removed by addition of plasma apo-VLDL band 4, the other by addition of band 2.

Thus, the liver Golgi apparatus contains and secretes a very low density lipoprotein particle containing the same major apoproteins seen in circulating plasma VLDL. The function of these different apoproteins remains to be demonstrated.

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Hormone-Sensitive Adenyl Cyclase: Cytochemical Localization in Rat Liver

Abstract. *An electron microscopic procedure has been developed, using rat liver, for the localization of hormone-sensitive adenyl cyclase. Isoproterenol-sensitive adenyl cyclase is located almost exclusively in the parenchymal cells. In contrast, glucagon-sensitive adenyl cyclase is located primarily in the reticuloendothelial cells but is also present in parenchymal cells. Sodium fluoride–sensitive adenyl cyclase is found in both cell types.*

The effects of many hormones on their target cells appear to be mediated through activation of adenyl cyclase, the enzyme which catalyzes the conversion of adenosine triphosphate (ATP) to adenosine 3′,5′-monophosphate (cyclic AMP) and inorganic pyrophosphate (PPi). The cyclic AMP so formed is believed to bring about changes characteristic of the hormone (*1*). Adenyl cyclase can be considered in such cases to be the hormone receptor. A proper understanding of the mechanism of action of the hormone requires a knowledge of the precise location of adenyl cyclase in tissues and cells. This information is not available at present for any tissue although biochemical studies of broken-cell systems from pigeon erythrocytes and rat liver indicate that adenyl cyclase is associated with the plasma membrane (*2*). However, even the simplest organs are composed of several cell types, and the isolation of pure cell fractions uncontaminated with other organelles is very difficult to achieve, and considerable caution must be exercised in interpreting biochemical data obtained from such studies. Cytochemical demonstration of adenyl cyclase activity should, in contrast, permit discrete localization of the enzyme in specific cell types as well as in specific cell organelles. We report here the development of such a cytochemical method at a fine structural level with rat liver as a tissue source. The hormones studied were isoproterenol (a synthetic analog of the naturally occurring hormones epinephrine and nonepinephrine) and glucagon, because both of these agents stimulate liver adenyl cyclase. Sodium fluoride was also studied because adenyl cyclase is the only enzyme known to be stimulated by this compound. Parallel cytochemical observations and biochemical measurements were made on samples from the same liver.

The basis of the cytochemical reaction was the precipitation of a heavy metal salt of the PPi produced by the action of adenyl cyclase on ATP. Lead ions (Pb^{2+}) precipitated PPi from the medium with the final product easily visualized in the electron microscope.

Adult male albino rats, anesthetized by intraperitoneal injection of 3.1 percent chloral hydrate (1 ml/100 g of body weight), were perfused through the left ventricle with 400 ml of 1 percent glutaraldehyde (*3*) in 0.05*M* cacodylate-nitrate buffer (*p*H 7.4) containing 4.5 percent dextrose. The fixed liver was removed, cut into slices 2 to 3 mm thick, and washed overnight in the same buffer. The fixed tissue was then cut into blocks less than 1 mm^3 which were used in both the cytochemical and biochemical studies.

For the cytochemical studies, the small blocks of fixed tissue were incubated (five blocks per milliliter of medium) for 30 minutes at 30°C in the standard medium (Table 1) to which 4.8 m*M* lead nitrate had been added together with one of the following additions: (i) none; (ii) isoproterenol; (iii) glucagon; and (iv) NaF. After the tissue blocks were incubated, they were washed briefly in 0.05*M* tris(hydroxymethyl) aminomethane (tris) maleate buffer (*p*H 7.4) containing 8 percent dextrose; they were then refixed in 1 percent osmium tetroxide in 0.05*M* veronal acetate buffer (*p*H 7.4) containing 7.5 percent sucrose, dehydrated in ethanol, and embedded in an Epon-Araldite mixture. Thin sections were cut on an LKB ultramicrotome and examined in an Hitachi 11B electron microscope.

Tissue incubated in the standard medium contained an electron-dense reaction product only in the bile canaliculi (Fig. 1A, insert), presumably due