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

to the action of bile canalicular adenosine triphosphatase (*4*). Deposits of reaction product were rarely associated with the surface of parenchymal cell microvilli in the space of Disse. No deposits were present intracellularly (Fig. 1A).

Isoproterenol appeared to cause deposition almost exclusively on the parenchymal cell surface, with little or no deposit on the surface of the reticuloendothelial cells (Fig. 1B). In contrast, the predominant effect of glucagon was to cause deposition of reaction product on the reticuloendothelial cell surface; however, deposits were also present on the parenchymal cells (Fig. 1C). In the presence of NaF, there were substantial deposits on the surface of both types of cells (Fig. 1D). Regardless of the activating agent used, the reaction product associated with parenchymal cells was confined to the external surface of microvilli in the space of Disse, which is in continuity with the lumen of the liver sinusoid. No deposits of reaction product occurred in relation to any of the other liver cell surfaces, except in the bile canaliculus, as mentioned above. In control experiments in which ATP was omitted, no deposits occurred, either on parenchymal cells or on reticuloendothelial cells, even when hormone was present. Electron microscopic observations after 5 minutes of incubation were similar to those after 30 minutes; however, the longer times were selected for the illustrations in order to visualize the reaction product more easily. The experiment was repeated a number of times, and several tissue blocks were sampled from each experiment. A large number of random pictures of sinusoids were taken with the only provision being that the tissue examined be close to the surface of the block used. The random sampling of tissue with regard to both the blocks examined and the micrographs taken should have been sufficient to eliminate sampling errors.

Biochemical experiments showed that some of the activity of the hormone-sensitive adenyl cyclase survived the fixative procedure (*5*). The effect of varying the concentrations of isoproterenol, glucagon, and ATP on the activity of adenyl cyclase in samples of fixed liver was determined, with the result that $4 \times 10^{-6} M$ isoproterenol and $1.5 \times 10^{-7} M$ glucagon were optimum, higher concentrations of the hormones causing a lower degree of activation of enzyme activity. Moreover, the stimulating effect of the hormones was greatest when the ATP concentration was 0.5 mM. Some of the results of the biochemical measurements of adenyl cyclase activity are shown in Table 1. Results qualitatively similar to those shown in Table 1 were

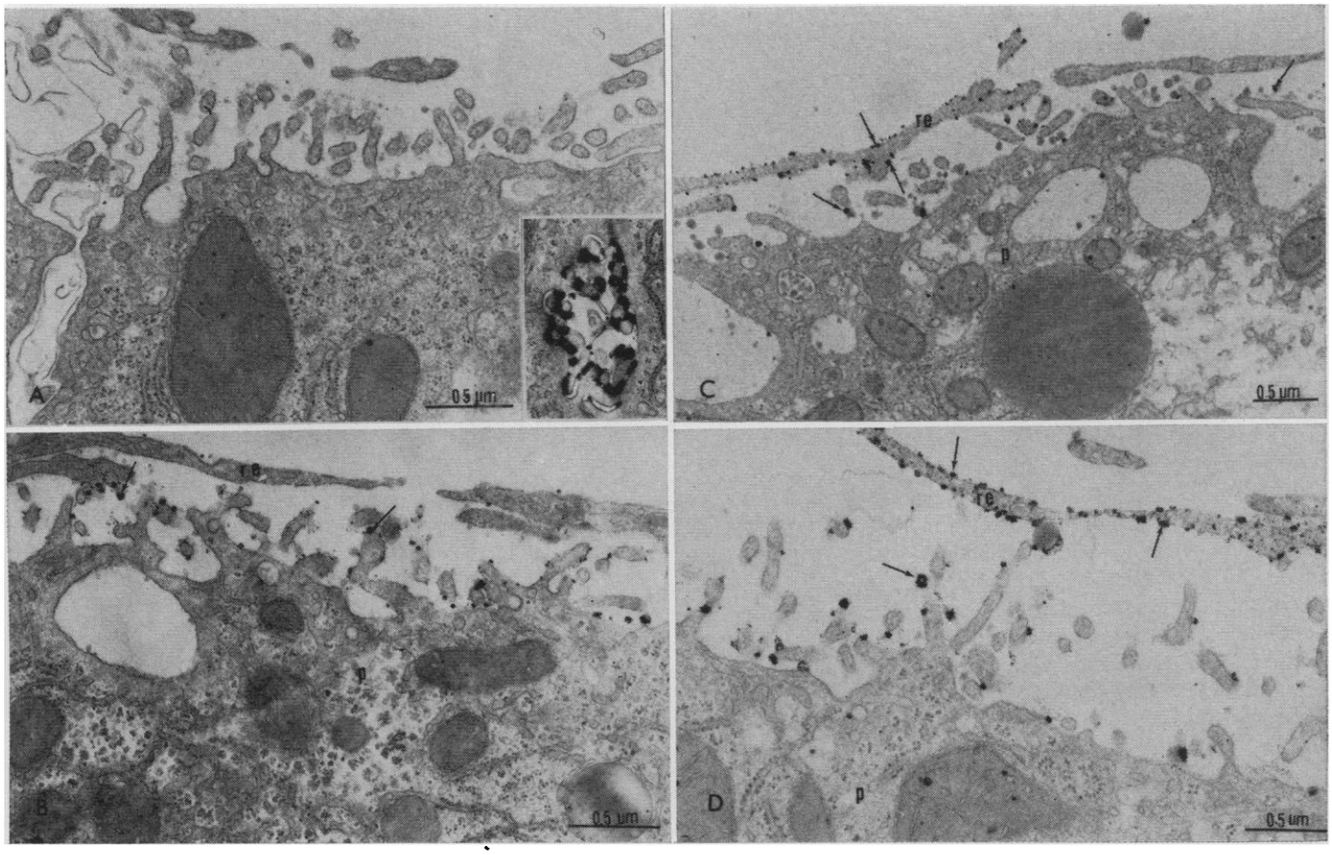

Fig. 1. (A) Liver incubated in the standard medium (Table 1), without additives, for 30 minutes at 30°C. Note the absence of reaction product associated with the microvilli of the liver cell plasma membrane. Insert, reaction product is present in the bile canaliculi of liver (× 22,500). (B) Liver incubated in the standard medium plus isoproterenol ($4 \times 10^{-6} M$) for 30 minutes at 30°C. Reaction product (arrows) is confined to the surface of the parenchymal cell (*p*) microvilli. Note the absence of reaction product in association with the reticuloendothelial cell (*re*) (× 22,500). (C) Liver incubated in the standard medium plus glucagon ($1.5 \times 10^{-7} M$) for 30 minutes at 30°C. Reaction product (arrows) is associated with the surface of the reticuloendothelial cells and to a lesser extent with the parenchymal cell (*p*) microvilli (× 22,500). (D) Liver incubated in the standard medium (Table 1) plus NaF ($12.5 \times 10^{-3} M$) for 30 minutes at 30°C. Reaction product (arrows) is present on surfaces of reticuloendothelial cells (*re*) as well as on the microvilli of the parenchymal cells (*p*) (× 22,500).

Table 1. Effect of hormones on adenyl cyclase activity of glutaraldehyde-fixed tissue. The fixed liver (50 mg) was sliced into blocks 0.5 to 1.0 mm^3 and incubated at 30°C in 80 m*M* tris-maleate (*p*H 7.4), 8 percent dextrose, 2.0 m*M* theophylline, 4.0 m*M* $MgSO_4$, and 0.5 m*M* [^{3}H]ATP (625 $\mu c/\mu$mole) in a volume of 0.5 ml (*10*). The reaction was carried out for 5 minutes, with shaking, in a water bath at 30°C and was terminated by placing the tubes in a boiling water bath for 2 minutes. Nonradioactive cyclic AMP (0.5 mg) was then added to the incubation mixture as carrier, and the cyclic AMP was determined by a slight modification of the method of Krishna *et al.* (*11*).

Addition	Cyclic AMP formed	
	Amount (pmole)	Percentage of control
None	10	100
Isoproterenol ($4 \times 10^{-6}M$)	73	730
Glucagon ($1.5 \times 10^{-7}M$)	31	310
NaF ($12.5 \times 10^{-3}M$)	38	380

found with incubation times up to 30 minutes. However, linearity with time was observed for only about 5 minutes.

Comparison of our biochemical and cytochemical data indicates that the reaction product observed on the surface of parenchymal cells and reticuloendothelial cells is due to adenyl cyclase activity. The amount of precipitate observed in the electron micrographs roughly parallels the amount of enzyme activity determined biochemically; such discrepancies as do exist between the two types of observations will be explained later. It seems logical for the enzyme to be confined to the plasma membrane of cells in contact with the blood plasma, where hormones are circulating; this was found here. The evidence that the final product is a lead salt of pyrophosphate is indirect; apart from the correlation between cytochemical and biochemical results, the precipitate observed at the cell surface is coarser than the lead phosphate present in the bile canaliculus (compare Fig. 1A, insert, with Fig. 1, B, C, and D).

One might have predicted that the parenchymal cells of the liver would have both glucagon-sensitive and epinephrine-sensitive adenyl cyclase systems, since glucagon and epinephrine are each capable of almost completely depleting glycogen stores in perfused liver (*6*). However, it is not possible to decide whether individual enzyme molecules on a single parenchymal cell can respond both to glucagon and to isoproterenol. Although studies of broken-cell preparations of rat liver have provided evidence that the glucagon-sensitive and epinephrine-sensitive adenyl cyclases might be separate systems (*7*), it now appears possible that the two systems were derived from separate cells of origin: our results demonstrate that distinct enzyme systems are present in parenchymal cells and reticuloendothelial cells. The parenchymal cell system is more responsive to isoproterenol, and the reticuloendothelial cell system is more responsive to glucagon. As the isoproterenol-stimulated enzyme in whole tissue is least inhibited by fixation (*5*), it is unlikely that the absence of isoproterenol-stimulated activity in the reticuloendothelial cells is due to inhibition by the fixative. In addition, the pattern of deposition of cytochemical reaction product was the same in various experiments regardless of the extent of survival of the enzyme found biochemically. Therefore, it is unlikely that the qualitative differences observed in the location of the isoproterenol-, glucagon-, or NaF-stimulated enzymes are attributable to a selective pattern of enzyme inactivation.

No theories concerning the function of adenyl cyclase in the liver have considered its role in the endothelial cells which act mainly as phagocytes. These littoral cells constitute 33 percent of the cells in the liver (*8*) and would be expected to contribute substantially to biochemical measurements of adenyl cyclase activity, and of cyclic AMP concentrations, when whole liver slices, homogenates, or cell fractions constitute the enzyme source. The interpretation of the results of such studies should take into account the cellular heterogeneity of the liver adenyl cyclase system as indicated here.

Some of the activity of the hormone-sensitive adenyl cyclase from rat liver survived glutaraldehyde fixation; presumably some of the enzyme retained its natural configuration in this fixed tissue. It is therefore of interest that the reaction product was found only on the outer surface of plasma membranes. Since this probably represents the site of release of pyrophosphate, and since biochemical evidence indicates that cyclic AMP is released on the inside of the cell (*9*), it is conceivable that adenyl cyclase may release its two products on opposite sides of the plasma membrane.

This paper represents the first instance of the cytochemical localization of a hormone-activated enzyme and therefore the localization of the site of action of a hormone.

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10. Lead was not included in the biochemical experiments because a significant conversion of ATP to cyclic AMP occurred, in the absence of tissue in the incubation medium, in the presence of the heavy metal. This nonenzymic conversion was several times greater than that observed in the normal incubation medium in the presence of tissue. Therefore, in the biochemical experiments, stimulation due to the hormone could not be observed in the presence of the lead. The nonenzymic production of cyclic AMP did not present a problem in the cytochemical studies, very probably because the nonenzymic breakdown of ATP occurred uniformly throughout the tissue and incubation medium, so that a significant concentration of reaction product was not formed at any location.
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