The stellate cells\(^1\) of the liver, which were first described by von Kupffer (1876) with the gold impregnation method, are quite different from the so-called phagocytic Kupffer cells in the sinusoids, and vitamin A is stored in these stellate cells (see Wake, 1971).

The stellate cells are star-shaped perisinusoidal cells located in the space of Disse. Their cytoplasm contains well-developed, rough-surfaced endoplasmic reticulum and a number of lipid droplets. These lipid droplets produce intense vitamin A-fluorescence in the fluorescence microscope (Nakane, 1963; Wake, 1964, 1971), and they increase in number remarkably after the administration of excess vitamin A, whereas after the administration of detergents they fail to increase (Wake, 1971; Kobayashi et al., 1973). The localization of vitamin A in the lipid droplets of the stellate cells has also been demonstrated with the gold chloride reaction (Wake, 1973) and with autoradiography after the injection of tritiated vitamin A (Hirosawa and Yamada, 1973). However, the mechanism of the formation of the vitamin A-rich lipid droplets in the stellate cells remained to be investigated.

The purpose of the present paper is to report the development of vitamin A-rich lipid droplets in the multivesicular bodies (mvbs) of the stellate cells after the administration of excess vitamin A and, thus, to propose another function of the mvbs other than their being phagolysosomes as first suggested by de Duve and Wattiaux (1966).

\(^1\) In view of the deep-rooted misconception, for a long period, regarding the designation of the stellate cells (\textit{Sternzellen}), it is necessary to mention briefly the appropriate terminology. The cells that react with gold chloride are designated as \textit{stellate cells} (Wake, 1971), following the first report of von Kupffer (1876), while the phagocytic cells in the sinusoids are designated as \textit{Kupffer cells}. The \textit{fat-storing cells} (Itô, 1951) are identical to the stellate cells (see Wake, 1971), but the term "fat-storing" does not appropriately reflect the function of these cells, because these cells store vitamin A (Nakane, 1963; Wake, 1964; Hirosawa and Yamada, 1973) and not "fat" (glycerides).

\textbf{MATERIALS AND METHODS}

Sprague-Dawley rats (200–250 g) of both sexes, which were fed laboratory chow and water \textit{ad libitum}, were used in the present experiments. Animals received vitamin A acetate (Chocola A, Eisai Co., Ltd., Tokyo) in four subcutaneous injections on alternate days. The total dosage of vitamin A administrated was 1,320,000 IU/kg. At 2 wk after the last injection, the animals were sacrificed.
**Fluorescence Microscopy**

Animals were perfused via the heart with 5% Formalin. The fixed liver was removed, sliced, and fixed for 16 h in calcium Formalin (Baker, 1958) in the dark at 4°C. Frozen sections (15-µm thick) were mounted in physiological saline and examined with a fluorescence microscope.

**Electron Microscopy**

Liver blocks were removed from animals under Nembutal anesthesia and fixed in cold 2% osmium tetroxide in 0.1 M phosphate buffer (Millonig, 1962) at pH 7.4 for 2 h. After a brief wash in the same buffer the tissue was postfixed in unbuffered 12% glutaraldehyde for 2 h and then treated with 2% uranyl acetate in a water solution for 20 min before dehydration (Kanaseki and Kadota, 1969). The osmium-aldehyde-uranyl method gave better preservation of the ultrastructure of the tissues than the ordinary method (aldehyde-osmium-uranyl). This method has the advantage of clearly delineating the unit membrane structure (Gray and Willis, 1970). The tissue was dehydrated in a graded series of ethanol, and embedded in Epon (Luft, 1961). The sections were stained with Millonig’s lead procedure (Millonig, 1961) and examined in a Hitachi HU-11Ds electron microscope.

**Gold Reaction in Electron Microscopy**

1-mm thick liver blocks were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 2 h. After washing in distilled water, the tissue was treated with 0.02% gold chloride solution (pH 2.8) for 16 h in the dark at room temperature (Wake, 1971). A short rinse in unbuffered 1% OsO₄, before incubation, is recommended for preservation of fine structure. The tissue was post-fixed for 2 h in 2% OsO₄, dehydrated, and embedded in Epon.

**OBSERVATIONS**

Since the light and electron microscope structure of the stellate cells both in control rats and in rats with hypervitaminosis A has been previously described in general (Wake, 1971), attention will be given here to only the lipid droplets and the related organelles.

After the administration of excess vitamin A to rats, lipid droplets increased in number in the cytoplasm of the stellate cells of the liver. In control animals the stellate cells containing lipid droplets were distributed only in the peripheral zone of the liver lobule, whereas in rats with hypervitaminosis A these cells were found not only in the peripheral zone but also in the intermediate and central zones. These lipid droplets produced intense vitamin A fluorescence in the fluorescence microscope (Fig. 1), and they reacted with gold chloride to form a metallic gold deposition on the surface (Fig. 2). Gold-reactive chylomicrons appeared in the phagosomes in the Kupffer cells (Fig. 2), while no such chylomicrons and phagosomes were observed in the stellate cells.

Two kinds of gold-reactive lipid droplets could be morphologically differentiated in the stellate cells (Figs. 2 and 3); type I lipid droplets were electron dense, variable in size but always smaller than type II lipid droplets which were not only larger (ca. 2 µm in diameter) but almost equal in size. Type II lipid droplets were located in the matrix of the cytoplasm, and when these lipid droplets were situated very close to one another a thin cytoplasmic wall was interposed between them (Fig. 3). Most lipid droplets in the stellate cells in control animals were type II. In rats with hyper-

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**FIGURE 1** A fluorescence micrograph showing vitamin A fluorescence from the lipid droplets of the stellate cell 2 wk after subcutaneous injections of excess vitamin A acetate. × 2,800.

**FIGURE 2** An electron micrograph of the stellate cell from the same rat with hypervitaminosis A. The liver specimen was treated with 0.02% gold chloride solution en bloc for 16 h at room temperature. Reaction product is seen on the surface of the well-preserved lipid droplets. Note two kinds of lipid droplets: type I (L₁) is smaller and more electron dense than type II (L₂). The nucleus (N) is indented by the lipid droplets. Gold-reactive chylomicrons (arrows) are seen in the phagosomes (P) of the Kupffer cells (KC). HC, hepatic parenchymal cell; C, collagen bundle in the Disse’s space. × 11,000.

**FIGURE 3** Part of a stellate cell from a rat with hypervitaminosis A. The liver specimen was treated by the osmium-aldehyde-uranyl method. Type I lipid droplet (L₁) is round and clearly delineated by a membrane, whereas type II lipid droplets (L₂) are located in the cytoplasmic matrix. Granular endoplasmic reticulum (ER) and a multivesicular body (MV) are seen in the narrow rim cytoplasm. A thin cytoplasmic bridge (arrow) is seen between two lipid droplets. HC, hepatic parenchymal cell; DS, Disse’s space; E, endothelial lining cell; RB, red blood corpuscle in the sinusoid. × 38,000.
vitamin A, type II droplets occurred throughout the lobule, whereas type I were frequently observed especially in the central and intermediate zones of the lobule.

Type I lipid droplets were characterized by the presence of a unit membrane (80-90 Å) surrounding them (Fig. 4). This unit membrane does not have any direct contact with the surface of the lipid droplet, but a light layer (40-80 Å thick) was intercalated between the inner leaflet of the unit membrane and the dense surface layer (60 Å thick) of the lipid droplet. In this work, this light layer is called the "intercalated layer." Along the margins of type I lipid droplets one could observe a pentalaminar layer (Fig. 4, inset) consisting of (1) the dense outer leaflet of the unit membrane, (2) the light lipid layer of the unit membrane, (3) the dense inner leaflet of the unit membrane, (4) the light intercalated layer, and (5) the dense surface layer of the lipid droplet. Frequently, in some regions the intercalated layer protruded externally to form a thicker area (Fig. 5). These protruded portions were characterized by the presence of a limited number of vesicles within them. These areas of large type I lipid droplets usually contained a few vesicles (Fig. 5), while in the small lipid droplets many vesicles were clustered in a group (Fig. 6). The diameter of those vesicles measured ca. 700 Å. The size and the nature of these vesicles were similar to those of the mvbs of the stellate cells (Fig. 7).

The mvbs were often observed in the vicinity of the Golgi complex of the stellate cells of control animals. After the administration of excess vitamin A, these organelles appeared to increase in number in the cytoplasm. They contained many vesicles including occasional dense vesicles and filamentous structures (Fig. 7). After the administration of vitamin A, small lipid droplets often appeared in the mvbs (Fig. 8). These small lipid droplets were also reactive with gold chloride and were well preserved after gold treatment (Fig. 9). However, with this method it was difficult to know whether the small lipid droplets existed in mvb vesicles or in the matrix. In the matrix of the mvbs, larger lipid droplets appeared to be formed by the fusion of small lipid droplets (Fig. 9).

DISCUSSION

The present investigation shows that in the stellate cells of rat liver there exist two kinds of lipid droplets after the administration of excess vitamin A: the first kind are membrane-bounded lipid droplets (type I), and the second are larger and are present in the matrix of the cytoplasm (type II). Both types of lipid droplets in the stellate cells as well as the chylomicrons in the phagocytic Kupffer cells react with gold chloride, resulting in a precipitation of fine grains of metallic gold on their surface, while lipid droplets in the parenchymal cells fail to react with gold chloride. The intensity of the gold reaction in the stellate cells increases remarkably in parallel with the intensity of vitamin A fluorescence after the administration of excess vitamin A (Wake, 1971). Since no reaction occurs after irradiation with ultraviolet light, as a result of destruction of vitamin A, and since the reaction occurs only below pH 4.2 and increases with the lowering of the pH, the gold reaction is believed to be one of reduction of gold chloride by vitamin A (Wake, 1973). The cytochemical analysis of both types of lipid droplets in the stellate cells gives evidence of their enrichment in vitamin A.

In the liver of the normal rat, vitamin A-rich lipid droplets are observed only in the stellate cells located in the peripheral zone of the lobule, but after the administration of excess vitamin A these lipid droplets are demonstrated in the central and intermediate zones as well as in the peripheral zone (Wake, 1971). Type I lipid droplets are found more frequently in the central and intermediate zones than in the peripheral zone. On the basis of these findings and the sizes of both lipid droplets, it is likely that type II droplets are derived from type I droplets after losing their surrounding unit membrane. Studies to explore this possibility are in progress.

After the administration of excess vitamin A, the number of mvbs in the stellate cells increases. These mvbs have dense plaques on their surface, a fibrillar density, and many vesicles. They are designated as dense mvbs according to the classification proposed by Rosenbluth and Wissig (1964) and Nicander (1966). The term mvb is commonly used to describe a vacuole containing small vesicles (Frield, 1969). According to this definition, the membrane-bounded body containing a large lipid droplet and a number of vesicles, shown in Figs. 5 and 6, can be designated as mvb. Moreover, this body is surrounded by a limiting membrane equal in thickness to the plasma membrane and the membrane of the mvbs. Dense plaques are frequently seen on their surface. It is evident, therefore, that vitamin A-rich lipid droplets are located in the matrix of the mvbs in the stellate cells.
FIGURE 4 Pentalaminar layers surrounding type I lipid droplets ($L_1$) in a stellate cell from a rat with hypervitaminosis A. A light layer, termed intercalated layer in the present paper (inset, 4), occurs between the surrounding unit membrane (inset, 1, 2, and 3) and the dense surface layer (inset, 5) of the lipid droplet. Granular endoplasmic reticulum (ER) is located in the narrow strip of cytoplasm between two lipid droplets. $HC$, parenchymal cell; $DS$, Disse's space. $\times 122,000$. Inset. $\times 366,000$. 

$HC$, parenchymal cell; $DS$, Disse's space. $\times 122,000$. Inset. $\times 366,000$. 

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FIGURE 5 Section of a type I lipid droplet (L₁). Note the limited number of vesicles (arrows) in the thick portion of the intercalated layer. L₁, type I lipid droplet. × 65,000.

FIGURE 6 A less developed type I lipid droplet (L₁) (as compared to that shown in Fig. 5) and a cluster of many vesicles are surrounded by a membrane. The size and shape of these vesicles are similar to those of the vesicles in the multivesicular body shown in Fig. 7. × 72,000.

FIGURE 7 A multivesicular body in a stellate cell from a control rat. Small vesicles, dense vesicles, and filamentous structures are seen in the matrix. A dense plaque (arrow) is seen on the surface membrane. × 140,000.

FIGURE 8 Two small lipid droplets (L) in the matrix of a multivesicular body of a stellate cell from a rat with hypervitaminosis A. Cytoplasmic vesicles are associated with the multivesicular body. Arrow, dense plaque. × 160,000.
FIGURE 9 Developing type I lipid droplets as revealed by the gold reaction. Lipid droplets (*) of various sizes are well preserved. Small lipid droplets seem to fuse with one another to form larger droplets. L2, type II lipid droplets. × 165,000.

The presence of lytic enzymes (Holtzman et al., 1967; Friend and Farquhar, 1967; Friend, 1969) as well as sequestered protein (Friend and Farquhar, 1967; Locke and Collins, 1968) have confirmed the view that the mvb is a phagolysosome (de Duve and Wattiaux, 1966). On the other hand, Farquhar (1969) suggested that lipid droplets in the mammotrophs in the anterior pituitary gland develop from mvbs. Recently, Schulze (1973) also reported the development of lipid droplets, which were suggested to contain steroid hormone and related substance, in the mvbs of Leydig cells in the frog. However, to our knowledge, the presence of such large lipid droplets in the mvbs as observed in the stellate cells has not been reported previously. On the basis of the various morphological steps involved in the transformation of small lipid droplets (Fig. 8) to larger ones (Fig. 4), it is difficult to believe that the mvbs in the stellate cells are a type of autophagic vacuole. These bodies contain no sequestered cytoplasmic material within the limiting membrane. It is suggested, therefore, that the lipid droplets develop in the matrix of the mvbs. Small lipid droplets seem to fuse with one another to form large lipid droplets in the matrix of the mvbs (Fig. 9).

The present observations are consistent with the view expressed in biochemical reports (Mahadevan and Ganguly, 1961; Mahadevan et al., 1963; Futterman and Andrews, 1964). The biochemical data on vitamin A uptake in the liver, obtained using fractions from the organ homogenate, seem to indicate that this uptake is a function of the stellate cells, because vitamin A in the liver is mainly stored in the lipid droplets of the stellate cells (Nakane, 1963; Wake, 1964, 1971; Hirosawa...
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