# EFFECTS OF *IN VIVO* DECALCIFICATION ON ULTRASTRUCTURE OF ADULT RAT LIVER

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ABSTRACT

A method of *in situ* perfusion of rat liver via the portal vein is described, by which osmium-tetroxide fixative can be introduced rapidly and uniformly to all parts of the tissue. Previous perfusion with balanced physiological saline solution, under the conditions described, of itself causes minimal change in the liver cell and liver architecture. Perfusion with a chelating agent causes no further detectable alteration within the liver cell but results in separation of the cells, even to the extent of producing a free cell suspension. The separation of cells is not accompanied by any recognizable damage to the plasma membrane but there is a striking tendency to pseudopod formation on the newly exposed surface. These findings provide direct evidence to support the classical concept of the importance of calcium to the adhesiveness of the plasma membrane in the tissues of the adult rat.

## INTRODUCTION

The importance of calcium in the cohesion of metazoan and plant cells has long been accepted as an established fact (1). Evidence for this role is abundant, and ranges from the classic experiments with dividing sea urchin eggs to the use of ethylenediaminetetraacetic acid (EDTA) for preparing free cell suspensions from tissue explants growing *in vitro*. In these experiments, the removal of calcium appears to affect only the adhesiveness of the cell surfaces, the metabolic activities of the cells and their reproductive capabilities apparently remaining intact.

In adult mammalian tissues, however, the significance of calcium for the integrity of cellular structure and function is much less clear. Anderson (2) described a method of preparing suspensions of isolated parenchymal cells from the livers of adult rats by perfusion of the liver with solutions of EDTA or citrate, which presumably remove the calcium. These suspensions, however, have been found to show abnormal metabolic activity *in vitro*, as compared with liver tissue slices. They show no endogenous respiration, and greatly increased stimulation of oxygen uptake by added succinate (3, 4). Anaerobic glycolysis is sharply depressed (4), and there is no incorporation of acetate into cholesterol (5). Such alterations do not depend upon gross separation of the cells, as in a suspension, because they are already detectable in slices cut from the livers immediately after perfusion with EDTA or citrate (4, 5). These metabolic disturbances appear to be related to a loss of selective permeability of the cell walls, with inability to retain differential intra- and extracellular concentrations of $Na^+$, $K^+$ and other substances (6).

Because of these alterations of function, it is important to know whether the perfusion with a chelating agent results in any detectable structural alteration other than loss of cohesion of the cells, especially in relation to the morphological integrity of the plasma membrane. The metabolic anomalies would be readily explained by gross breakage of cells. However, if no such breakage occurred, it would suggest the possibility that calcium was somehow essential for the *functional*

integrity of the cell wall, in a way as yet unexplained. As reported previously (2, 4), the isolated cell suspensions appear normal when viewed by phase contrast and other light microscopic methods. Coman, however, has reported that electron microscopy of liver perfused with EDTA reveals, in addition to separation of cells, lifting of the cell membranes from the underlying cytoplasm, or partial to complete destruction of the membrane (7). In comparison with the electron micrographs of normal liver published by Fawcett (8) and others, even the unperfused controls used by Coman show numerous alterations which can be considered fixation artefact.

In contrast, Easty and Mutolo claim that perfusion of adult rat liver with EDTA solutions via the thoracic aorta failed to produce any separation of liver cells. They, therefore, question the importance of calcium in cellular adhesiveness (9).

The problem has, therefore, been reinvestigated, with close attention to the techniques of perfusion and fixation. In this report are presented the findings in adult rat liver following perfusion with a balanced physiological salt solution, or with the same solution plus added EDTA.

## METHODS

### *Perfusion of Liver*

Albino rats of about 300 *g* body weight were used. Under sodium pentobarbital anaesthesia (0.5 mg. per 100 *g* body weight, intraperitoneally), the abdominal cavity was opened widely, exposing the liver and portal vein. A polyethylene cannula filled with heparinized saline was inserted cephalad into the portal vein, and a ligature was placed around the portal structures including the cannula, blocking the flow of mesenteric and pancreatic venous blood and hepatic arterial blood into the liver. Perfusion via the cannula was started immediately. The inferior vena cava was clamped above the diaphragm, and drainage was permitted through a small nick in the vena cava below the liver.

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### *Explanation of Plates*

All plates are electron micrographs of rat liver, prepared as described in the text and in the descriptions of the individual figures. The following explanatory letters are used in the illustrations:

*B* Bile canaliculus
*C* Cell (plasma) membrane
*E* Endoplasmic reticulum
*F* Fat
*K* Kupffer (reticulo-endothelial) cell
*L* Lumen of sinusoid
*M* Mitochondrion
*N* Nucleus
*O* Nucleolus
*P* Parenchymal cell
*R* Red blood cell
*S* Separation of adjacent cell membranes
*V* Microvilli or pseudopodial protrusions
*W* White blood cell

FIGURE 1

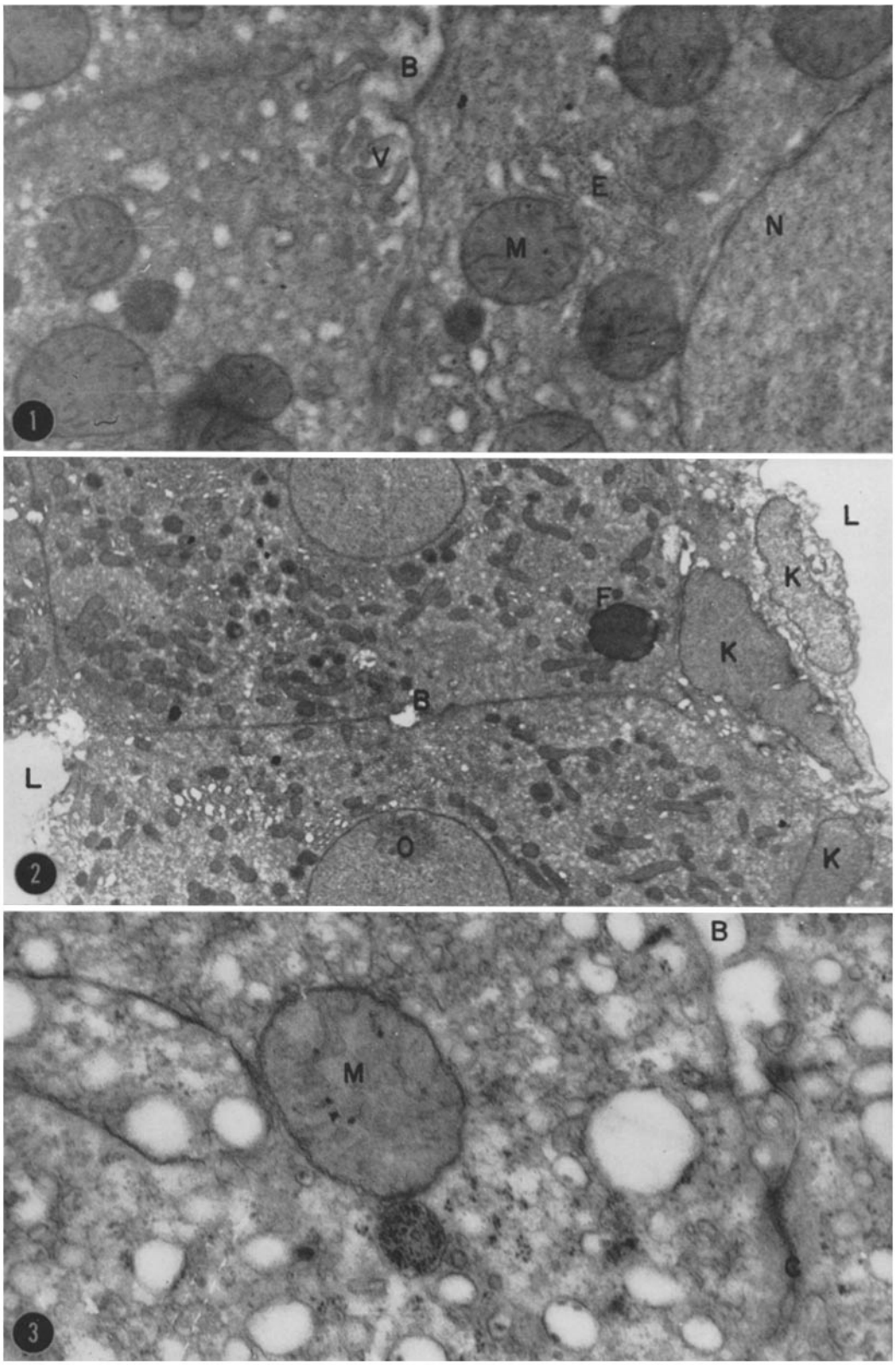
Unperfused control, fixed by simple immersion. Parts of three liver parenchymal cells are seen, meeting at a bile canaliculus (*B*), the lumen of which is partly filled with microvilli (*V*). Part of a nucleus (*N*), mitochondria (*M*), and endoplasmic reticulum (*E*) seen. × 20,500.

FIGURE 2

Control, perfused with Krebs-Ringer-phosphate solution containing no EDTA, then fixed by infusion of osmium tetroxide. Parts of two parenchymal cells are shown. Nuclei of three Kupffer cells (*K*) are seen, while the lumen of another sinusoid (*L*) is also visible. × 2600.

FIGURE 3

Perfused control. A small bile canaliculus (*B*) is shown, situated between two adjacent parenchymal cells. The plasma membranes (*C*) of the two cells are shown. × 35,000.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The perfusion fluid consisted of Krebs-Ringer phosphate solution (10), either with or without added di-sodium salt of EDTA in a concentration of 0.5 per cent (*w/v*), and adjusted to pH 7.4. The perfusion fluid, previously saturated with oxygen, was passed from a raised reservoir through a glass coil immersed in a thermostatically controlled bath at 39–40°C., and then through a polyethylene screw valve into the portal cannula. Care was taken to exclude all air bubbles from the perfusion tubing. The reservoir was at a height of 75 cm. above the table, and the screw valve was set to maintain the flow rate at approximately 5 to 6 ml. per minute. This flow rate was too slow to distend the liver by itself but intermittent digital pressure on the vena cava, damming back the perfusate, produced intermittent slow distension of the liver which flushed it out without injuring it mechanically. By means of a manometer in the vena cava, it was found that this degree of distension of the liver was achieved with a back pressure of only 15 cm. of water, or approximately 12 mm. of mercury. The total volume perfused was about 125 ml., taking 20 to 25 minutes to run through.

### *Fixation*

When most of the perfusion fluid had passed through the liver, all the liver lobes except one were ligated at their pedicles and removed. As soon as the rest of the perfusion fluid had passed through the remaining lobe, 5 ml. of the fixative solution, 1 per cent osmium tetroxide in Palade's buffer at pH 7.4 (11), was infused via the portal cannula over a period of about 2 minutes; the vena cava was occluded during this infusion, so that the fixative slowly distended the liver. Almost immediately, the remaining liver tissue turned a patchy black and became appreciably firmer to the touch. At this stage, it was removed rapidly from the animal, and blocks of approximately 1 mm.$^3$ were cut from it. Some of these blocks were further fixed by immersion in the fixative solution for periods of 30, 45, 60, 90, or 120 minutes. Other blocks were placed immediately in 70 per cent ethanol. Comparison of these various blocks indicated that optimal fixation was produced by immersion for 45 minutes after the infusion.

As a basis of comparison for both the non-chelated and chelated perfused livers, samples of unperfused fresh normal liver were fixed in the conventional manner. Blocks of 1 mm.$^3$ were dropped directly into the fixative without any prior treatment, and immersed for 120 minutes.

After fixation, all blocks were dehydrated rapidly through graded ethanols, infiltrated, and embedded in a mixture of *n*-butyl and methyl methacrylate (87½ to 12½) containing 1 per cent benzoyl peroxide as catalyst. Polymerization of the methacrylate was performed at 58°C. for 24 hours. The blocks were trimmed and sectioned with a Porter-Blum ultramicrotome using a diamond knife, and the sections were collected by flotation on a 2 per cent acetone solution. The sections were then picked up on formvar-coated grids and examined in a Philips 100 B electron microscope, using the small objective aperture at 60 kv.

## RESULTS

Four complete experiments were carried out, and identical results were found in all. The accom-

FIGURE 4

Chelated (*i.e.*, perfused with EDTA solution before infusion of fixative). Early separation of the parenchymal cells is seen. The complete cell in the centre illustrates the standard of fixation obtained after perfusion and chelation. × 1700.

FIGURE 5

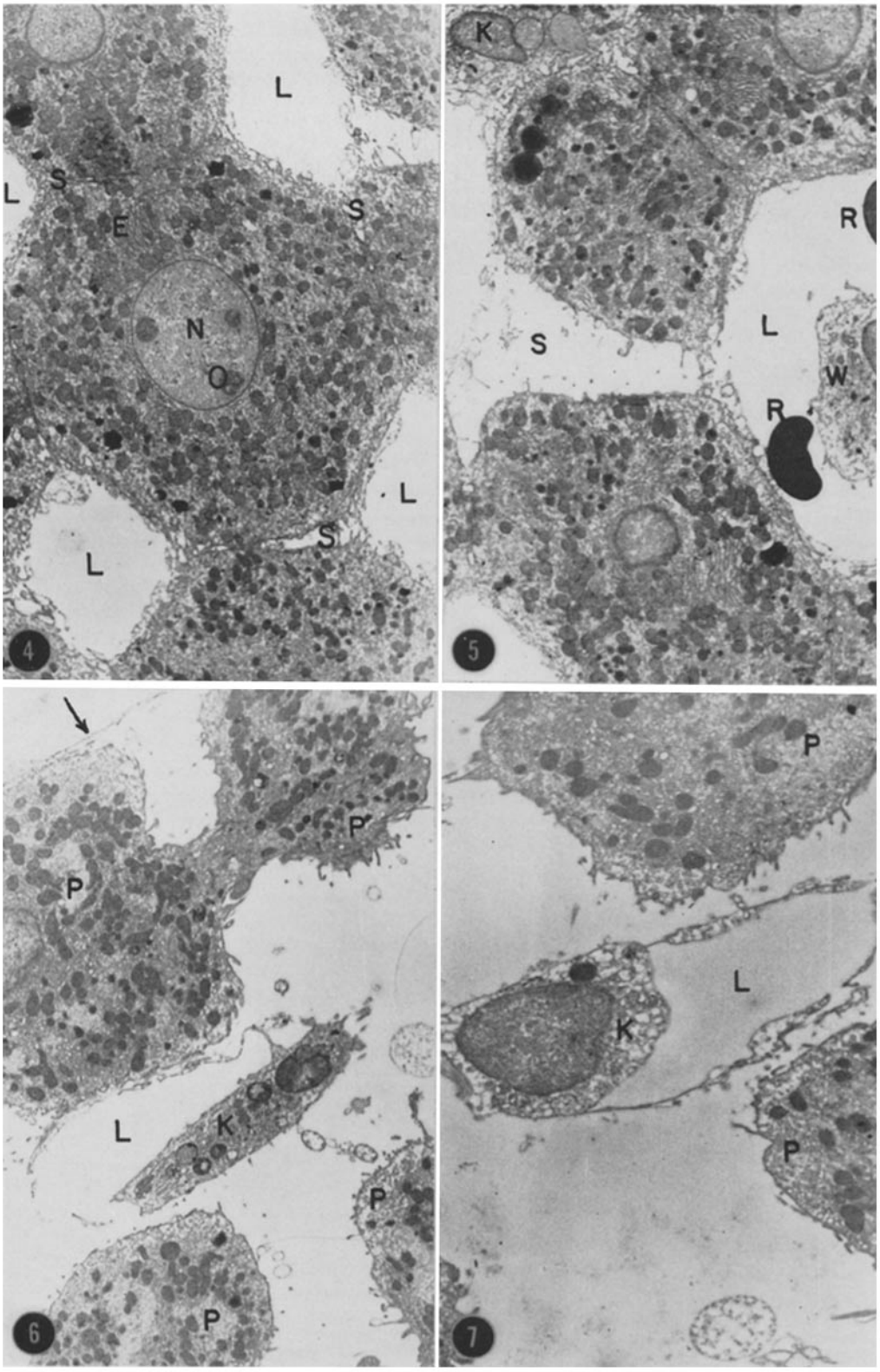
Chelated. Separation of parenchymal cells is farther advanced than in Fig. 4. The sinusoid (*L*) to the right still contains red (*R*) and white (*W*) blood cells. × 1700.

FIGURE 6

Chelated. In the lower centre is a reticuloendothelial cell (*K*) lining a nearly intact sinusoid (*L*). Parenchymal cells (*P*) are virtually completely separated. Attenuated cytoplasm of another reticulo-endothelial cell lining an adjacent sinusoid is indicated by arrow. × 1700.

FIGURE 7

Chelated. A further example of an intact sinusoid (*L*) from which the parenchymal cells are completely separated. × 1700.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panying micrographs were selected from all four experiments to illustrate the principal findings. In each experiment, the standard of fixation was judged by the control material; *i.e.*, that perfused only with the physiological saline. Well fixed material showed a normal architecture; the cells were arranged in cords between blood sinusoids, and each cell showed the usual organelles with minimal distortion (Figs. 2 and 3). The material fixed by immersion without any perfusion (Fig. 1) showed appearances identical to those of the control perfused material. Shrinkage and "crinkling" of the nuclear membrane, vesiculation of the endoplasmic reticulum, and enlargement and a decrease in the electron-density of the mitochondria with poorly seen cristae mitochondriales, all are indicative of postmortem degeneration and poor fixation. Such appearances occasionally were seen, but the majority of the control blocks showed these abnormalities only to a minor degree. In each experiment, at least four blocks of "normal," un-chelated liver and at least ten blocks of chelated material were examined. In each case, the material showing the most nearly normal, undistorted organelles was used in the final assessment.

The changes resulting from chelation were few. In adequately fixed material the cell-organelles appeared virtually normal (Figs. 8, 9, and 12), showing the same appearance as those of the control-perfused tissue. The only additional changes were to be found at the cell surfaces, and these were similar in all experiments although they varied in degree from block to block, obviously depending on the extent of the chelation. The most gross change was a complete disruption of the liver architecture, with cells completely separated from each other but each showing essentially normal organelles (Figs. 10 and 11). The plasma membrane of these cells showed small, stubby pseudopodia (Fig. 12). In such tissue, liver sinusoids could be readily identified (Figs. 6, and 7), each limited by the thin, attenuated cytoplasm of a reticulo-endothelial (Kupffer) cell, and some still contained the occasional blood cell (Fig. 5). With less marked change, the liver architecture perhaps appears unaltered when the tissue is viewed at low power, but higher magnification showed a commencing separation at the cell interfaces with pseudopod formation at the adjacent plasma membranes (Figs. 4 to 9).

## DISCUSSION

The results obtained in the present work must be compared with those of Coman (7) and of Easty and Mutolo (9). Liver tissue is not easily fixed for electron microscopy and we consider that the partial destruction of plasma membranes described by Coman as consequent upon calcium-chelation is, in fact, due to postmortem degeneration associated with slow diffusion of the fixative into the tissue. Extensive degenerative changes in the cytoplasm and organelles, seen in his illustrations, are in keeping with this view. When the fixative is introduced rapidly by perfusion through the portal vein the risk of such postmortem change is greatly decreased. Pease (12) has recently stated that liver is one of two tissues which can be well fixed by vascular perfusion. In the present experiments, the perfusion technique was not physiologically optimal, since the perfusion fluid contained no colloid, and oxygenation was impaired by the absence of hemoglobin. Despite these limitations, only relatively minor degenerative changes were seen after 20 to 25 minutes of control perfusion at 38°C. Under the same conditions, the only visible effect attributable to the addition of the chelating agent to the perfusion fluid was the separation of cells at cell interfaces.

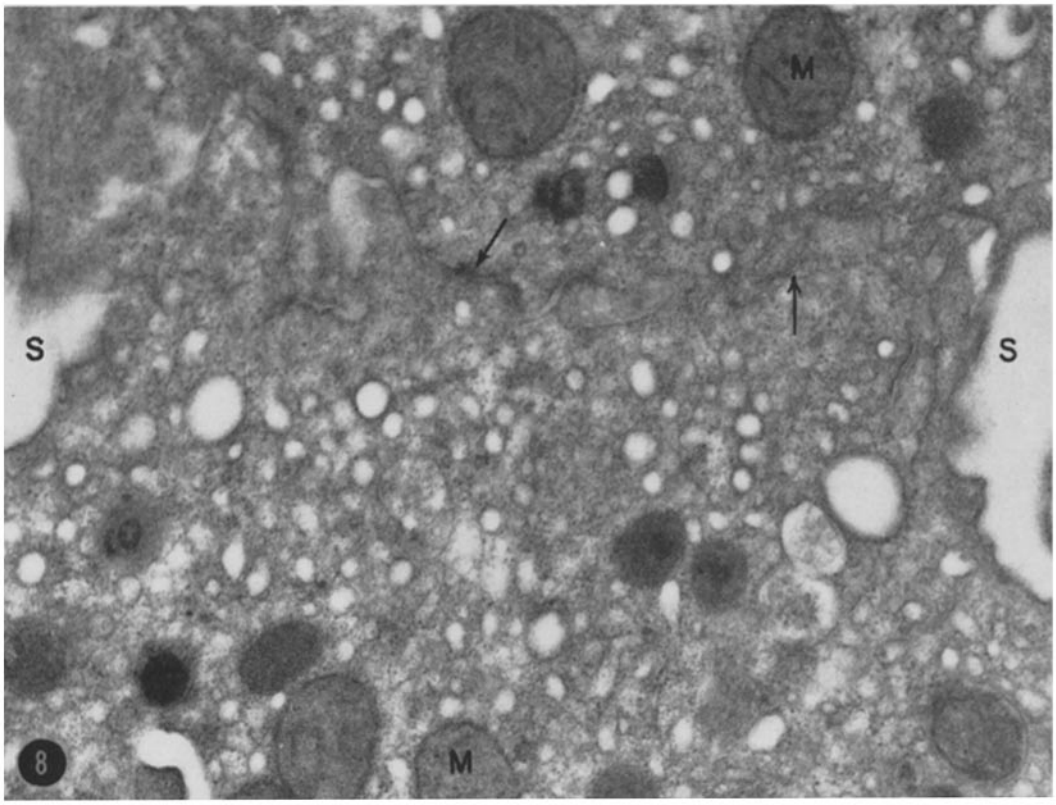
The findings of Easty and Mutolo (9) are diametrically opposed to those of Coman and of

FIGURE 8

Chelated. Two parenchymal cells are shown which have separated (*S*) at each side but are still attached in the centre of the micrograph. Note the interdigitating apposed plasma membranes (arrows). × 29,800.

FIGURE 9

Chelated. Parts of two parenchymal cells are shown, with adjacent plasma membranes separated above (*S*), with formation of pseudopodial protrusions of the cell surface (*V*), but still in contact below (arrow). × 36,500.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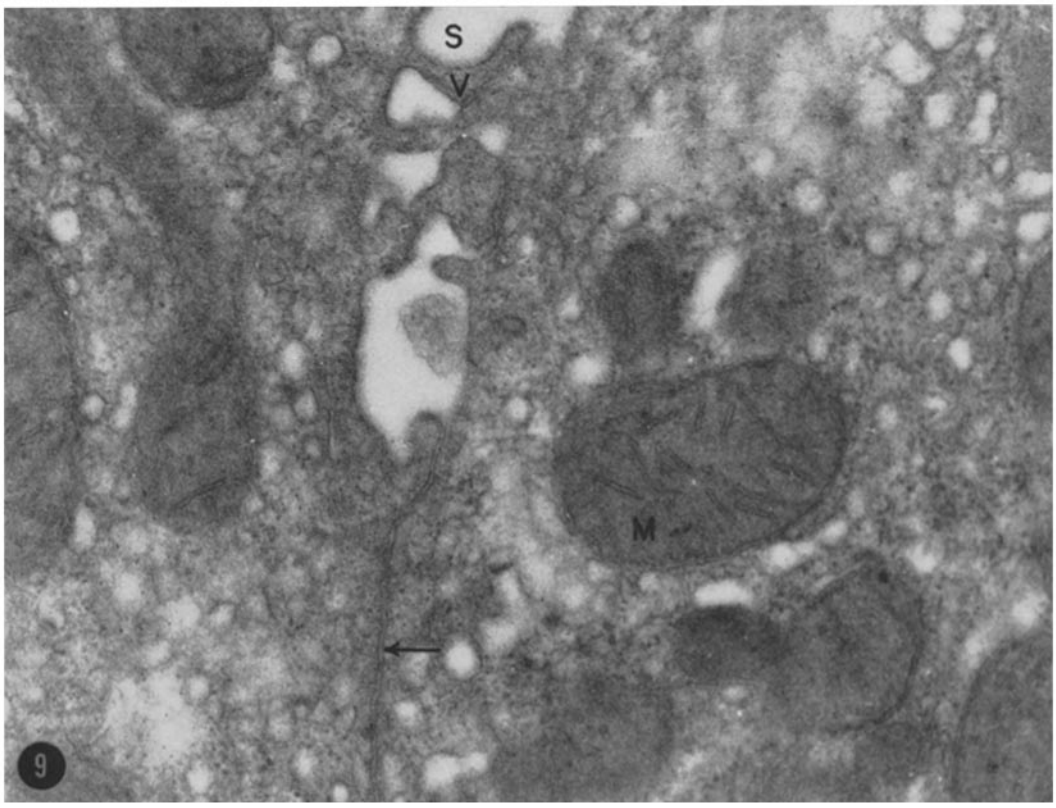
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ourselves. While the reason for this difference can only be conjectured, we believe the likeliest explanation to be that Versene simply did not reach the liver in adequate amount in Easty and Mutolo's preparations. In our experience, perfusion of the liver via the thoracic aorta is unreliable, particularly with cold perfusion solutions which appear to induce vasospasm. Easty and Mutolo do not state the temperature of the fluid, nor the perfusion pressure. In addition, small air bubbles can block perfusion through local areas of the liver. Despite gross blanching of the liver, it is entirely possible that the blocks of tissue which they examined by electron microscopy came from inadequately perfused portions.

In some of the separated cells (*e.g.*, Figs. 10 and 11) small sections of the plasma membrane appear to be missing. If these defects had been produced during the course of the perfusion, the cell contents would inevitably have been damaged or washed out. The fact that the cytoplasmic structure and organelles are intact even in the immediate vicinity of the membrane defects can only mean that these breaks were produced during the handling of the tissues *after* fixation. The formation of pseudopodia at the cell surface is interesting. Adhesion of adjacent cells by "jigsaw" interlocking of cytoplasmic processes has been described in the liver (7, 8) and was seen in the present experiments (Fig. 8, arrows). However, the great majority of interfaces in the liver appear as relatively straight, parallel plasma membranes, the two membranes being separated normally only at the site of bile capillaries (Fig. 2). It is noteworthy that the plasma membrane of a parenchymal cell where it lines a bile capillary shows the presence of microvilli (Fig. 1). After chelation and separation of cells, the remainder of the plasma membrane also shows projections which bear considerable resemblance to the microvilli in the bile capillaries, though they tend to be more irregular than the latter in size and shape (Figs. 9 and 12).

This experiment indicates that perfusion of liver tissue with a chelating agent, presumably resulting in removal of calcium and other divalent ions, results only in separation of the cells at their interfaces. The cells themselves appear to have been no more affected by the procedure than by perfusion with a balanced physiological salt solution alone. It is, of course, conceivable that the perfusion pressure might have contributed to the disruption of liver architecture, but in the presence of still intact sinusoids lined by the attenuated cytoplasm of reticulo-endothelial cells (Figs. 6 and 7) it seems scarcely reasonable to consider that any such mechanical disruption occurred. Therefore, it is concluded that the calcium bond is extremely important in cell adhesion in the liver of the adult rat. It is interesting to note that no residual points of attachment, corresponding to desmosomes, were observed in liver cells which had been separated by perfusion with EDTA solution. This suggests that if liver cells are normally held together by such structures, the integrity of the latter may depend in some way upon the presence of calcium or other divalent ions.

In addition, the previously reported metabolic alterations in EDTA-treated liver tissue (3–6), together with the morphological integrity of the plasma membranes in such tissue as described above, suggest that calcium plays some essential, though unexplained, role in the normal permeability regulation by the cell wall. These functions

FIGURE 10

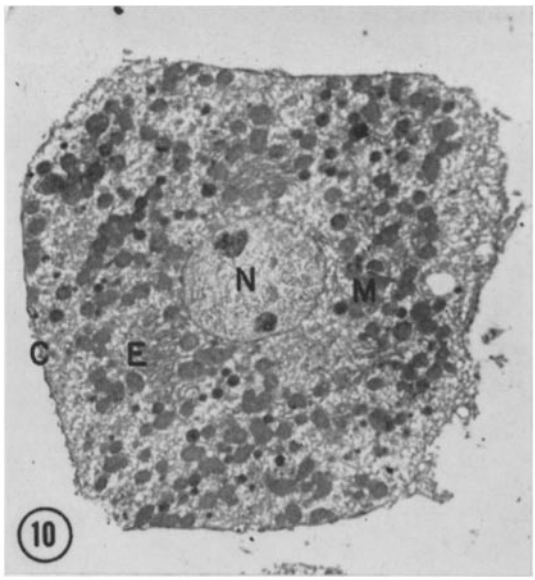
Chelated. A final stage showing a completely separated and isolated parenchymal cell with intact plasma membrane (*C*), nucleus (*N*), mitochondria (*M*), and endoplasmic reticulum (*E*). × 1600.

FIGURE 11

Chelated. Another single cell as above. × 1600.

FIGURE 12

Chelated. This micrograph shows pseudopodial protrusions (*V*) at the surface of a completely separated parenchymal cell with an intact plasma membrane (*C*) and well preserved mitochondria (*M*). × 54,000.

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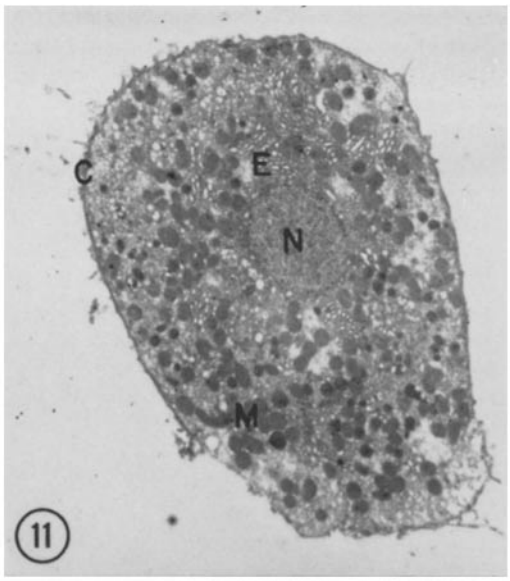


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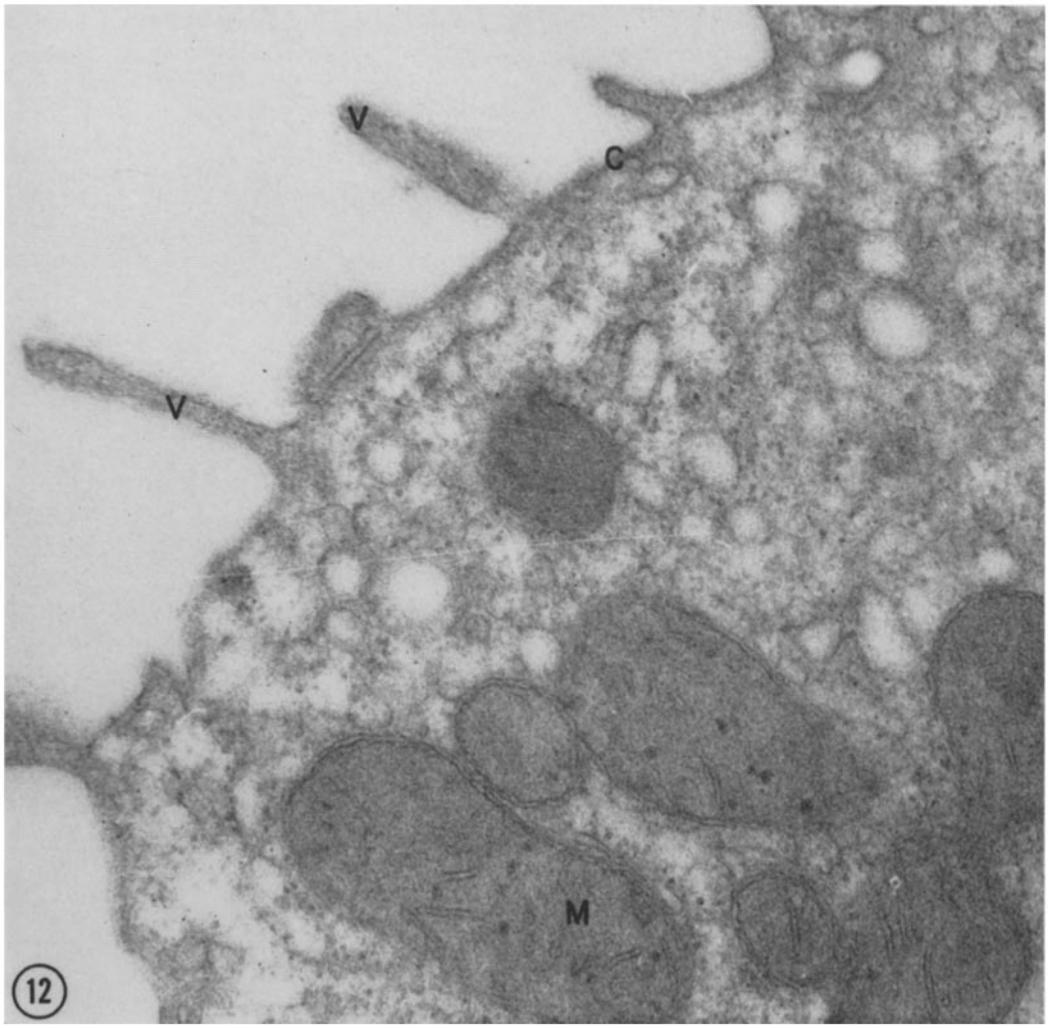


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of calcium have long been known in relation to foetal tissues, plant cells, and to cells of lower orders of metazoa. Their applicability to adult mammalian tissues has been largely assumed by analogy; the present work provides more direct evidence.

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