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Distribution of retinol in rat liver cells: effect of age, sex and nutritional status

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1. We have recently shown that the stellate cells, under normal conditions, contain a majority (more than 80%) of the total store of retinol in liver (Blomhoff *et al.* 1985).

2. In the present work we have studied the role of the various liver cells in rats of different ages, sex and vitamin A status.

3. In most of these groups of rats, storage of retinol in parenchymal cells was proportional to the liver store of retinol, and less than 10% of total retinol in the liver could be recovered in the parenchymal cells. The only exception was parenchymal cells isolated from vitamin A-deficient rats. In rats containing 5 nmol retinol/g liver, about 16% of total retinol could be recovered in parenchymal cells, while in rats with only 1 nmol retinol/g liver, about 40% of total retinol could be recovered in parenchymal cells.

4. These results indicate that parenchymal cells played a minor role in liver storage of retinol, and that stellate cells stored more than 90% of liver retinol in most instances. Only in rats with a low retinol status did the percentage of retinol in parenchymal cells increase.

In a previous report (Blomhoff *et al.* 1985) we have shown that hepatic perisinusoidal stellate cells (also called fat-storing cells) are the main storage site for retinol in the rat. Our findings suggested that under normal conditions as much as 80% or more of the total liver retinoids might be stored in stellate cells with the rest stored in parenchymal cells. Isolated parenchymal and stellate cells also contained enzymes and intracellular binding proteins involved in retinol metabolism. Isolated endothelial cells and Kupffer cells contained very low levels of these components (Blomhoff *et al.* 1985).

The method for isolating parenchymal liver cells most often involves collagenase perfusion of the liver and differential centrifugation of the total liver cell suspension (Seglen, 1976; Tolleshaug *et al.* 1977; Olson & Gunning, 1983). However, by analysing the parenchymal-cell fraction in the fluorescent microscope, one becomes aware of the numerous stellate cells which contain lipid droplets with autofluorescent retinol. We found that parenchymal cells isolated by this method are contaminated with 5–10% stellate cells (Blomhoff *et al.* 1985). Since stellate cells contain 100–200 times more retinol than parenchymal cells, it is difficult to quantify the exact amount of retinol in parenchymal cells using traditional methods.

In the present study we have used a refined method which includes centrifugal elutriation to purify the parenchymal liver cells. After centrifugal elutriation, the parenchymal-cell fraction contains less than 0·1% stellate cells. Based on this method we have determined the quantitative role of parenchymal cells in the hepatic storage of retinol in rats of different ages, sex and nutritional status.

MATERIALS AND METHODS

Chemicals

Percoll and Nycodenz were purchased from Pharmacia Fine Chemicals AB, Sweden and Nycomed A/S, Oslo, Norway respectively. Collagenase (type IV, *EC* 3.4.24.3) and pronase (type XXI) we obtained from Sigma Chemical Co., Poole, Dorset.

Animals

Wistar rats of both sexes (initial age 4 weeks) were fed on an ordinary pelleted diet (no. 3155; AREX, Møllesentralen, Norway) which contained about 9·4 μmol retinol (50% as retinyl acetate and 50% as retinyl palmitate)/kg. A group of male rats (4 weeks old) was fed on a retinol-deficient diet (EWOS A/B, Södertälje, Sweden) for 8–10 weeks, at which time they stopped gaining weight. Another group of male rats (6 weeks old) were given 2·4 mg retinol/kg body-weight per d for a period of 45 d.

Preparation of parenchymal liver cells

Total liver cell suspensions were prepared by a modified (Tolleshaug *et al.* 1977) collagenase perfusion technique (Seglen, 1976). Parenchymal cells were isolated from the total liver cell suspension by differential centrifugation (Tolleshaug *et al.* 1977), and by centrifugal elutriation. Cells were introduced into the elutriation chamber at a flow-rate of 18 ml/min and a rotor speed of 1200 rev/min (Blomhoff *et al.* 1984*b*). Pure parenchymal cells were washed out at a rotor speed of 45 ml/min. About 98% of the isolated parenchymal cells were viable as determined by the trypan blue exclusion test. The parenchymal-cell suspension was contaminated with less than 0·1% endothelial cells, Kupffer cells or stellate cells. Endothelial cells, Kupffer cells and stellate cells were identified as described below. About 40% of the parenchymal cells estimated to be present in intact liver (Munthe-Kaas *et al.* 1976) were recovered in the isolated parenchymal-cell suspension. Parenchymal cells were identified by light microscopy and by transmission electron microscopy (Plate 1(*a*)).

Preparation of non-parenchymal liver cells

Non-parenchymal cells were prepared from total liver cell suspensions by differential centrifugation (Berg & Boman, 1973), followed by centrifugation in 200 g Nycodenz/l (Blomhoff *et al.* 1984*c*) to remove cell debris. The yield of cells by this method was about 32%, and the non-parenchymal-cell suspension contained about 72% endothelial cells, 17% Kupffer cells and 8% stellate cells.

Preparation and identification of endothelial liver cells

Total liver-cell suspensions were treated with enterotoxin from *Clostridium perfringens* as described by Blomhoff *et al.* (1984*c*). The enterotoxin rendered the parenchymal cells leaky, and these cells were separated from the non-parenchymal cells by centrifugation in a HEPES-buffered minimal salt solution (Tolleshaug *et al.* 1977) containing 200 g Nycodenz/l. Endothelial cells were separated from the other non-parenchymal cells by centrifugal elutriation (Blomhoff *et al.* 1984*c*). Endothelial cells were identified following incubation with fluoresceinamine-conjugated ovalbumin, a compound which is selectively taken up by the endothelial cells (Smedsrød *et al.* 1982), and by transmission electron microscopy (Plate 1(*b*)). None of the endothelial cells showed a positive cytochemical peroxidase reaction (Wisse, 1974). The endothelial-cell fractions were contaminated with 7% (mean of fifteen preparations) Kupffer cells and 2% (mean of seven preparations) stellate cells. About 55% of the endothelial cells estimated to be present in intact liver were recovered in the final endothelial-cell preparation.

Preparation and identification of Kupffer cells

Kupffer cells were purified by centrifugal elutriation (Blomhoff *et al.* 1984*b*) of non-parenchymal cells prepared from the total liver-cell suspension by incubation with pronase (Berg & Boman, 1973). They were identified cytochemically by positive peroxidase reaction

(Wisse, 1974), and by transmission electron microscopy (Plate 1(*c*)). The fraction containing the Kupffer cells was contaminated with 0·2% (mean of six preparations) parenchymal cells, 7% (mean of nine preparations) endothelial cells and 3% (mean of nine preparations) stellate cells. The yield of Kupffer cells using this method was estimated to be about 45%.

Preparation and identification of stellate cells

Non-parenchymal cells were prepared from the total liver-cell suspension by differential centrifugation (Nilsson & Berg, 1977). The stellate cells were isolated further by Percoll density-gradient centrifugation (Blomhoff *et al.* 1984*b*). The fractions with densities between 1·025 and 1·035 g/ml contained exclusively stellate cells. The stellate cells were identified both by transmission electron microscopy (Plate 1(*d*)) and by fluorescence microscopy (Blomhoff *et al.* 1984*b*). Of the total number of stellate cells estimated to be present in liver, 8% were recovered in the final cell suspension.

Quantification of total retinol

For determination of total retinol (i.e. retinol plus retinyl esters) the fractions were saponified for 30 min at 57° with 2 ml potassium hydroxide (100 g/l) in ethanol (910 ml/l) in the presence of pyrogallol (10 g/l). Total retinol was then extracted into 5 ml hexane and 2 ml water. A 4 ml portion of the hexane phase was transferred to new vials, and the internal standard (retinyl acetate) was added. The hexane was evaporated under nitrogen. The residue was dissolved in a small volume of methanol, and a portion was injected on to a high-performance liquid chromatographic (HPLC) column. The column used was a 5 μm Supelcosil LC-8 column with a Waters model 440 detector monitoring absorbance at 313 nm. Retinol was eluted in water:methanol (7·5:92·5, v/v) at a flow rate of 1·5 ml/min. Quantification of total retinol was performed as described by Blomhoff *et al.* (1985).

Analysis of free retinol and retinyl esters

Whole liver and cell homogenates were extracted with ethanol and hexane. The homogenates (0·1–0·5 ml) were deproteinized with 96% ethanol, mixed thoroughly and, after a period of 30 min, 5 ml hexane was added for extraction. The procedure was thereafter essentially as described for 'Quantification of total retinol' (above). The HPLC column (LC-8) was eluted with water:methanol (7·5:92·5, v/v) at a flow rate of 1·5 ml/min. Retinyl ester standards (Sigma Chemical Co.) were injected separately on to the same column to determine the position of the three main ester peaks, i.e. retinyl palmitate, retinyl oleate and retinyl stearate. A System L Computing Integrator from Spectra Physics, Santa Clara, CA, was used for calculating percentage distribution, assuming an equal chromatographic area per mol retinol and its esters.

RESULTS

Total retinol in liver cells isolated from 3-month-old male rats

Total retinol (retinol plus retinyl ester) contents in different liver-cell fractions of rats were determined by HPLC. The results are presented in Table 1. These findings show that isolated parenchymal cells contained about 0·2 nmol total retinol/10^6 cells. Of the non-parenchymal cells, only stellate cells contained considerable amounts of retinol (about 34 nmol total retinol/10^6 cells). Assuming that 1 g liver contains 125×10^6 parenchymal cells (Munthe-Kass *et al.* 1976) and 9×10^6 stellate cells (Blomhoff *et al.* 1984*b*), it follows that less than 10% of the total retinol in liver is present in parenchymal cells, with more than 90% of the total retinol contained in stellate cells.

Table 1. *Total retinol in liver cells isolated from 3-month-old male rats*

(Total retinol (retinol plus retinyl esters) was quantified in liver cells isolated from 3-month-old male rats fed on a control pelleted diet. Values represent means, with their standard errors, for the number of rats given. When converting values from a per 10^6 cells to a per g liver basis, it was assumed that liver contains 190×10^6 cells/g wet weight, and parenchymal cells, endothelial cells, Kupffer cells and stellate cells constitute 56, 19, 10 and 6% of the hepatic cells respectively (Munthe-Kass *et al.* 1976; Blomhoff *et al.* 1984*c*)

		Total retinol		
		nmol/10^6 cells		
	No. of rats	Mean	SE	nmol/g liver
Parenchymal cells	5	0·2	0·04	25
Non-parenchymal cells	4	4·5	0·45	293
Endothelial cells	4	< 0·1	—	< 4
Kupffer cells	4	< 0·1	—	< 2
Stellate cells	5	34·1	7·31	307

Table 2. *The effects of age, sex and diet on total retinol in rat liver and isolated parenchymal cells*

(Total retinol (retinol plus retinyl esters) was quantified in total liver and parenchymal liver cells isolated from different groups of rats. Rats in group 4 were given an ordinary pelleted diet until they were 6 weeks of age, and then given a diet supplemented with 2·4 mg retinol/kg body-weight per d for a period of 6 weeks. Rats in groups 6 and 7 were fed on the ordinary pelleted diet until they were 4 weeks of age, and then on a vitamin A-deficient diet for 8 weeks and 10 weeks respectively (for further details, see p. 234). Values represent means, with their standard errors, for the number of rats given. When estimating the percentage of total hepatic retinol in parenchymal cells, it was assumed that 1 g liver contains 125×10^6 cells)

					Total retinol					
					nmol/g liver		nmol/10^6 parenchymal cells		nmol/125×10^6 parenchymal cells	Percentage estimated in parenchymal cells
Group	Sex	Age (months)	Retinol in diet	No. of rats	Mean	SE	Mean	SE		
1	Male	3	Adequate	10	539	20	0·15	0·026	19	4
2	Male	7	Adequate	6	777	107	0·50	0·058	62	8
3	Male	12	Adequate	9	1761	239	0·67	0·044	84	5
4	Male	3	Excess	5	6710	109	3·41	0·270	427	6
5	Female	3	Adequate	4	1730	432	0·42	0·073	53	3
6	Male	3	Deficient	5	5	0·8	0·006	0·001	0·8	16
7	Male	3.5	Deficient	3	1	0·3	0·003	0·001	0·4	40

Quantification of free retinol and retinyl esters in liver cells isolated from 3-month-old male rats

The percentage of free retinol and retinyl esters in parenchymal and stellate cells was determined by HPLC. Of the total retinol detected in isolated parenchymal cells, 30·8% (mean of five preparations) was recovered as free retinol, while the rest was recovered as retinyl esters. In stellate cells, nearly all the total retinol was present as retinyl esters, and less than 1% was recovered as free retinol.

Total retinol in liver and parenchymal cells isolated from rats of different ages, sex and nutritional status

In the next series of experiments we quantified the amount of total retinol in liver and parenchymal cells isolated from 3-, 7- and 12-month-old male rats, 3-month-old female rats, 3-month-old male rats fed on a retinol-deficient diet, and 3-month-old male rats fed on excess retinol (Table 2). The total retinol content of the liver increased considerably with age, from about 540 nmol/g liver in 3-month-old rats to about 1760 nmol/g liver in 12-month-old male rats. The 3-month-old female rats contained about three times more total retinol than their male counterparts. While male rats fed on a retinol-deficient diet for 8–10 weeks contained less than 10 nmol total retinol/g liver, 3-month-old male rats given 2·4 mg retinol/kg body-weight per d for 45 d contained more than 6000 nmol total retinol/g liver.

In most of these groups of rats, the amount of total retinol found in parenchymal cells was approximately proportional to the total retinol content in the liver (Table 2). Less than 10% of total retinol in the liver was recovered in the parenchymal cells. The only exception was parenchymal cells isolated from retinol-deficient rats. In rats having 5 nmol retinol/g liver, about 16% of the total retinol was estimated to be present in parenchymal cells, while in rats having only 1 nmol retinol/g liver, about 40% was present in parenchymal cells.

DISCUSSION

Retinol absorbed in the intestine is esterified and transported via the lymphatic route in chylomicrons and their remnants (Goodman *et al.* 1965). The retinyl esters remain with the chylomicron remnant particles, and are removed from the circulation by the liver (Blomhoff *et al.* 1982, 1984*a*).

Studies have indicated that chylomicron-remnant retinyl esters are mainly taken up by parenchymal liver cells (Blomhoff *et al.* 1982). The newly endocytosed vitamin A is, however, transferred from the parenchymal cells to the perisinusoidal stellate cells in the liver shortly after uptake (Blomhoff *et al.* 1982, 1984*b*). Furthermore, recent work in our laboratory (Blomhoff *et al.* 1985), and by others (Blaner *et al.* 1985), has shown that the stellate cells under normal conditions contain a majority (more than 80%) of the total store of retinol in liver.

In agreement with previous reports (Batres & Olson, 1987*b*; Blaner *et al.* 1985; Blomhoff *et al.* 1985) our findings showed that most of the reserve (more than 90%) of retinol was found in stellate cells in normal rats. The retinol in stellate cells was almost exclusively present as retinyl esters, and less than 1% was recovered as free retinol. In previous studies, it was, however, difficult to quantify the relative amounts of retinol and retinyl esters in parenchymal cells due to the contamination of stellate cells in the isolated parenchymal-cell fraction. Using a refined method to prepare parenchymal cells enabled us, in the present study, to quantify the amounts of retinol and retinyl esters in parenchymal cells. Of the total retinol detected in isolated parenchymal cells, about 30% was recovered as free retinol, while the rest was recovered as retinyl esters.

In rats given an excess of retinol, only 6% of the massive amount of total retinol (about 6·7 nmol/g liver) stored in liver was recovered in parenchymal cells. This is in agreement with a recent report from Batres & Olson (1987*a*). In rats storing high amounts of retinol (about 1·4 nmol/g liver) they found that parenchymal cells isolated by centrifugal elutriation contained less than 5% of the total liver retinol.

In the present study we also investigated the role of the various liver cells in rats of

different ages and sex, and in vitamin A-deficient rats. In most of these groups of rats, the amount of retinol found in parenchymal cells was roughly proportional to the total amount in the liver, and less than 10% of total retinol in the liver could be recovered in the parenchymal cells. This latter statement, however, did not apply to parenchymal cells isolated from retinol-deficient rats. In rats containing 5 nmol retinol/g liver, about 16% of the total liver retinol was present in parenchymal cells, while in rats with only 1 nmol retinol/g liver, about 40% of the total retinol was present in parenchymal cells.

Batres & Olson (1987*b*) reported that parenchymal cells isolated from vitamin A-deficient Sprague–Dawley rats storing 5 nmol retinol/g liver contained more than 80% total liver retinol. Although this value does not agree with ours, both studies suggest that the relative importance of parenchymal cells increases in vitamin A-deficient rats. The discrepancy between our values and those reported by Batres & Olson (1987*b*) may be due to the difference in rat strain or feeding procedure.

Taken together, these results indicate that parenchymal cells play a minor role in liver storage of retinol, and that stellate cells store more than 90% of liver retinol, in most instances. Only in rats with a low retinol status does the percentage of retinol in parenchymal cells increase.

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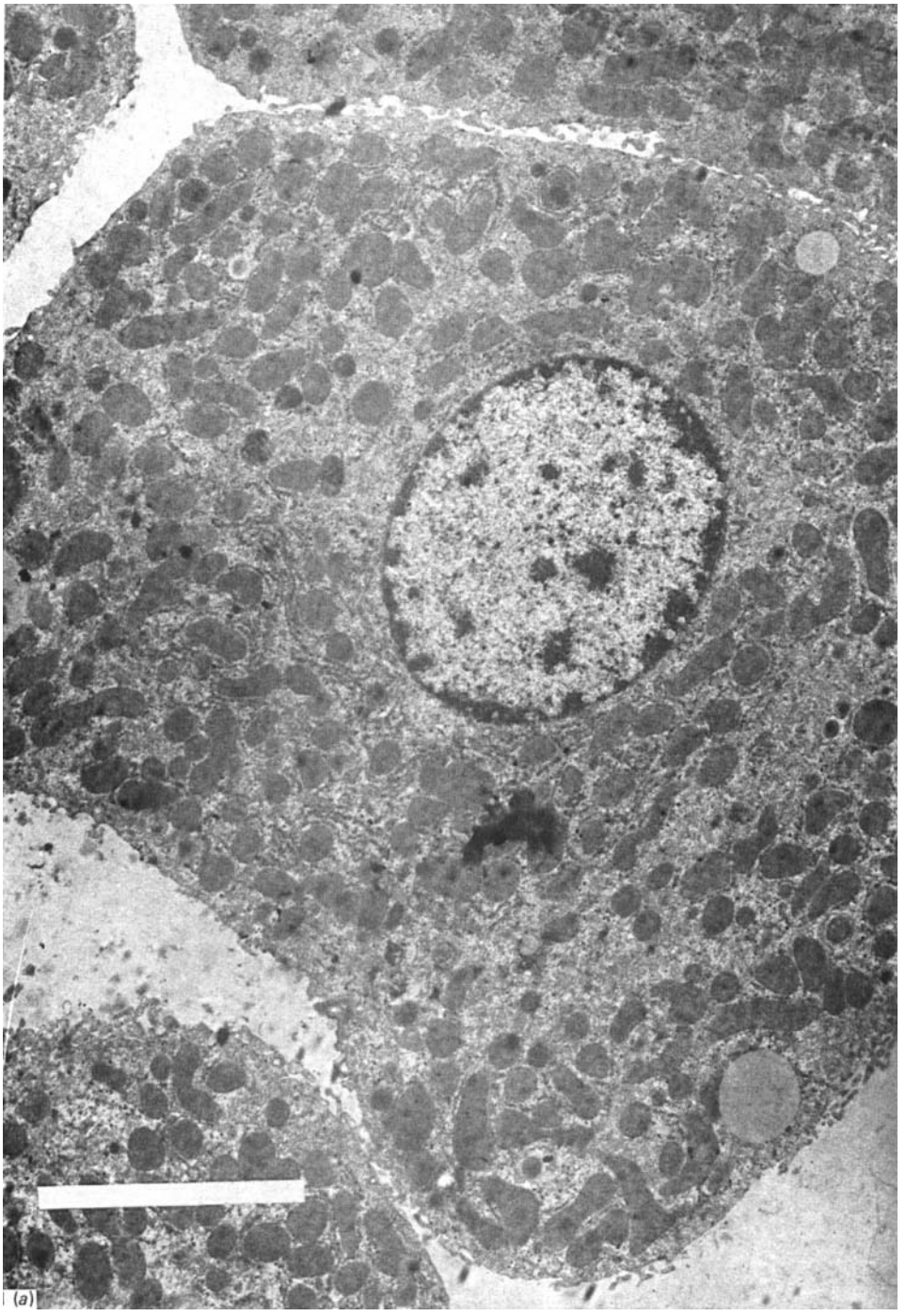
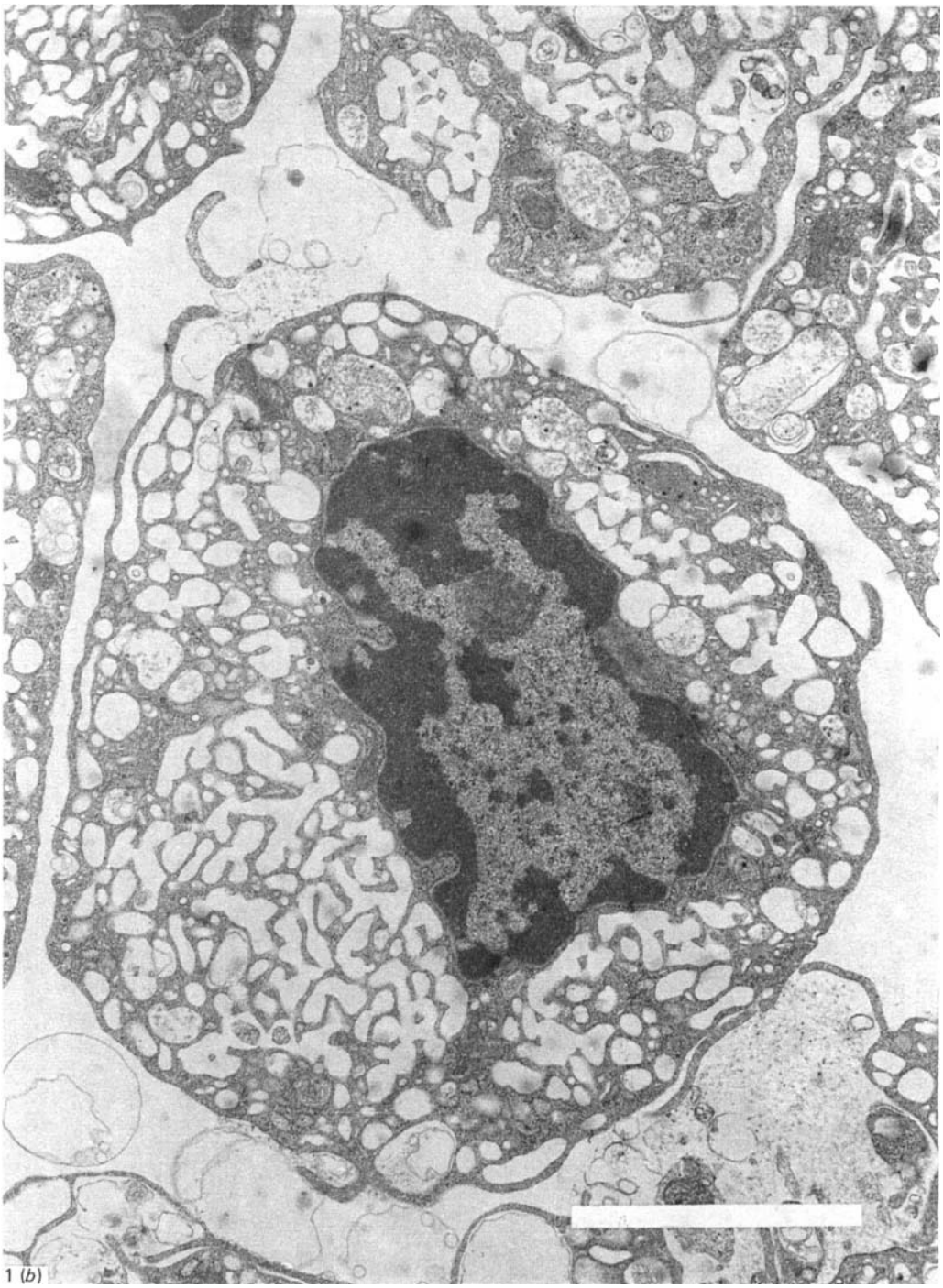
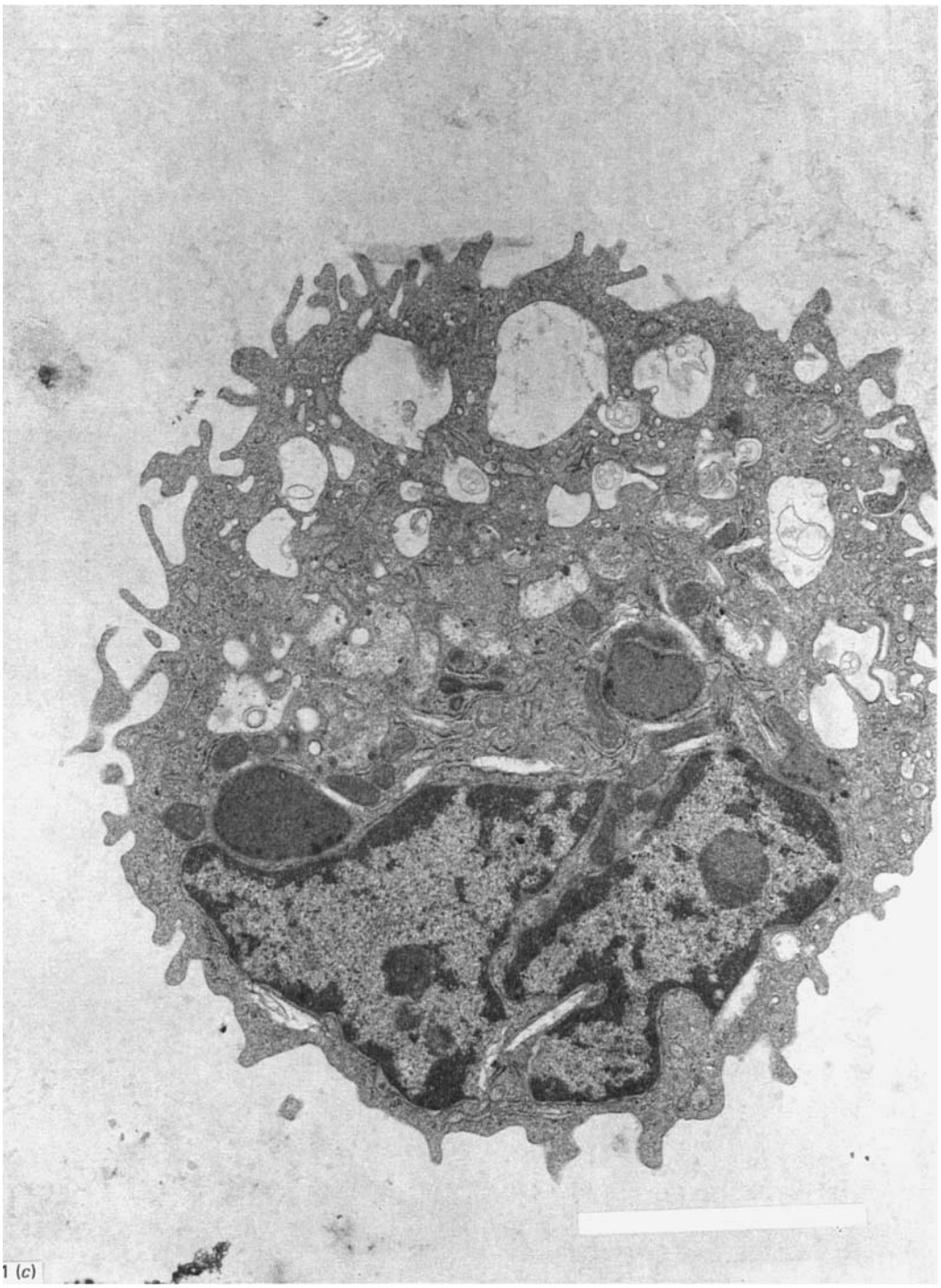
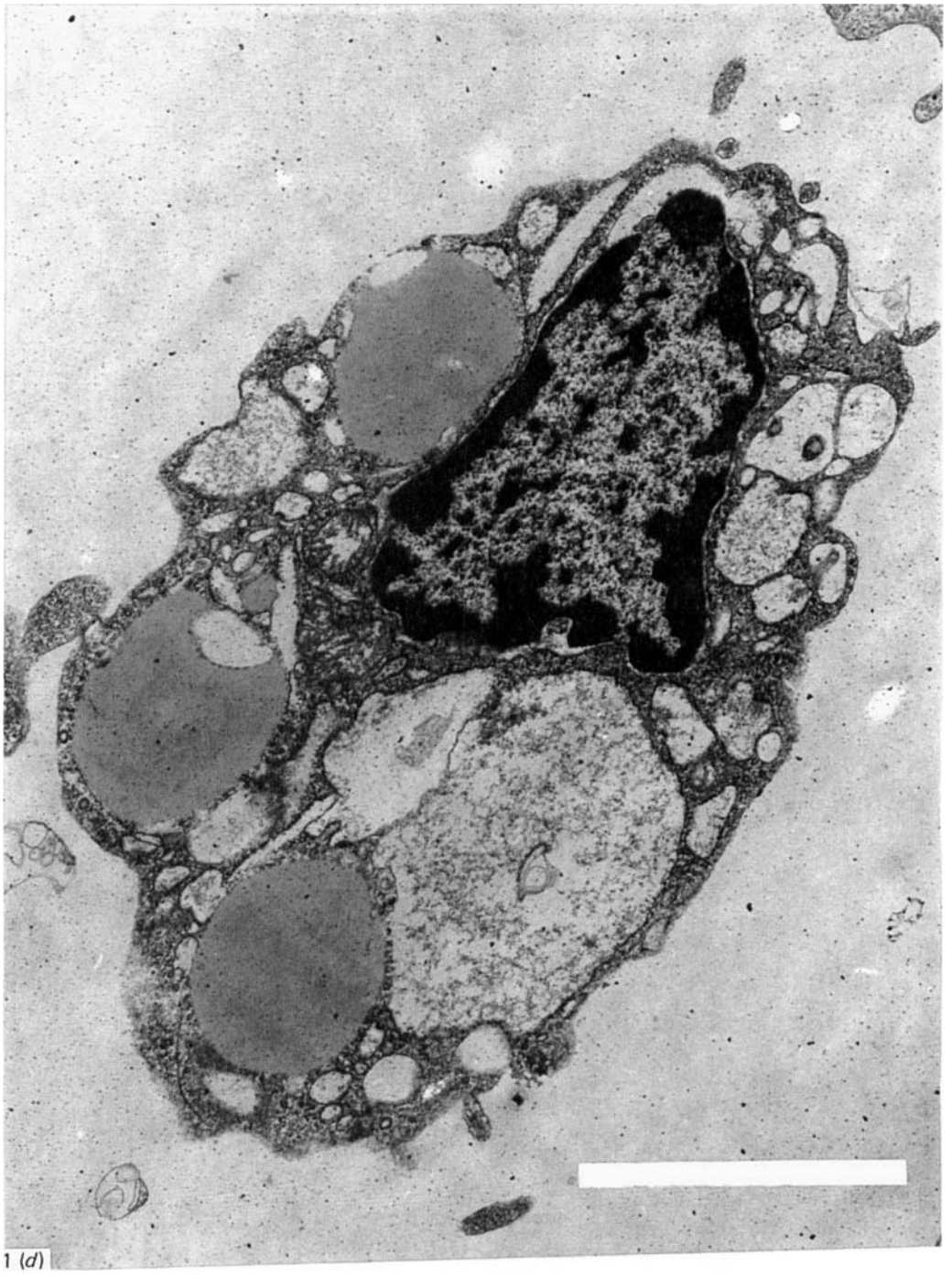


For description of Plate see page 239







1 (*d*)

EXPLANATION OF PLATE

Plate 1. Transmission electron micrographs of freshly isolated rat liver parenchymal cells (*a*), endothelial cells (*b*), Kupffer cells (*c*) and stellate cells (*d*). Cell fractions were fixed with glutaraldehyde (20 g/l) in 0·17 M-cacodylate buffer, pH 7·2, for 2 h at 4° and post-fixed in osmium tetroxide (10 g/l) at 4° overnight. Cells were then incubated with uranyl acetate (5 g/l) for 1 h at room temperature and processed further for transmission electron microscopy. Bars = 5 μm.

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